Season, not long-term warming, affects the relationship between ecosystem function and microbial diversity

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1 Abstract

Across biomes, soil biodiversity promotes ecosystem functions. However, whether this relationship will be maintained under climate change is uncertain. Here, using two long-term warming experiments, we investigated how warming affects the relationship between ecosystem functions and microbial diversity across seasons, soil horizons, and warming duration. The soils in these warming experiments were heated +5 °C above ambient for 13 or 28 years. We measured seven different ecosystem functions representative of soil carbon cycling, soil nitrogen cycling, or nutrient pools. We also surveyed bacterial and fungal community diversity. We found that the relationship between ecosystem function and bacterial diversity and the relationship between ecosystem function and fungal diversity was unaffected by warming or warming duration. Ecosystem function, however, was significantly affected by season, with autumn samples having higher function than summer samples. Our findings further emphasize that season is a consistent driver of ecosystem function and that this is maintained even under simulated climate change.

Importance

Soils perform a variety of ecosystem functions, and soil microbial communities with higher diversity tend to promote the performance of these functions. Yet, biodiversity loss due to climate change threatens this relationship. Long-term global change studies provide the opportunity to examine the trajectory of the effects of climate change. Here, we utilized two long-term warming experiments, where soils have been heated +5 °C above ambient temperature for 13-28 years, to understand how increased temperatures affect the relationship between ecosystem function and soil microbial diversity. We observed that the effects of increased temperature on the relationship between bacterial diversity and ecosystem function were season-specific. This work emphasizes the role that environmental conditions, such as season, have on modulating effects of climate change. Additionally, these findings demonstrate the value of long-term ecological research in furthering our understanding of climate change.

2 Introduction

Climate change driven losses in biodiversity threaten the relationship between diversity and ecosystem function in soils (Isbell et al. 2023). Generally, biodiversity promotes higher ecosystem productivity (Tilman, Reich, and Isbell 2012) and more stable ecosystem function rates (Naeem and Li 1997; Tilman, Reich, and Knops 2006; Yachi and Loreau 1999), through mechanisms such as complementarity (Cardinale et al. 2007; Loreau and De Mazancourt 2013). This relationship has been well-validated in plant diversity-ecosystem

function studies (Tilman 1996; van der Plas 2019). Recent field studies (Delgado-Baquerizo et al. 2016a; Wagg et al. 2014) and laboratory incubations (Delgado-Baquerizo et al. 2020; Osburn et al. 2023; Wagg et al. 2019) have documented a similar relationship between microbial diversity and ecosystem function, although the strength of this relationship varies from study to study (van der Plas 2019). While there is evidence that microbial diversity promotes ecosystem function, less is known about how increased temperatures will affect this relationship. The studies that have examined the effects of warming have revealed decreases in both diversity and ecosystem function (Wu et al. 2022), a weaker relationship between diversity and ecosystem function (Hong et al. 2021), or even abiotic modulators of the diversity-ecosystem function relationship (Domeignoz-Horta et al. 2020). Increased temperatures also alter interactions between taxa in microbial communities (Anthony et al. 2021; Van Nuland et al. 2020), which might explain why at higher temperatures more diverse microbial communities are required to maintain ecosystem functions (García et al. 2018).

To assess overall ecosystem functioning, an ecosystem multifunctionality (EMF) index can be calculated by aggregating ecosystem function or property measurements (Byrnes et al. 2014; Hector and Bagchi 2007). Compared to a single fucntion approach, these aggregated indices are more sensitive to biodiversity changes (Gamfeldt, Hillebrand, and Jonsson 2008) and can better represent the inherent tradeoffs (Gamfeldt et al. 2013; Raudsepp-Hearne, Peterson, and Bennett 2010). While there are fewer studies investigating how increased temperature will impact EMF, the effects of increased temperature on individual ecosystem functions or properties are more widely studied. Ecosystem functions that are sensitive to kinetic effects, and therefore directly influenced by increased temperatures, such as enzyme activity or soil respiration, tend to be positively affected by warming (Hicks Pries et al. 2017; Melillo et al. 2011; Meng et al. 2020; Pold et al. 2017; Romero-Olivares, Allison, and Treseder 2017). Other ecosystem properties which are indirectly affected by increased temperatures, like microbial biomass or total soil carbon, tend to be negatively impacted by warming (Frey et al. 2008; Pold et al. 2017; Verbrigghe et al. 2022; Domeignoz-Horta et al. 2022). Overall when aggregated into a (multi)functionality index and compared across studies, warming tends to have positive effects on EMF (Zhou, Wang, and Luo 2020). This is perhaps due to the larger effect size that warming has on ecosystem functions that are sensitive to kinetic effects (García-Palacios et al. 2015), increased enzyme activity in substrate limited warmed soils (Pold et al. 2017), or changes to microbial growth/carbon use efficiency that results in higher respiration relative to biomass accumulation (Li et al. 2019).

Soil warming does not seem to have consistent effects on microbial diversity across different types of soils. At the temperate forest site sampled in this study, increases in bacterial diversity have been observed in plots heated +5 °C above ambient relative to control plots, due to changes in community evenness (DeAngelis et al. 2015). Studies in other forest ecosystems have similarly noted increases in bacterial diversity (Wang

et al. 2019a), and meta-analyses have further supported this observation (Yang et al. 2021; Zhou, Wang, and Luo 2020). However, warming does not necessarily have positive effects on microbial diversity in all soils, and the effects of warming on diversity may be ecosystem and organism dependent. Studies in tropical forests and grasslands, for example, have found that warming negatively impacted bacterial diversity (Nottingham et al. 2022; Wu et al. 2022). Additionally, compared to bacterial diversity, fungal diversity tends to be more sensitive to warming, irrespective of biome (Anthony et al. 2021; Frey et al. 2008; Nottingham et al. 2022; Wu et al. 2022). Previous work at the temperate forest site sampled in this study observed a decline in fungal diversity due to the fungal community becoming more uneven, with a single ectomycorrhizal taxon coming to dominate (Anthony et al. 2021). Overall, warming produces shifts in the microbial community diversity and/or composition, but bacterial diversity appears to be more positively affected by warming than fungal diversity.

While EMF typically positively correlates with microbial diversity, long-term warming has uncertain impacts on both EMF and microbial diversity. Further complicating predictions, the response of ecosystem functions to warming is also season-dependent (Contosta, Frey, and Cooper 2011; Domeignoz-Horta et al. 2022; Pold et al. 2017). Ultimately, this calls into question whether the positive relationship between EMF and microbial diversity will be altered by global warming. Long-term global change experiments, like those at Harvard Forest Long-Term Ecological Research (LTER) site, which was sampled for this study, provide a unique opportunity to examine long-term warming in a realistic field setting. Here, we examined how 13 or 28 years of long-term soil warming affects ecosystem multifunctionality and microbial diversity in two different seasons. We hypothesized that warming would increase microbial diversity, thereby promoting higher ecosystem multifunctionality.

3 Results

3.1 Fungal diversity

For overall fungal Chao1 estimated richness, warmed plots had 30% lower estimated richness than the control plots (negative binomial GLM, $p = 0.0015$, Table 1), and fall had a significant 27% decrease in Chao1 estimated richness compared with summer (negative binomial GLM, $p = 0.0035$). There was a significant interaction between heating treatment and season, resulting in heated soils in fall having 80% higher Chao1 estimated richness compared to control soils in fall (negative binomial GLM, $p = 0.0003$).

3.2 Bacterial diversity

Bacterial diversity responded differentially across warming duration. The organic horizon at the younger site had a 12% increase in Chao1 estimated richness (negative binomial GLM, $p = 0.0190$, Table 2) compared to the organic horizon at the older site. We observed a minor interaction between warming and season in the organic horizon Shannon diversity (Table 2). There was no warming treatment effect in summer, but in fall, the heated plots had slightly higher Shannon diversity compared to the control plots (0.2466 ± 0.1120, $F_{1,28} = 4.8390$, $p = 0.0725$). We did not see a similar season dependent effect of warming in the mineral horizon. Indeed, neither season nor warming treatment affected the mineral horizon Shannon diversity.

3.3 Ecosystem multifunctionality

Ecosystem multifunctionality was influenced more by season than by warming treatment or duration. Due to known differences between the organic horizon and mineral soil (Pold et al. 2017), ecosystem multifunctionality was calculated for the horizons separately. In the organic horizon, EMF was significantly higher in fall compared to summer (0.0886 ± 0.0645, $F_{1,35} = 7.252$, $p = 0.0108$, Figure 1A). EMF in either the 13 year warmed plots or the 28 year warmed plots was not different from control plots.

In the mineral soil, EMF was somewhat higher in fall compared to summer (0.0668 ± 0.1839, $F_{1,36} = 4.0764$, $p = 0.0526$, Figure 1B). Similar to what was observed in the organic horizon, neither the plots warmed for 13 years nor the plots warmed for 28 years had an EMF index that was significantly different from the control EMF. We did not observe decreasing EMF with longer duration of heating. Rather the 28 year warmed plots had slightly higher EMF than the 13 year warmed plots, although the difference ultimately was not significant.

3.4 Ecosystem multifunctionality-microbial diversity relationship

Ecosystem multifunctionality did not have a significant relationship with fungal Chao1 estimated richness ($p = 0.8414$) (Figure 1A). While the intercept of the relationship between Chao1 and EMF did decrease with prolonged warming duration, the warmed treatment intercepts were not significantly different from the control treatment intercept ($p = 0.7224$).

Season had a significant effect on the EMF-bacterial Chao1 estimated richness relationship in the organic horizon, where fall had significantly higher intercept for EMF than summer (0.1081 ± 0.0741, $F_{1,31} = 8.1710$, $p = 0.0203$, Figure 2B). However, ecosystem multifunctionality did not have a relationship with bacterial Chao1 estimated richness in either the organic or mineral horizon. The relationship between EMF and Chao1 estimated richness had a progressively lower intercept as warming duration increased. However, neither the

13-year warmed nor the 28-year warmed plots showed a relationship between EMF and Chao1 estimated richness that was significantly different from the control plots ($p = 0.4550$, $p = 0.2576$, respectively).

In the mineral horizon, Chao1 estimated richness was not correlated with EMF ($p = 0.9463$), and the intercept for the relationship between EMF and Chao1 estimated richness did not change as warming duration increased.

4 Discussion

In this study, we observed no significant relationship between bacterial diversity and EMF or between fungal diversity and EMF, irrespective of season, soil horizon, warming treatment, or warming duration. Typically, ecosystem multifunctionality positively correlates with both fungal and bacterial diversity (Chen et al. 2020; Delgado-Baquerizo et al. 2016b; Delgado-Baquerizo et al. 2020; Jing et al. 2015; Luo et al. 2018; Mori et al. 2016; Wagg et al. 2019), but neutral or marginally positive relationships are not uncommon (Hagan, Vanschoenwinkel, and Gamfeldt 2021; van der Plas 2019). In fact, abiotic or environmental factors, such as soil moisture or pH, are consistently identified as stronger drivers of EMF than diversity itself (Delgado-Baquerizo et al. 2016a; Delgado-Baquerizo et al. 2020; Jing et al. 2015; van der Plas 2019). Additionally, active microbial diversity (Bastida et al. 2016) or functional trait diversity (Hillebrand and Matthiessen 2009; van der Plas 2019; Steudel et al. 2016; Trivedi et al. 2019) may be a better predictor of ecosystem multifunctionality than total or taxonomic microbial diversity. This may explain why we did not see a relationship between microbial diversity and EMF. Our DNA-based sequencing approach captured total taxonomic diversity, which comprises both the active and inactive community, and did not provide any functional trait information. Moreover, in soils many bacteria are dormant, with minimal metabolic activity (Papp et al. 2018) and these dormant may not contribute to EMF. Altogether, this suggests that when microbial diversity positively correlates with ecosystem function, this correlation tends to be weak, mainly driven by active members of the community or specific functional groups, and ultimately can be better explained by abiotic variables.

We observed that ecosystem multifunctionality was not altered by heating, contrary to our hypothesis. Changes in microbial community functions could be underlying the shift in ecosystem multifunctionality. Previous work at the Harvard Forest has noted changes in microbial community functions under warming. Higher levels of CAZymes involved in degrading complex carbohydrates (Roy Chowdhury et al. 2021) and an expanded number of bacteria able to degrade lignin (Pold, Melillo, and DeAngelis 2015) point to altered bacterial community functions in the heated plots. This shift in metabolism towards decomposing more recalcitrant soil organic carbon (SOC) seems to be a common occurrence under soil warming (Cheng et al.

2017). Additional studies at the Harvard Forest also found that fungal hydrolytic gene abundance is higher in response to chronic soil warming and negatively correlated with total soil carbon (Anthony et al. 2021) suggesting that fungi may have increased decomposition capacity under warming. Together, all of these studies point to changes in the underlying microbial functions, without associated changes in the microbial diversity.

Season is a critical driver of ecosystem function, especially in deciduous forests. We observed that ecosystem multifunctionality was significantly higher in fall compared to summer. Indeed, season had a larger effect on EMF than warming treatment, which had no effect on EMF. The seasonal input of fresh litter could explain the difference in EMF between fall and summer. With leaf-fall, a priming effect could be initiated, resulting in higher microbial activity (Lyu et al. 2018; Zhang et al. 2020; Domeignoz-Horta et al. 2022). This priming could be substantial enough to mitigate the negative impact of warming on EMF. Further supporting a priming effect, we observed a significant increase in microbial biomass in fall, and this increase has also been noted following the addition of fresh carbon in priming experiments (Jilling et al. 2021; Lyu et al. 2019; Zhou et al. 2021). In the heated plots at Harvard Forest, SOM is more depleted in simple sugars and is lower in quantity compared to SOM in the control plots (VandenEnden et al. 2021), and the influx of litter in the fall might help alleviate this resource limitation (Domeignoz-Horta et al. 2022). Accordingly, seasonal influx of nutrients cancels out the warming-induced substrate limitation which can be observed by the response of the microbial biomass carbon (Domeignoz-Horta et al. 2022). This could subsequently increase enzyme production and activity and microbial biomass, both of which were included in our EMF index.

An additional contributor to the higher EMF observed in fall could be a two-day rainfall event that occurred two days prior to the fall sampling (Boose and Gould 2022). This rainfall could have initiated a Birch effect, which is an increase in respiration that occurs after rewetting a dried soil (Birch 1958). Furthermore, the rainfall could have resulted in a flush of dissolved organic matter. Drying-rewetting cycles can increase microbial biomass (Dong et al. 2021; Evans and Wallenstein 2012; Fierer and Schimel 2003), which is in line with the significantly higher microbial biomass that we observed in fall. Warmed soils have significantly lower soil moisture compared to the control plots (Contosta, Frey, and Cooper 2011), and the combination of warming and lower moisture can further increase CO_2 efflux after rewetting (Schindlbacher et al. 2012). Yet, we did not observe a significant increase in respiration in fall (Domeignoz-Horta et al. 2022), as would be expected with a Birch effect. This could be due to the fact that respiration was measured after the soil was taken back to the lab, up to six days after the rainfall. This falls outside of the typical 1.5 days when the respiration spike that is characteristic of the Birch effect is typically observed (Canarini, Kiær, and Dijkstra 2017). Enzyme activity can also increase after rewetting (Ouyang and Li 2020). While

we did observe increased phenol oxidase activity in the fall, we did not observe similar increases in activity for β-glucosidase and N-acetyl glucosaminidase (Domeignoz-Horta et al. 2022). The higher EMF observed in fall is likely partially due to the rewetting, but the ecosystem function responses we observed are not entirely consistent with a rewetting response.

Similar to EMF, the effects of warming on fungal communities are season dependent. We found that fungal diversity was positively affected by warming, but only in fall (Table 1). Fungi appear to be more sensitive overall to warming compared to bacteria (Anthony et al. 2021; Frey et al. 2008; Zhou et al. 2022), and particularly in deciduous forests, fungal diversity is responsive to season (He et al. 2017; Xie and Yin 2022). In deciduous forests, like Harvard Forest, the addition of the fresh leaf litter in the fall could spur an increase in fungal diversity, since fungi are the primary decomposers in these ecosystems. At Harvard Forest, the relative abundance of saprotrophic fungi is positively correlated with lignin abundance (Pec et al. 2021). If the fresh input of leaf litter in fall is initiating a priming effect, then that could also coincide with an increase in fungal diversity (Zhou et al. 2021). While the issues with sample prep and sequencing reduced our sample size and limited the analysis to only the organic horizon, previous work at the site found fungal abundance was highest in the organic horizon and not the mineral horizon (DeAngelis et al. 2015).

Generally, bacterial diversity is less sensitive to warming compared to fungal diversity. Studies at other sites found that experimental warming does not appear to result in decreases in bacterial community richness (Wang et al. 2019b; Yu, Han, and Fu 2019; Zhou, Wang, and Luo 2020; Zi et al. 2018). Seemingly, a more widespread trend is warming driven shifts of community evenness (Anthony et al. 2021; Deslippe et al. 2012; Nottingham et al. 2022), due to either losses of rare taxa or decreases in dominant taxa. We observed experiment-dependent differences in bacterial diversity, but no differences based on warming treatment (Table 2). Prior studies at the Harvard Forest also revealed increased bacterial diversity in the heated plots, driven by increased evenness (DeAngelis et al. 2015). Additionally, the effects of warming on soil ecosystems are nonlinear over decadal time scales at Harvard Forest (Melillo et al. 2017), where there are years when there is a large treatment effect of heating compared to controls and years where there is no differences between heated and control plots. Soils for DeAngelis et al. 2015 were sampled in a phase of larger fluxes of soil C lost as CO_2 from heated plots relative to the control plots. In this current study, we sampled during a phase when there was no difference in soil CO_2 flux between the heated and control (Melillo et al. 2017). The nonlinear nature of warming effects over three decades might also apply to the microbial community responses to warming, but this phenomenon requires further study.

This study investigated if long-term soil warming influenced the relationship between ecosystem multi-functionality and bacterial and fungal diversity. We found that warming treatment or warming duration had no effect on the relationship between EMF and fungal diversity and EMF and bacterial diversity. Indeed, we

found that EMF had no relationship with bacterial or fungal diversity, which is not uncommon in natural or non-laboratory systems. We also observed increases in fungal richness in the heated plots in fall, as well as increased bacterial richness in the organic horizon at the younger experimental site. Overall, we found that season exerts a strong influence on ecosystem functionality in temperate deciduous forests and that cross-season sampling is needed to capture the variability in EMF.

5 Methods

5.1 Site description and sample collection

Samples were collected as described in Domeignoz-Horta et al. 2022 from two long-term warming soil experiments located at the Harvard Forest in Petersham, MA 42°30′30″N, 72°12′28″W). The Prospect Hill Soil Warming Study was established in 1991 (Melillo et al. 2002), and the Soil Warming x Nitrogen Addition (SWaN) Study was established in 2006 (Contosta, Frey, and Cooper 2011). Warmed plots are heated continuously +5 °C above ambient using buried resistance cables placed 10 cm below the soil surface and spaced 20 cm apart. Soil samples were taken on July 15th and October 19th, 2019 from Prospect Hill and SWaN, which had been warmed for 28 and 13 years, respectively, at the time of sampling. Both experiments are located adjacent to one another and have the same dominant plant overstory (*Acer rubrum, Betula lenta, Betula papyrifera, Fagus grandifolia, Quercus velutina, Quercus rubra*) and soil type (coarse-loamy incepitsols). Duplicate 10 cm cores were taken from each plot using a tulip bulb corer. Cores were separated into the organic and mineral horizon, duplicate cores were pooled, roots and rocks were removed, and then the soil was sieved <2 mm. After sieving, samples were taken back to the lab for further analyses. The full sampling design was 2 sites (Prospect Hill, SWaN) x 2 treatments (control, heated) x 2 seasons (summer, fall) x 2 sampling depths (organic horizon, mineral soil) x 5 replicate plots for a total of 79 samples (one sample from the SWaN experiment had a mineral horizon that was beyond the reach of the corer for the July sampling).

5.2 Ecosystem functionality measurements and multifunctionality calculation

To assess ecosystem functionality, we measured seven different soil properties or ecosystem functions: total soil carbon, total nitrogen, microbial biomass carbon, respiration, and the potential activites of four enzymes: phenol oxidase, peroxidase, β-glucosidase (BG), and N-acetyl-glucosaminidase (NAG).

To measure total C and N, soils were dried in a 65 °C oven until they reached a constant mass and then ground using a mortar and pestle. The soil was then weighed and packaged in duplicate into tins, which

were run on a Perkin Elmer 2400 Series II CHN Elemental Analyzer with acetanilide as a standard at the University of New Hampshire Water Quality Analysis Lab.

For microbial biomass carbon measurements, four replicate soil samples were each split into two subsamples, one group which served as a control and one group which was fumigated with chloroform vapors under vacuum pressure for 24 hours. Dissolved organic carbon (DOC) was then extracted from both the unfumigated and fumigated samples using 15 mls of 0.05 M K_2SO_4 and quantified on a Shimadzu TOC analyzer. Microbial biomass carbon was determined by subtracting the DOC concentration in the unfumigated subsample from the fumigated subsample.

Soil respiration was measured on triplicate subsamples (0.15 or 0.3 g for the organic horizon or mineral soil, respectively) that were placed into Hungate tubes. Tubes were then sealed and incubated at 15 °C for 24 hours. A 30 ml headspace sample was then taken and injected into an infrared gas analyzer (Quantek 906) to measure CO_2.

Potential extracellular enzyme activity was measured using fluorescent substrates. Soil slurries were prepared with 1.25 g wet weight soil and 175 mls of 50 mM pH 4.7 sodium acetate in a Waring blender. For the BG and NAG assays, 200 µls of soil slurry was pipetted into black 96 well plates, and for the oxidative enzyme assay, 500 µls of soil slurry was pipetted into deep well plates. Plates were then placed in a 15 °C incubator for 25 minutes to allow for temperature acclimation. This temperature reflects the average air temperature between the summer sampling and the fall sampling. After temperature acclimation, either 50 µls of 4000 µM 4-methylumbelliferyl β-D-glucopyranoside, 50 µls of 2000 µM 4-methylumbellifery N-acetyl-glucosaminidase, or 500 µls of 25 mM L-DOPA + 0.03% H_2O_2 were added to each well. Each plate contained a standard curve as well as a slurry-only control. All plates were read on a SpectraMax M2 plate reader. BG and NAG plates were measured at 360/450 nm excitation/emission wavelengths after substrate addition. Oxidative enzyme plates were incubated for 4 hours after substrate addition, and then 100 µls were removed, transferred to a clear 96 well plate, and read at 460 nm.

Multifunctionality was calculated for the organic horizons and mineral soil using the multifunc package in R (Byrnes et al. 2014). We included microbial biomass carbon, respiration, total carbon, total nitrogen, N-acetyl glucosaminidase activity, β-glucosidase activity, and oxidative enzyme activity in the ecosystem multifunctionality index. These functions were included since they represent both process rates (respiration, potential enzyme activity) and nutrient pools (total C, total N, microbial biomass) (Garland et al. 2021). The soil layers were analyzed separately due to documented differences in soil parameters (Pold et al. 2017). Prior to multifunctionality calculations, measured parameters were z-score transformed. A multifunctionality index was calculated for each sample by taking the average of the z-score transformed ecosystem functions. While there are different ways of assessing diversity-multifunctionality relationships, we selected the averaging

approach for ease of interpretation. Individual ecosystem functions' relationship with diversity was also investigated. For this approach, the raw untransformed function measurements were used.

5.3 DNA extraction and library preparation

Bacterial and fungal diversity were measured using 16S rRNA gene or ITS2 region amplicon sequencing. DNA was extracted from soils using the Qiagen Powersoil kit following the manufacturer's protocol, and DNA concentration was measured using the Picogreen dsDNA kit (ThermoFisher Scientific).

We sequenced both the bacterial and the fungal community. The fungal ITS gene was sequenced using the primers 5.8S-Fun (5' - AAC TTT YRR CAA YGG ATC WCT - 3') and ITS4-Fun (5'- AGC CTC CGC TTA TTG ATA TGC TTA ART- 3') (Taylor et al. 2016). Samples were amplified in triplicate prior to barcoding using a 25 µl PCR reaction containing 0.25 µl Invitrogen recombinant Taq, 1.25 µl of 10 µM forward primer, 1.25 µl of 10 µM reverse primer, 2.5 µl 10X Taq buffer, 0.75 µl 50 µM $MgCl_2$, 0.5 µl 10 µM dNTP mix, 17.5 µl molecular grade H_2O, and between 1-5 ng DNA. Samples were run on an eppendorf Mastercycler Pro thermocycler with the program parameters of 96 °C for 3 min, 27 cycles of 94 °C for 30 s, 58 °C for 40 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. After verification of successful PCR amplification using gel electrophoresis, samples were purified using AMPure XP beads (Beckman Coulter) and barcoded using the IDT xGen DNA MC Library Prep kit and IDT UDI primers. Barcodes were added using the following thermocycler parameters of 98 °C for 2 min, 9 cycles of 98 °C fo 20 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 1 min. Barcoded samples were quantified using the Qubit dsDNA kit (ThermoFisher Scientific), normalized to a concentration of 7 nM, and then equal volumes of each sample were pooled. Samples were spiked with PhiX prior to sequencing at the UMass Genomics Facility on an Illumina MiSeq with 2x300 bp paired end reads.

For the bacterial sequencing, we sequenced the 16S rRNA gene V4 region using the primers 515F (5' - GTG YCA GCM GCC GCG GTA A- 3') and 806R (5' - GGA CTA CNV GGG TWT CTA AT - 3') following the Earth Microbiome Project protocol (Caporaso et al. 2012), with minimal modifications. Forward primers contained the 5' Illumina adapter, 12 basepair unique Golay barcode, forward primer pad and linker, and 515F. Reverse primers contained the 3' reverse complement Illumina adapter, reverse primer pad and linker, and 806R. Templates were amplified in duplicate prior to sequencing using a 25 µl reaction containing 10 µl 2X Invitrogen Platinum Hot Start master mix, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 13 µl molecular grade H_2O, and 1 µl template. Samples were amplified in an eppendorf Mastercycler Pro thermocycler with the program parameters of 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. Replicated amplicons were combined and visualized

on a 1% agarose gel. We quantified the amplicons with the PicoGreen Assay for dsDNA (ThermoScientific) and pooled 300 ng of each sample. We used AMPure XP magnetic beads (Beckman Coulter) to clean the pooled library. Samples were spiked with PhiX prior to sequencing at the UMass Genomics Facility on an Illumina MiSeq (Reagent Kit V2) with 2x150 bp paired end reads. For both the bacteria and the fungi, no template controls were sequenced as well.

5.4 Sequencing processing and analysis

Raw FastQ files were demultiplexed, and primers were trimmed using cutadapt (Martin 2011). Both bacterial 16S rRNA gene sequences and fungal ITS2 sequences were processed with the DADA2 pipeline (v.1.18.0) (Callahan et al. 2016). Due to the variable length of the ITS gene, fungal reads were not trimmed to a specific length, but were filtered for a minimum length of 200 bp. This minimum length is below the reported shortest amplicon length in Taylor et al. 2016. Bacterial forward reads were trimmed to 150 bp, and reverse reads were trimmed to 140 bp. For both bacteria and fungi, reads with expected error higher than 2 were discarded, and all PhiX sequences were also removed. Taxonomy for fungi was assigned using UNITE (v 8.3) (Nilsson et al. 2019), and taxonomy for bacteria was assigned using SILVA (v 138.1) (Quast et al. 2012). All mitochondrial and chloroplast sequences were removed from the final bacterial ASV table. To account for differences in sequencing depth between samples, raw ASV counts were normalized with library size estimation factors calculated in DESeq2 (Love, Huber, and Anders 2014; McMurdie and Holmes 2014).

Since a variety of diversity metrics have been correlated with EMF, Simpson diversity, Shannon's H, Chao1 estimated richness, community structure (PCoA axes), and evenness (Pielou's J) were calculated for bacteria and fungi. Fungal and bacterial diversity metrics were calculated using the packages vegan (Oksanen et al. 2007) and phyloseq (McMurdie and Holmes 2013). We found that the different fungal diversity metrics and the different bacterial diversity metrics performed similarly based on their ΔAICc, so we elected to report the Shannon's H and Chao1 estimated richness metrics. Fungal ecological guilds were also assigned using FUNGuild (Nguyen et al. 2016), and only assignments classified as "highly probable" or "probable" were used.

We sequenced the ITS gene to assess fungal diversity. We started with 80 samples, but due to sample preparation and sequencing issues, we ended up with 23 samples, all from the organic horizon. Following sequence processing, we had 536,580 total reads, with an average read count of 23,368 per sample, and 2652 total ASVs. We elected to apply a sample specific library size estimate correction factor that was calculated in DESeq2 (Love, Huber, and Anders 2014) to account for differences in sequencing depth between samples

(McMurdie and Holmes 2014). After applying the correction factor, the total read count was 555,419, with an average read count of 24,149 per sample. Additionally, we did not combine the control plots from the 13 year warmed experiment and the 28 year warmed experiment because their Chao1 estimated richnesses were significantly different ($p = 0.02621$).

We sequenced the 16S rRNA gene to assess bacterial diversity. Our final data set comprised 69 out of 80 samples. At the end of sequence processing, this data set had 4,492,339 reads, with an average depth of 62,394 reads per sample, and 8,754 total ASVs. After correcting with a sample specific library size estimate calculated in DESeq2 (Love, Huber, and Anders 2014), the total read count was 4,159,476, with an average read count of 60,282 per sample. The control plots from the two warming experiments (13 year warmed and 28 year warmed) had significantly different Chao1 estimated richness ($p = 0.0260$) so they were not combined for the analysis.

Rarefaction analyses for both the fungi and the bacteria indicated that for the samples that were successfully sequenced, the sequencing efforts were adequate. For both the fungal and bacterial sequencing, both of the negative controls had fewer than 500 reads each.

5.5 Statistical analysis

5.5.1 Model construction and model selection

We examined the effect of warming, soil horizon, season, and site on EMF, fungal diversity, and bacterial diversity. We evaluated whether we could combine warming treatment and site into one variable, where both Prospect Hill and SWaN plot control samples were combined into a single "control", Prospect Hill warmed samples were "28 year warmed", and SWaN warmed samples were "13 year warmed". This was done after checking for significant differences between the control plot samples from Prospect Hill and SWaN using either a Wilcoxon rank sum test or a t-test. We found no significant differences between the control plots' EMF in Prospect Hill and SWaN. However, we did find significant differences in fungal and bacterial diversity between the control plots from the two experiments. As a result, the control plots were combined for the EMF analyses, but not for the bacterial or fungal diversity analyses.

We then investigated how warming treatment, warming duration, soil horizon, and season affected the relationship between EMF and bacterial or fungal diversity. For all of these analyses, we constructed a set of candidate generalized linear models. In these sets of candidate models, we considered all predictors and possible interactions between warming and diversity, warming and horizon, warming and season, or warming and warming duration. All model residuals, except for models where Chao1 estimated richness was the response variable, were checked for normality, and if necessary a log transformation was applied to

the response variable. All GLMs used a Gaussian distribution, except for the models with Chao1 estimated richness as the response variable, which used either a Poisson distribution or negative binomial distribution. Poisson models for Chao1 estimated richness were checked for overdispersion, and if overdispersion was observed, a negative binomial distribution was used with the R package MASS (Venables and Ripley 2002). Model fit was assessed using sample size corrected Akaike Information Criterion (AICc) in the package (Mazerolle 2020). If models were within 2 ΔAICc units, we considered the models equally supported, and used a likelihood ratio test for the nested models to select a model. If the models with similar ΔAICc were not nested, we selected the model that explained more variance in the data. Results from the models are reported in percent change if the model used a log-link function. For the mineral horizon, any model where EMF was the response variable had a log-transformation applied to EMF to make the residuals normally distributed. The results from those models are reported without back-transformation. To account for multiple comparisons, a Tukey HSD test was utilized or p-values had a Benjamini-Hochberg correction applied to them. All estimates are reported with a 95% confidence interval.

6 Data availability

The raw fungal sequencing data and primer-trimmed bacterial sequencing data are available at the NCBI Sequence Read Archive under the BioProject accession number PRJNA957454 and PRJNA957454 for the fungi and bacteria, respectively.

Ecosystem function data is available in the Harvard Forest Data Archive under the Dataset ID HF431.

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Melissa S. Shinfuku and Kristen M. DeAngelis conceptualized the study. Melissa S. Shinfuku, Luiz A. Domeignoz-Horta, and Mallory J. Choudoir conducted experiments and collected the data. Serita D. Frey maintains the long-term soil warming experiments at Harvard Forest, facilitated sample collection, and

provided input on data interpretation. Melissa S. Shinfuku analyzed the data and wrote the first draft of the manuscript. All authors contributed to editing and revising the manuscript.

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Table 1: Summary of fungal diversity in the organic horizon. Chao1 richness used a negative binomial generalized linear model. Shannon diversity model used a linear model.

Organic Horizon					
		Estimate	SE	z-value	p-value
Chao1 richness	Warming	-0.3511	0.1077	-3.261	0.0015
	Site	-0.3133	0.1075	-2.916	0.0035
	Warming:Season	0.5873	0.1548	3.793	0.0003
		Estimate	SE	t-value	p-value
Shannon diversity	Warming	0.0557	0.1709	0.326	0.9246
	Season	-0.2355	0.1709	-1.378	0.2040

Table 2: Summary of bacterial diversity in the organic and mineral horizon. Chao1 richness used a negative binomial generalized linear model. Shannon diversity model used a linear model.

Organic Horizon					
		Estimate	SE	z-value	p-value
Chao1 richness	Warming	0.0170	0.440	0.869	0.3851
	Site	0.1097	0.0440	2.4920	0.0190
		Estimate	SE	t-value	p-value
Shannon diversity	Warming	-0.1139	0.0766	-1.7490	0.1217
	Season	-0.0759	0.0802	-0.9470	0.3520
	Warming:Season	0.2466	0.1121	2.2000	0.0725
Mineral Horizon					
		Estimate	SE	z-value	p-value
Chao1 richness	Warming	0.0170	0.0623	0.273	0.7845
	Site	0.04657	0.0623	0.748	0.6816
		Estimate	SE	t-value	p-value
Shannon diversity	Warming	0.0008	0.0766	0.011	0.9915
	Season	0.0114	0.0766	0.149	0.9915

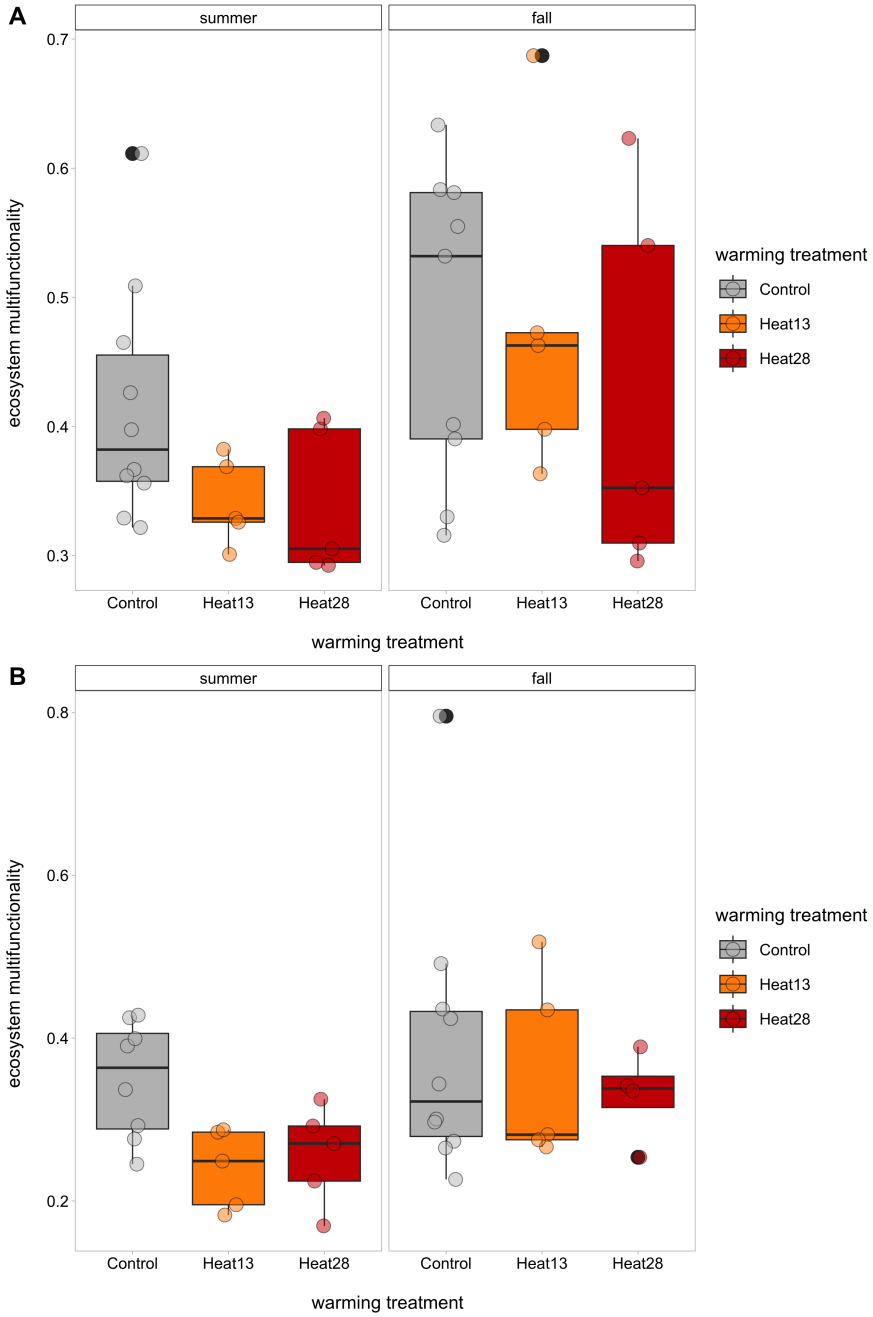

Figure 1: EMF by soil horizon and season Ecosystem multifunctionality (EMF) in the organic horizon (A) and the mineral horizon (B). EMF was calculated by averaging z-score standardized function measurements for each sample. In both horizons, there was a seasonal trend where EMF was higher in the fall, but this was only significant in the organic horizon (A) (0.0886 ± 0.0645, $F_{1,35} = 7.252$, $p = 0.0108$). In the organic horizon (A), as warming duration increased, EMF tended to decrease, but this was not significant. A similar trend was not observed in the mineral horizon (B).

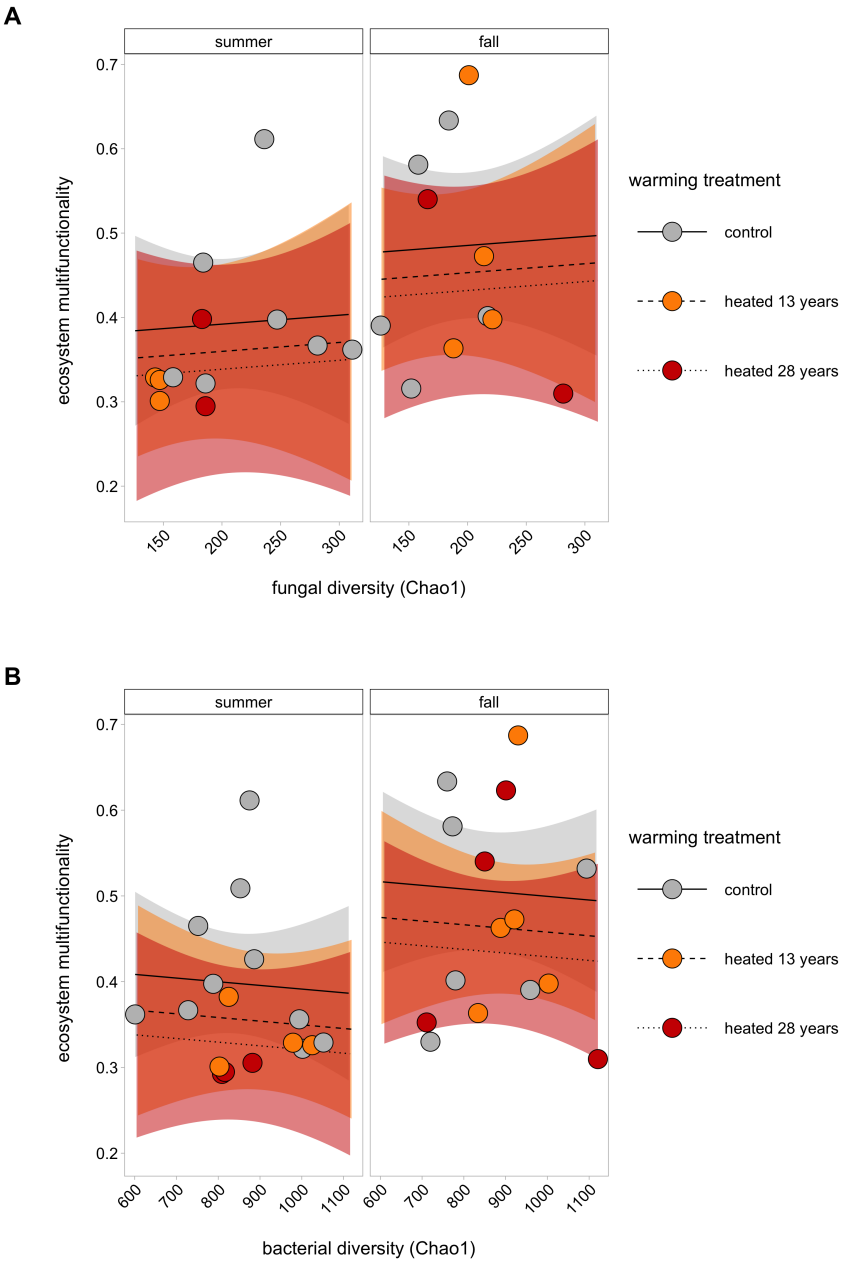

Figure 2: EMF and microbial diversity relationship The relationship between ecosystem multifunctionality (EMF) and fungal Chao1 richness (A) and EMF and bacterial Chao1 richness (B) in the organic horizon. EMF was calculated by averaging z-score standardized function measurements for each sample. Shaded regions represent a 95% confidence interval. There was no significant difference between the heated 13 years, heated 28 years, and control plots for either of the relationships. There was also no significant relationship between bacterial diversity (B) and EMF or fungal diversity and EMF (A). For EMF and bacterial Chao1 richness (B), there was a significant effect of season, where fall had an overall higher baseline EMF than summer (0.1081 ± 0.0741, $F_{1,31} = 8.1710$, $p = 0.0203$).