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Belowground Impacts of Alpine Woody Encroachment are determined by Plant Traits, Local Climate and Soil Conditions

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Running Title: *Alpine Woody Encroachment Impacts Soil Microbes*

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Abstract

Global climate and land use change are causing woody plant encroachment in arctic, alpine, and arid/semiarid ecosystems around the world, yet our understanding of the belowground impacts of this phenomenon is limited. We conducted a globally distributed field study of 13 alpine sites across 4

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continents undergoing woody plant encroachment and sampled soils from both woody encroached and nearby herbaceous plant community types. We found that woody plant encroachment influenced soil microbial richness and community composition across sites based on multiple factors including woody plant traits, site level climate, and abiotic soil conditions. In particular, root symbiont type was a key determinant of belowground effects, as Nitrogen-fixing woody plants had higher soil fungal richness, while Ecto/Ericoid mycorrhizal species had higher soil bacterial richness and symbiont types had distinct soil microbial community composition. Woody plant leaf traits indirectly influenced soil microbes through their impact on soil abiotic conditions, primarily soil pH and C:N ratios. Finally, site level climate affected the overall magnitude and direction of woody plant influence, as soil fungal and bacterial richness were either higher or lower in woody encroached versus herbaceous soils depending on mean annual temperature and precipitation. All together, these results document global impacts of woody plant encroachment on soil microbial communities, but highlight that multiple biotic and abiotic pathways must be considered to scale up globally from site and species level patterns. Considering both the aboveground and belowground effects of woody encroachment will be critical to predict future changes in alpine ecosystem structure and function and subsequent feedbacks to the global climate system.

Keywords: Woody encroachment, plant-soil interactions, alpine, global change, soil microbes, leaf traits

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Introduction

Global climate and land use change are altering the distributions of organisms worldwide (Chen, Hill, Ohlemüller, Roy, & Thomas, 2011; Parmesan, 2006; Walther et al., 2002) and this is particularly true in arctic and alpine tundra ecosystems where warming is accelerated (Elmendorf et al., 2012; Walker et al., 2006; Wilson & Nilsson, 2009). One prevalent change in tundra ecosystems is the encroachment of woody plants (shrubs and dwarf trees) into areas previously dominated by non-woody grasses, sedges and forbs (Myers-smith & Hik, 2018; Rundqvist et al., 2011; Sturm et al., 2005). Woody plant encroachment can strongly impact aboveground productivity, the redistribution of snow by wind, and water and nutrient cycling in the tundra (Demarco, Mack, & Bret-Harte, 2014; Myers-Smith et al., 2011; Myers-Smith & Hik, 2013; Weintraub & Schimel, 2005). However, few studies have considered the biotic impacts of woody encroachment, particularly belowground effects on soil microbial communities (Myers-Smith et al., 2011). Some case studies, primarily from the Arctic, show that encroachment alters soil microbial community structure and function via woody litter inputs, leading to increased soil organic matter mineralization and soil carbon C:N ratios (Eskelinen, Stark, & Männistö, 2009; K. Rousk, Michelsen, & Rousk, 2016; Wallenstein, McMahon, & Schimel, 2007). However, we lack a general understanding of how woody encroachment affects soil microbial communities at the global scale, or whether observed impacts are species and site specific (Donhauser & Frey, 2018; Myers-Smith et al., 2011).

To fill this knowledge gap, we conducted a coordinated global study of alpine woody encroachment on soil microbial communities. We assessed a diverse set of pathways by which plants can impact soil microbes, including changes in the quality and quantity of litter inputs (J. H. C. Cornelissen et al., 2007;

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Santonja et al., 2017), alteration of soil abiotic conditions such as soil chemistry, moisture and pH (Eskelinen et al., 2009; Schimel, Bilbrough, & Welker, 2004; Yannarell, Menning, & Beck, 2014), or through interactions with rhizospheric microbes such as dinitrogen (N_2)-fixing bacteria or mycorrhizae (Bengtson, Barker, & Grayston, 2012). Due to fluctuating environmental conditions and extreme spatial heterogeneity, alpine soil microbial communities are highly specialized, and can vary greatly across vegetation types, soil properties, and microclimates (Donhauser & Frey, 2018). Also, the effects of woody plant encroachment may interact with the direct effects of climate change (e.g. soil warming or drought) on soil microbes, making net outcomes difficult to predict (Classen et al., 2015; Kardol, Cregger, Campany, & Classen, 2010). Thus understanding how woody plant encroachment directly and indirectly influences soil microbial communities is key to predicting long-term changes in the structure and function of alpine ecosystems (Hagedorn, Gavazov, & Alexander, 2019).

Direct effects of woody plant encroachment on soil microbial communities include shifts in both the quality and quantity of leaf and root litter (Wardle et al., 2004, Eldor Alvin Paul, 2007;) as well as interactions with microbial symbionts in their roots for nutrient and resource uptake (Smith & Read, 1997a; Wookey et al., 2009). A shift from primarily herbaceous (grasses, sedges, forbs) to woody plant cover generally increases the quantity and decreases the quality of litter inputs, and may result in slower decomposition of organic matter (J. H. C. Cornelissen et al., 2007). However this pattern can differ across woody plant species based on chemical and morphological litter traits such as leaf carbon: nitrogen ratio (C:N), leaf dry matter content (LDMC) and specific leaf area (SLA) (Cornwell et al., 2008; Gavazov, 2010; Urbina, Grau, Sardans, Ninot, & Peñuelas, 2020). Litter mixing between woody and herbaceous plants can increase the chemical complexity of the substrate pool, enhancing both microbial niche space and diversity (Chapman & Newman, 2010; McGuire, Zak, Edwards, Blackwood, & Upchurch, 2010). Additionally, different types of microbial symbionts engage in distinct resource use strategies, and can greatly influence the resource economy of their plant host (J. Cornelissen, Aerts, Cerabolini, Werger, & van der Heijden, 2001; Gerz, Guillermo Bueno, Ozinga, Zobel, & Moora, 2018; Smith & Read, 1997b, 1997c). For example, Ecto- and Ericoid mycorrhizal fungi (ECM, ERM) have a higher affinity for organic forms of N and phosphorus (P) than arbuscular mycorrhizal fungi (AMF) which primarily scavenge inorganic nutrients (Read, 2003; Wookey et al., 2009), while N_2-fixing bacteria directly convert elemental N_2 into plant available forms of N (van der Heijden, Bardgett, & van Straalen, 2008). Differences in leaf litter chemistry across plant symbiont types may further select for faster (Cheeke et al., 2017; M. K. Taylor, Lankau, & Wurzburger, 2016) or slower (McGuire et al., 2010) decomposition by saprotrophic soil microbes. Furthermore, root symbionts can directly interact in numerous ways with

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saprotrophic fungi and bacteria in the rhizosphere. For example, mycorrhizal fungi release organic acids, hyphal exudates and provide hyphal necromass, which can enhance bacterial growth and serve as a food source for free-living soil biota (Bending, Aspray, & Whipps, 2006; Liang, Schimel, & Jastrow, 2017). Alpine soils usually have very low organic matter, and therefore changes in the quantity and quality of litter inputs, hyphal exudates, and microbial necromass as a result of woody encroachment have the potential to create major changes in free-living soil microbial communities and belowground ecosystem functioning (Donhauser & Frey, 2018; Körner, 2003).

Woody plant encroachment can also indirectly influence soil microbes through changes in the abiotic soil environment (Collins, Carey, Aronson, Kopp, & Diez, 2016; Grau et al., 2019) and via interactions with local climate (Classen et al., 2015). Woody encroachment can alter C and nutrient cycling, water availability and pH, and can also drastically alter the spatial distribution of resources across a landscape (Eldridge et al., 2011; Myers-Smith et al., 2011). Shading under woody plant canopies retains soil moisture higher in the soil profile in addition to physical trapping of snow, that concentrates snowmelt (Gómez-Aparicio, Gómez, Zamora, & Boettinger, 2005; Sturm et al., 2005). Enhanced soil moisture and thermal insulation from snow can promote decomposition and biogeochemical cycling (Schimel et al., 2004), while leaching of organic acids from woody litter can directly influence soil pH (Jobbagyl & Jackson, 2003), which is a key driver of microbial community composition (Lauber, Hamady, Knight, & Fierer, 2009; J. Rousk et al., 2010). Overall, resource accumulation below woody plant canopies can lead to increased microbial biomass (Cable, Ogle, Tyler, Pavao-Zuckerman, & Huxman, 2009; Liao & Boutton, 2008), diversity (Hollister, Schadt, Palumbo, James Ansley, & Boutton, 2010) and shifts in community composition (Yannarell et al., 2014). In addition, impacts of woody plant encroachment may be more or less severe depending on ambient temperature and precipitation, which are changing rapidly in alpine environments (Rammig, Jonas, Zimmermann, & Rixen, 2010). Interactions between plant growth form (i.e. woody or herbaceous) and experimental shifts in air temperature, soil moisture and CO_2 influenced soil microbial enzyme production and nematode community composition (Kardol et al., 2010). Similarly, soil temperature and moisture determined whether arctic soils became net sources or sinks of CO_2 in woody but not herbaceous plant communities (Cahoon, Sullivan, Shaver, Welker, & Post, 2012). Because of these complexities, we lack a clear understanding of how specific abiotic conditions or climate patterns will influence woody plant-soil interactions. Thus, assessing woody plant encroachment across multiple sites spanning diverse climates and environmental conditions is crucial (Wookey et al., 2009).

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The objectives of this research were to determine: 1) Is there a consistent global signature of woody plant encroachment on soil microbial communities in alpine ecosystems? and 2) What are the major abiotic and biotic drivers mediating the observed changes in soil microbial communities? We conducted this study across 13 alpine sites all undergoing woody plant encroachment, spanning four continents and ten mountain ranges (Table 1). We hypothesized that woody plant encroachment will: 1) alter soil microbial diversity and microbial community composition via changes in litter quality. Such changes are likely driven by differences in leaf functional traits and their influence on soil abiotic conditions; 2) impact soil microbial communities differently depending on root symbiont types (AMF, ECM and, N_2-fixers) and associated resource use strategies; 3) influence soil microbial communities indirectly through changes in abiotic soil conditions; 4) have climate-dependent effects on soil microbial communities due to high microbial sensitivity to temperature and moisture.

Materials and Methods

Site selection

This study took place at 13 sites (Fig 1, Table 1) across North and South America, Europe and Asia. We selected sites based on the following criteria: 1) woody plant encroachment into alpine plant communities dominated by herbaceous species, was observed within the last 50 years. We confirmed that woody plants were not previously present using aerial photography, historical records, and personal knowledge or information from local groups. See citations in Table 1 for further details regarding woody encroachment at each site. 2) Sites were alpine or subalpine (close to or above treeline), not Arctic (one site in Abisko, Sweden was considered 'subarctic' alpine). 3) Sites were not actively grazed or managed for agriculture (low intensity grazing did occur at our sites on the Tibetan Plateau in China and in the Swiss Alps and pine (*Pinus mugo*) silviculture occurred historically around our site in the Czech Republic). 4) International shipping speeds allowed samples to arrive in 72 hours or less on dry ice so that soils would stay frozen (this requirement affected our choice of study sites that excluded the Southern Hemisphere, Africa, and remote parts of Asia in our study). Finally, while we use the term 'woody' to describe primarily shrubs and dwarf trees at our study sites, one site (Japan) has a dwarf bamboo species (*Sasa kurilensis*) which is technically a 'woody graminoid. ' This and other species of bamboo are common woody encroachers across Asia (Xu et al., 2020).

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Soil sampling

We sampled soils from both directly under and outside woody plant canopies (~1.5-3.0 m outside) in the herbaceous plant interspace in areas where woody shrubs and dwarf trees were newly established (not present > 50 years). Soils were sampled during the growing season in either 2017 or 2018 (depending on site). All soils were sampled using an aseptic technique and sampling protocol as described in the USEPA/USGS Sample Collection Protocol for Bacterial Pathogens in Surface Soil (EPA, 2014). We collected ten soil samples from each vegetation type (woody and herbaceous) at each site for a total of 20 samples per site (20 x 13=260 soil samples). For each soil sample, three replicate soil cores were taken at a depth of 10-15 cm, combined into one sample with all excess rocks, roots, leaves or twigs removed and placed in sterile Whirlpak bags (Uline, Pleasant Prairie, WI, USA). Sampling locations within sites (individual woody plants and paired herbaceous soils) were at least 5 m apart. Soils were frozen within 24 hrs after sampling and remained in the freezer (-20° C) until being shipped. Soils were shipped on dry ice via expedited shipping to the University of California, Riverside, USA. All soils were sampled from within the same parent material and 100 m elevation differential or less at each site.

Soil abiotic parameters

At each soil sampling location (N=10 woody + 10 herbaceous=20 per site), we measured soil volumetric water content (VWC %) and soil pH *in situ* using handheld probes (Vegetronix VG-Meter-200 basic or equivalent; EXTECH Model PH100 or equivalent). For soil chemistry, shipped soils were thawed at room temperature (half of each sample, other half remained frozen for microbial analyses) sifted through a 2mm mesh sieve and ground via mortar and pestle. Soils were then oven dried at 60 ºC for 72 hours, weighed into tin capsules and measured for total C and N on a Flash EA 112 analyzer at the University of California Riverside Environmental sciences research laboratory, U.S.A.

Leaf sampling and traits

Ten leaves were sampled from the encroaching woody species at each study site (n=10 x 13 sites= 130 leaves). Leaves were kept moist and weighed within 24 hours of sampling on a microbalance to obtain fresh weight (g). Leaves were then placed in paper envelopes and left to air dry until shipping.

We measured the following leaf functional traits for each woody plant species: leaf dry matter content LDMC (g/g), specific leaf area SLA (cm^2/g), leaf N (%), leaf C (%), δ13C, and δ15N. Leaves were scanned on a flatbed scanner to calculate leaf area (cm^2) using ImageJ software (https://imagej.nih.gov/ij/). Leaves were dried (60°C, 72 hours) and then weighed for dry weight (g). LDMC was calculated as the ratio of fresh weight (g) to dry weight (g) and SLA was calculated as leaf area

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238 (cm^2) to dry weight (g). Leaf chemical (C, N) and isotope ($\delta^{13}C$, and $\delta^{15}N$) content were measured from
239 dried leaf subsamples at the University of Wyoming Stable Isotope Facility (Laramie, WY, USA.).

240 *Soil microbial analyses*

241 We extracted microbial DNA from 0.25 g of soil (±0.025 g) of each sample using a Qiagen DNeasy
242 PowerSoil Kit (Qiagen Inc., Germantown, MD, USA) and quantified the extracted DNA using a NanoDrop
243 2000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). After quantification, we standardized DNA
244 extracts to 10 ng/µL. We performed PCR amplification using the 515F/806R primer set targeting V4
245 region of the 16S rRNA gene for bacteria (Caporaso et al., 2011) and the 5.8S-Fun/ITS4-Fun primer set
246 targeting the ITS-2 region for Fungi (D. L. Taylor et al., 2016). PCR was run in 25 µl reactions including
247 1.25 µl of 1 µM for each primer (forward and reverse), 1 µl DNA template, 12.5 µl of Phusion Green Hot
248 Start 2X Master Mix (Thermo Fisher Scientific Inc., USA), 1.5 µl of 3 µM $MgCl_2$ and 7.5 µl PCR grade
249 water. Thermocycler settings were 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds,
250 55°C for 30 seconds, and 60°C for 4 minutes (ITS2) or 2:30 minutes (16S) with a 10°C hold. We then did
251 PCR clean-up using Agencourt AMPure XP beads (Beckman Coulter, Inc., Indianapolis, USA, IN 46268).
252 Purified PCR products (2.5 µl) were mixed with 2.5 µl of 100 nm custom universal tails indexing primers
253 (forward and reverse) developed at EnGGen Laboratory, Northern Arizona University (Flagstaff, AZ,
254 USA)(Colman et al., 2015) 12.5 µl of Phusion Green Master Mix, 1.5 µl of 3 µM $MgCl_2$ and 3.5 µl PCR
255 grade water and were amplified using thermocycler settings of 95°C for 2 minutes, followed by 15 cycles
256 of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute with a 10°C hold. We then ran
257 another round of cleanup and quantified PCR products using the Quant-iT PicoGreen dsDNA assay kit
258 (Life Technologies Inc., Grand Island, NY, USA). As a final step, the samples were pooled in equimolar
259 concentrations and sequenced in a multiplexed 2- x 300-bp paired-end sequencing run on the Illumina
260 MiSeq platform (Illumina Inc., San Diego, CA, USA) at the Genomics Core Facility, University of California
261 Riverside (USA).

262 *Bioinformatics*

263 ITS-2 sequences were analyzed using AMPtk: Amplicon Toolkit for NGS data (Palmer, Jusino, Banik,
264 & Lindner, 2018) (https://github.com/nextgenusfs/amptk). Demultiplexed paired-end sequences data
265 were pre-processed by trimming primer sequences, trimming forward and reverse reads to 250 bp
266 (reads length less than 100 bp were dropped), and merging paired-end reads using USEARCH v9.1.13
267 (Edgar, 2010). A total of 8,310,353 reads passed the preprocessing steps and reads were filtered based
268 on quality scores with a cutoff of an expected error less than 0.9 (Edgar & Flyvbjerg, 2015) to produce

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269 6,441,443 reads which passed quality filtering. The quality filtered reads were clustered into 19,790
270 Operational Taxonomic Units (OTUs) using UPARSE (Edgar, 2013) at 97% identity threshold. The OTUs
271 were further processed with VSEARCH (v 2.3.2)(Rognes, Flouri, Nichols, Quince, & Mahé, 2016) to
272 identify and remove 569 chimeras based on comparison to the UNITE database v8.0(Nilsson et al., 2019)
273 leaving 19,221 OTUs . We assigned taxonomy with the AMPtk "hybrid" approach which uses Global
274 Alignment, SINTAX, and UTAX. Lastly, sequences were rarefied to 10,000 sequences per sample and
275 processed with QIIME Core Diversity pipeline (Caporaso et al., 2010) to estimating Alpha (OTU richness)
276 and Beta diversity (Bray-Curtis dissimilarity).

277 16S sequences were analyzed using QIIME2 (Bolyen et al., 2018) (https://qiime2.org) following the
278 'Atacama soil microbiome' pipeline for demultiplexed paired-end sequences. We truncated sequences at
279 220 bp and trimmed the first 25 bp based on the interactive quality plots in QIIME2 and then denoised
280 sequences using DADA2 after truncating all sequences Chimeras were removed using the default method
281 in DADA2 (Callahan, Mcmurdie, Rosen, Han, & A, 2016). A total of 12,669,635 sequences passed quality
282 filtering. Unique sequences were aligned using MAFFT (Katoh & Standley, 2013), filtered using the masked
283 alignment file, and used to construct a Maximum LIkelihood phylogeny with FastTree (Price, Dehal, &
284 Arkin, 2010). Alpha (OTU richness) and Beta diversity measures (Weighted UniFrac distance) (Lozupone &
285 Knight, 2005) were estimated using a subsampled feature table containing 10,000 sequences per sample.
286 Taxonomy was assigned to 34,417 unique sequences using a Naïve Bayes classifier trained on the
287 GreenGenes database (McDonald et al., 2012) (version 13_8) using trimmed sequences pre-clustered at
288 99% similarity. After all sequence processing we retained N=224 unique samples for fungi and N=215
289 unique samples for bacteria.

290 *Climate data*

291 To test the interaction between site specific changes in climate and the influence of woody
292 plant encroachment, we acquired climate data for each site through the WorldClim v 2.1 database at 30
293 second resolution (Fick & Hijmans, 2017). We tested the influence of multiple climate parameters at
294 each site including: Mean Annual Temperature (MAT), Temperature Seasonality (standard deviation
295 x100), Maximum Temperature of Warmest Month, Minimum Temperature of Coldest Month, Mean
296 Annual Precipitation (MAP), and Precipitation Seasonality (Coefficient of Variation). We chose to use the
297 30-year climate normals (WorldClim) rather than annual climate data because our analyses aimed at
298 understanding climatic control over broad geographic variation in microbial communities**.** We found
299 substantial climate variability across sites and symbiont types (Fig S1), but found that overall MAT was

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the best univariate predictor of microbial diversity (Fig S2). Therefore, we included MAT, and for consistency MAP, as the primary climate variables in subsequent models.

Statistical methods

Leaf traits

We used Principal Components Analysis (PCA) to collapse the values of the six measured leaf traits into two PC axes to be used in hierarchical models (below). Prior to the PCA, we infilled missing leaf trait data (LDMC and leaf chemistry) for one site where only SLA could be measured (China) and any NA values using the package *mice* in R (R Core Team, 2019; van Buuren & Groothuis-oudshoorn, 2011), taking the average of 100 imputed values for each trait estimate. All data were logged prior to PCA. Leaf traits and principal components scores were averaged by (woody) plant species at each site.

We also tested for a difference in leaf N between root symbiont types, due to frequently higher N in tissues of N_2-fixing plants. We used one way ANOVA with leaf N (%) (logged) as the response and symbiont type (N_2-fix, ECM/ERM, AMF) as the predictor, followed by a Tukey's HSD test.

Alpha diversity (OTU richness)

We fit linear mixed-effects (hierarchical) models in a Bayesian SEM framework to test the impacts of woody plant encroachment on soil fungal and bacterial richness. First, we estimated the effects of vegetation type, climate, abiotic soil conditions, root symbiont type and their interactions on OTU richness. Next, we ran a second set of models to estimate the effects of woody plant leaf traits on soil abiotic conditions (soil C:N and soil pH), as we predicted that leaf traits would influence microbial richness via shifting abiotic soil conditions (Hypothesis 1). Thus, soil abiotic conditions were a predictor in the first set and a response in the second set of models (see General Model, Table S1). We did not hypothesize a relationship between leaf traits and soil moisture, however, so we simply used vegetation type as a predictor of soil moisture. Additionally, for the root symbiont type by vegetation interaction, we grouped symbiont types at the site level based on each woody plant species (see Table 1, Table S1), and thus we only estimate the effect of root symbionts for woody plants.

We fit Bayesian models using the *brms* package in R (Bürkner, 2017). All data were standard normalized prior to modeling to improve model convergence and we logged the bacterial response variable (16S OTU richness) for normality. All models contained a site level random intercept and hierarchical structure as described below and in Table S1. The Bayesian framework was convenient here due to the somewhat uneven design and multilevel structure of the data (Table S1), and was useful for

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predicting relationships with reasonable estimates of uncertainties. We used the posterior distributions of each parameter to calculate the probabilities that it was different from zero, and three probability levels are reported (85, 90 and 95% probabilities, respectively, that the parameter estimate is different from zero). We also used these parameter distributions to calculate pairwise post-hoc comparisons between root symbiont types.

General Model:

$Alpha\ Diversity$= (1|$site$)+ $Vegetation\ type$*$Root\ symbiont\ Type$+ $Vegetation\ type$*$Climate$+ $Soil\ abiotic$

$Soil\ abiotic$= (1|$site$)+$Woody\ leaf\ traits$

BRMS model syntax =

OTU richness ~ (1|site) + Symbiont*Vegetation type+ MAT*Vegetation type + MAP*Vegetation type + VWC + pH + soilC:N

soilC:N~ (1 | site) + PC Axis1 (leaf traits) + PC Axis2 (leaf traits)

pH ~ (1 | site) + PC Axis1 (leaf traits) + PC Axis2 (leaf traits)

VWC ~ (1 | site) + Vegetation type

Beta diversity (Community composition)

To assess the impacts of woody plant encroachment on bacterial and fungal community composition, we used non-metric multidimensional scaling (NMDS) of the Bray-Curtis (fungi) and weighted Unifrac (bacteria) dissimilarity metrics and permutational multivariate analysis of variance (perMANOVA) with the 'adonis' function in the *Vegan* package in R (Oksanen, Blanchet, Kindt, Legendre, & O'Hara, 2016) (999 permutations). We ran three perMANOVA models, first with vegetation type (woody versus herbaceous) as a predictor and site as a strata variable to restrict permutations within sites; next we used root symbiont type, climate, and soil abiotic parameters as predictors with vegetation type as a strata; third we ran a leaf trait model for woody soils only using leaf trait PCA axes 1 and 2 as predictors and no strata variable. All perMANOVA models had either bacterial or fungal community composition as the response variable.

General Model:

$Beta\ Diversity$ = $Vegetation\ type$

$Beta\ Diversity = Root\ symbiont\ Type$+ $Climate$+ $Soil\ abiotic$

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$Beta\ Diversity = Woody\ leaf\ traits$

Adonis model syntax =

Bray Curtis/Unifrac distance ~ Vegetation type, strata=site

Bray Curtis/Unifrac distance ~ Symbiont + MAT + MAP + VWC + pH + soilC:N , strata=vegetation type

Bray Curtis/Unifrac distance ~ PC Axis1 (leaf traits) + PC Axis2 (leaf traits)

Taxonomic analyses

To assess differences in the relative read abundance of microbial taxa between woody and non-woody vegetation, we used linear mixed effects models (for normally distributed data) or generalized linear models with a Gamma distribution in the 'lmer' and 'glmer' functions in the *lme4* package in R (Bates, Mächler, Bolker, & Walker, 2014). Read abundances (logged, zeroes removed) of microbial phyla were the response variable, vegetation type (woody/herbaceous) was a fixed effect and site was included as a random effect.

General Model:

$Phylum\ reads \sim (1|site) + Vegetation\ type$

We also used indicator species analysis to determine which taxa characterized soils from different vegetation types (woody versus herbaceous) using the function 'multipatt' in the *indicspecies* package in R(De Cáceres, Legendre, Wiser, & Brotons, 2012). We calculated Indicator Values (Indvalg) based on species (OTU) abundance and considered indicator taxa significant at α=0.05 based on permutation tests (n=999) and an indicator value (stat) of 0.2 or greater.

Results

Leaf Traits

PCA analysis showed that SLA, leaf N, δ15N, and LDMC loaded on PC1 which explained 37.3% of the variation among species, and high PC1 values were associated with low SLA, leaf N and δ15N and high LDMC. Leaf C and δ13C loaded on the second axis (PC2), which explained 17.5% of the variation among species, and high PC2 values were associated with high leaf C and low δ13C (Fig S3).

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ANOVA and post hoc analysis revealed N_2-fixing woody plants had the highest leaf N content (%) overall, and significantly higher leaf N than AMF and ECM/ERM symbiont types (Fig S5).

Alpha diversity (OTU richness)

Woody plant encroachment influenced the richness of soil microbial communities, but interestingly, these impacts differed across sites, with woody plant soils having higher, lower or similar richness as herbaceous soil microbial communities (Fig 2 a, b). Bayesian hierarchical models showed that N_2-fixing woody plants had higher soil fungal richness and lower soil bacterial richness than herbaceous plant communities within sites (Fig 3, Table S2). Additionally, ECM/ERM woody plants had higher soil bacterial richness and lower soil fungal richness than herbaceous plant communities within sites (Fig 3, Table S2). Post-hoc comparisons also revealed that N_2-fixing woody plants had higher soil fungal richness than AMF and ECM/ERM woody plants across sites, while ECM/ERM plants had higher soil bacterial richness than AMF and N_2-fixing woody plants across sites (Table S2, FigS6).

Soil abiotic conditions also predicted fungal and bacterial richness, including a positive relationship between pH and both fungal and bacterial richness, a negative relationship between soil C:N and fungal richness (Fig 4, Table S2), and a positive relationship between soil water content (VWC) and bacterial richness (Table S2). Woody plant soils had lower VWC than herbaceous soils and woody plant leaf traits predicted soil abiotic conditions (Table S2). The first axis of a principal components analysis (PC1) of multiple leaf traits was negatively related to soil pH and soil C:N, while PC2 was negatively related to soil pH in the Bayesian hierarchical model (Fig 4, Table S2).

Finally, there were interactions between woody encroachment and climate, including a negative interaction between mean annual precipitation (MAP) and vegetation type on fungal richness, a positive interaction between mean annual precipitation (MAP) and vegetation type on bacterial richness and a negative interaction between mean annual temperature (MAT) and vegetation type on bacterial and fungal richness (Fig 3, Table S2).

Beta diversity (Community composition)

Microbial beta diversity was generally higher between rather than within sites, as communities clustered strongly by sampling site (Fig 2 c, d). Within sites, microbial community composition differed among vegetation types and this pattern was stronger for bacterial than fungal communities based on perMANOVA results and NMDS overlap (Fig 5 a, d, Table S3,). Within vegetation types, plant traits, climate and soil abiotic conditions were significantly related to both fungal and bacterial community

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417 composition (Table S3). Environmental variables such as climate and soil abiotic conditions explained up
418 to an order of magnitude more variation in bacterial than fungal community composition (maximum R^2
419 0.135 vs 0.012; mean R^2 0.06 vs 0.01, Table S3). Root symbiont type was a significant predictor of both
420 fungal and bacterial communities, with the highest community similarity within N_2-fixing soil fungal
421 communities (Fig 5 b,e). Mean annual precipitation (MAP) and soil pH were the best abiotic predictors of
422 fungal and bacterial community composition, respectively (Fig 5 c, f, Table S3). Woody plant leaf traits
423 were also significant predictors of microbial community composition with PC2 being most predictive of
424 fungal and bacterial communities (Table S3).
425

426 *Taxonomic analyses*

427 The soil fungal community comprised 10 phyla, with Ascomycota dominating (40.1%), followed by
428 Basidiomycota (26.6%) and Mortierellomycota (13.9%), Glomeromycota (0.8%) and Chytridiomycota
429 (0.5%) (Fig S4 a,b) . Six percent of the total ITS-2 sequences could not be assigned taxonomically, while
430 two percent were assigned as unknown Fungi (i.e. only to Kingdom level) (red color-Fig S4). The soil
431 bacterial community comprised 43 phyla with Proteobacteria making up the largest percentage (29.1%),
432 followed by Acidobacteria (16.4%), Actinobacteria (12.9%), Bacteroidetes (8.7%), Planctomycetes (6.5%),
433 Verrucomicrobia (6.5%), Chloroflexi (5.6%), unidentified bacteria (3.8%) and Firmicutes (1.5%) (Fig S4
434 c,d). Less than one percent of the total 16S sequences could not be assigned a taxonomy, while four
435 percent were assigned as unknown Bacteria (red color-Fig S4).

436 Taxa abundance models of the dominant microbial phyla showed a lower abundance of
437 Basidiomycota in woody versus herbaceous soils (Table S4, Fig S4 a,b). For bacterial phyla, soils from
438 herbaceous communities had a higher abundance of Acidobacteria, Actinobacteria, Proteobacteria,
439 Verrucomicrobia, and Planctomycetes than woody soils (Table S4, Fig S4 c,d).

440 Fifty-one fungal indicator OTUs (assigned to the species level) were found in woody plant soils and
441 23 indicator OTUs were in soils from herbaceous communities from Indicator species analysis. The six
442 most prevalent indicator species were from the *Mortierella*, *Penicillium*, *Vishniacozyma, Herpotrichia,*
443 *and Metapochonia* genera (OTUs 1585, 16274, 1203, 938, 101 and 1386) and were associated with soils
444 beneath woody plants from at least ten sites (Table S5a). Species in the *Penicillium, Clavaria, and*
445 *Pyrenochaetopsis* genera (OTUs 1611, 808, and 1271) were associated with soils from herbaceous
446 communities at seven sites (Table S5a). There were only nine bacterial indicator OTUs assigned to the
447 species level overall, but at the genus level, there were 32 bacterial indicator taxa (20 genera) for woody
448 soils and 35 indicator taxa (22 genera) for herbaceous soils. Members of the genus *Herminiimonas*

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(Proteobacteria) and *Segetibacter* (Bacteroidetes) were strongly associated with woody plant soils while the DA101 (Verrucomicrobia), *Rhodoplanes* (Proteobacteria), and GOUTA19 (Nitrospirae) genera were associated with soils from herbaceous communities. Indicator taxa from *Flavobacterium*, Candidatus *Koribacter*, Candidatus *Solibacter*, *Kaistobacter*, and *Pseudonocardia* genera were common in soils from both woody and herbaceous plants (Table S5b).

Discussion

One of the most striking ways that global change is restructuring alpine tundra plant communities is through the replacement of herbaceous plants by woody shrubs and dwarf trees (Brandt, Haynes, Kuemmerle, Waller, & Radeloff, 2013; Formica, Farrer, Ashton, & Suding, 2014; Hallinger, Manthey, & Wilmking, 2010). For example, conversion rates of alpine meadows to woody shrublands were estimated between 39-72% in the large portions of the southern Himalayas (Brandt et al., 2013). Here, using a global, coordinated field study we found that woody plant encroachment is influencing both richness and composition of soil microbial communities but that these changes depend on a combination of abiotic soil conditions, climate, root symbiont types and plant functional traits. This is an important first step in building a more predictive, functional understanding of how climate-driven shifts in woody plant cover will affect soil microbial communities and ecosystem processes worldwide.

Broadly, we did not find one 'global signature' of woody encroachment, but rather that woody encroachment was associated with increased, decreased, and no change in microbial alpha diversity (OTU richness) when comparing with soils of nearby herbaceous plant communities (Fig 2). This likely reflects the broad taxonomic and functional diversity of the woody plant species across these sites, leading to variable litter quality (Table 1, Fig S3). For example, study species included evergreen conifers, deciduous hardwoods, legumes and woody graminoids, highlighting the diversity of woody species expanding into different alpine ecosystems worldwide. However, when accounting for easily measurable characteristics, such as woody plant leaf traits and root symbiont types, consistent patterns emerged for effects of woody plants on both bacterial and fungal richness and community composition.

Woody plant leaf traits modulated shifts in soil microbial communities supporting our first hypothesis. Leaf traits predicted the community composition of both bacteria and fungi in woody plant soils and influenced soil microbial richness indirectly through changes in soil abiotic conditions (pH, soil C:N). Two distinct trait axes influenced microbial community structure. The first axis of the principal components analysis (PC1) was primarily characterized by low SLA, leaf N and δ15N and high LDMC and the second axis (PC2) was primarily characterized by high leaf C and low δ13C (Fig S3). Thus PC1

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481 represents variation in leaf economic traits and nitrogen acquisition strategies with low PC1 scores
482 representing more resource-acquisitive species with higher N content and SLA (Wright et al., 2004).
483 Moreover, PC2 represents variation in leaf C and water use with high PC2 scores representing species
484 with resource-conservative strategies including high leaf C content and water use efficiency (Moreno-
485 Gutiérrez, Dawson, Nicolás, & Querejeta, 2012). There was a negative relationship between PC2 and soil
486 pH (Fig 4), suggesting that woody plants with higher C content in leaves reduced soil pH, likely due to
487 leaching of organic acids into soil solution via recalcitrant litter (Eldridge et al., 2011; Jobbagyl & Jackson,
488 2003). Consistent with other studies, we also found that soil pH was a strong predictor of both bacterial
489 and fungal richness (Lauber et al., 2009; J. Rousk et al., 2010), providing a clear mechanism for how
490 woody plant litter chemistry can influence soil microbial diversity. Plant traits also influenced bacterial
491 and fungal community composition, but PC2 was a stronger predictor than PC1 (Table S3), further
492 suggesting that leaf C content is an important determinant of woody encroachment impacts on soil
493 microbial communities.

494 Woody plants with different root symbiont types (AMF, ECM/ERM, N_2-fixers) had distinct impacts
495 on soil microbial communities, supporting our second hypothesis. In particular, N_2-fixing woody species
496 had higher soil fungal richness and lower bacterial richness than both herbaceous soils (within sites) and
497 AMF, ECM/ERM woody plant soils (across sites)(Fig 3a, FigS6a, Table S2). Conversely, ECM-ERM
498 symbionts had higher soil bacterial richness but lower fungal richness than both herbaceous soils (within
499 sites) and N_2-fixing, AMF woody plant soils (across sites)(Fig 3a, FigS6b, Table S2). Root symbiont type
500 was also an important predictor of both fungal and bacterial community composition (Fig 5b,e, Table
501 S3). Root symbiont types can greatly influence plant resource use strategies, as well as litter chemistry
502 and thus the impact of woody plants on soil microbial communities (Cheeke et al., 2017; Wookey et al.,
503 2009). For example, N_2-fixing woody plants had higher leaf N content (%) than AMF symbiont types in
504 our study (Fig S5) and thus may be altering soil microbial richness through high N leaf litter. Previous
505 work has shown invasion of N_2-fixing woody species reduces soil microbial diversity (Lorenzo, Pereira, &
506 Rodríguez-Echeverría, 2013; Lorenzo, Rodríguez-Echeverría, González, & Freitas, 2010), which we find to
507 be true for bacteria, however we see the opposite response in fungi. Root symbionts, especially extra-
508 radical hyphal forming ecto- and ericoid mycorrhizas, may also interact directly with free-living microbes
509 (Bending et al., 2006). Woody plants utilizing ECM and ERM fungi had higher soil bacterial richness and
510 distinct soil microbial community composition (Fig 3a, 5b,e). ECM and ERM fungi release extracellular
511 enzymes and organic acids for decomposition into the rhizosphere which can select for specific bacterial
512 communities (Churchland & Grayston, 2014). In addition, mycorrhizal helper bacteria (MHB) (Frey-Klett,

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Garbaye, & Tarkka, 2007) and/or chitinophagous species that feed on dead fungal hyphae may be enhanced in the rhizosphere of ECM and ERM woody plants (Brabcová, Nováková, Davidová, & Baldrian, 2016), and several of these taxa were indicator species of woody plant soils in our analysis (Table S5).

While we designated root symbiont types based on current literature and site-specific information, several of the woody plant species in our study can utilize multiple types of root symbionts. For example, *Salix* spp. (Teste, Jones, & Dickie, 2019) and *Juniperus communis* (Thomas, El-Bargathi, & Polwart, 2007) can be dually colonized by ECM and AMF, and the relative abundance of each mycorrhizal type often differs across habitats, with alpine *Salix* varieties being more ECM dominant (Dhillion, 1994). In addition, Nitrogen fixers may utilize different bacterial symbionts; for example, *Alnus alnobetula* is an actinorhizal species which associates with bacteria in the genus *Frankia* (Richardson, Allsopp, D'antonio, Milton, & Rejmánek, 2000), while *Echinospartum horridum* is a legume which associates with bacterial species in the genus *Rhizobium* (Komac, Alados, & Camarero, 2011). Rhizobial strains are considered more host-specific than *Frankia*, and N_2-fixing plant species may also have co-occurring AMF or ECM fungi (Teste et al., 2019). Despite these discrepancies, these very broad categories still proved to be useful predictors of complex soil microbial communities undergoing woody plant encroachment.

Soil abiotic conditions influenced microbial communities, supporting our third hypothesis, and soil pH was the most consistent driver of soil microbial richness (Fig 3, Table S2) and community composition (Table S3). Further, abiotic conditions were influenced by woody plant leaf traits, suggesting that woody plants affect soil microbial communities indirectly through changes in abiotic soil conditions (Fig 4). For example, soil pH had a positive effect on both fungal and bacterial richness and was the best predictor of bacterial community composition (Fig 3a, 5f). As described previously, there was also a negative relationship between woody plant leaf traits, particularly leaf C content, and pH (Fig 4). Soil pH is a consistently strong predictor of microbial community structure (Lauber et al., 2009; J. Rousk et al., 2010), however it is often framed as an abiotic driver decoupled from plant litter chemistry. Soil C:N had a negative effect on fungal richness and also influenced fungal and bacterial community composition (Fig 3a,Table S3). On the other hand, Soil C:N was negatively associated with N related leaf traits (PC1) , however the direction of this relationship was the opposite of what we predicted (Fig 4). This may be due to the fact that in low N environments such as the alpine, N mineralization is very low and direct microbial uptake of organic N from is high (Schimel & Bennett, 2004), potentially weakening the link between leaf N traits and soil C:N. Finally, VWC had a positive effect on bacterial richness, and influenced microbial and fungal community composition (Fig 3a, Table S3), however unlike our initial

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prediction, soils from beneath woody plants had slightly lower VWC (Table S2). Thus, woody plants may be depleting soil moisture as compared to herbaceous vegetation through deeper roots, or via accessing water later into the growing season (Acharya, Kharel, Zou, Wilcox, & Halihan, 2018; Awada et al., 2013). Overall, these patterns highlight that woody plant effects on abiotic soil conditions are an important indirect pathway between woody plant encroachment and soil microbial community structure.

While changing climate is among the major drivers of woody plant encroachment, our results demonstrate that woody encroachment may also modulate climate effects on soil microbes. In support of our fourth hypothesis, the effects of woody plants interacted with climate at the site level, including interactions between vegetation type and MAP, MAT on fungal and bacterial richness (Fig 3, Table S2). This suggests that soil microbial communities undergoing woody encroachment are more distinct from those of herbaceous plants at the more extreme ends of temperature and precipitation gradients (Fig 3 b, c). Fungal richness was more sensitive to the precipitation by vegetation type interaction, which is consistent with previous work showing MAP to be the best predictor of fungal richness worldwide (Tedersoo et al., 2014). Bacterial richness was more sensitive to the temperature by vegetation type interaction, likely because bacteria tend to be less cold tolerant than fungi, and fewer strains can maintain their biomass under winter snowpack (Lazzaro, Hilfiker, & Zeyer, 2015; Zinger, Shahnavaz, Baptist, Geremia, & Choler, 2009). Furthermore, MAT was one of the best predictors of fungal richness overall and MAP was among the top predictors of both fungal and bacterial community composition (Fig 3a, Fig 5c, Table S3), emphasizing the strong influence of climate on soil microbial communities in alpine environments. All together, we find that woody encroachment can significantly influence how soil microbial communities respond to temperature and precipitation and may alter both the magnitude and influence of the climate driver. Thus, future predictions of climate impacts on alpine soil microbial communities must also consider co-occurring shifts in plant community structure.

Due to this study's observational rather than experimental approach, we cannot conclusively state that observed differences in soil microbial communities are in *response* to woody plant encroachment rather than a potential *cause* of woody plant establishment. However, there are several reasons why we believe the former to be true. First, soil microbial communities were highly correlated with attributes of the woody plants themselves, including leaf traits, root symbiont type, and soil abiotic conditions related to litter chemistry. In addition, we selected sites where woody plant encroachment began within the last 50 years, and at most sites, woody encroachment has been present for between 30-40 years. In a previous study, alpine soil microbial communities reflected the transition from a woody to herbaceous plant community in under 5 years (Collins et al., 2016) and thus we believe our sampling interval

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provides sufficient time for woody plants to have cultivated distinct soil communities. Next, our analysis of soil microbial community composition has focused on the saprotrophic, generalist species which are most abundant in bulk soil and unlikely to directly influence plant community composition (Fierer, 2017). This analysis does not test for species-specific soil mutualists or pathogens, the taxa which most strongly influence the success of plant establishment and range expansion (Mccarthy-Neumann & Ibáñez, 2012; Nuñez, Horton, & Simberloff, 2009; Tomiolo & Ward, 2018). Finally, while all soils were collected during the growing season (alpine summer), sampling times varied among sites due to differences in growing season length and snowmelt timing. Differences in sampling time can influence site-specific patterns in soil microbial communities (Bjork, Bjorkman, Andersson, & Klemedtsson, 2008; Lazzaro et al., 2015; Lipson & Schmidt, 2004), yet despite this, we observed many consistent patterns across sites in response to woody encroachment, suggesting that vegetation strongly influences soil microbial community structure in alpine ecosystems.

This study documents the global impacts of woody plant encroachment on soil microbial communities, but we emphasize that multiple pathways must be considered to disentangle these impacts. Specifically, divergent functional trait strategies and functional groups of woody plants based on root symbionts have consistent impacts belowground regardless of woody plant species or site. In addition, the influence of woody plants on soil microbes can be indirect through changes in the soil abiotic environment, such as reduced soil pH driven by high C content of woody plant litter. Finally, woody encroachment can influence both the direction and magnitude of direct climate effects on microbial richness, and bacteria and fungi respond to distinct climate and woody plant drivers. Our work highlights the complexity of plant-soil interactions in rapidly changing alpine ecosystems, an understanding that will influence our ability to predict feedbacks to terrestrial ecosystem function and climate, particularly the global C cycle, where soil microbes play an integral role.

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Data Availability

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632 All raw data and analysis scripts for thus study may be found at the following repository:
633 https://github.com/cour10eygrace/woody-encroachment-microbes.git. Raw Sequences may be found in
634 the NCBI Short Read Archive (SRA) accession # PRJNA659596.

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Figure legends

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Fig 1. Map and images of 13 alpine woody encroachment sites included in this study. Sites span 10 countries and 4 continents. See Table 1 for further information.

Fig 2. Box and whisker plots of soil a) fungal and b) bacterial OTU richness (logged) and NMDS ordination plots of soil c) fungal (stress =0.13) and d) bacteria (stress =0.11) beta diversity (community composition) at each site. For richness, box fill color designates whether the soil was sampled in woody encroached or herbaceous plant community and box outline color designates the root symbiont type of the woody plant at each site. Here both fungal and bacterial richness are plotted on the log scale for consistency but we only logged bacterial richness in Bayesian models. For beta diversity, colored ovals represent 95% confidence intervals of sample ordination grouped by sampling site and shapes represent the vegetation community (woody or herbaceous) of each soil sample.

Fig 3. a) Parameter estimates (points) and 95% credible intervals (lines) from Bayesian hierarchical models for the effects of root symbiont type (woody plants only), climate, and soil abiotic conditions associated with woody plant encroachment on alpha diversity (OTU richness) of fungi and bacteria. Asterisks denote probabilities that the effect of a parameter is greater or less than zero based on credible intervals (*** = probability> 95%; ** = probability> 90%; * = probability > 85%). Parameter estimates and credible intervals are listed in Table S2. All values are standard normalized as was done prior to modeling. b,c) Interactions between vegetation type and mean annual precipitation (MAP) and mean annual temperature (MAT) on fungal and bacterial richness. Points are raw data, lines are fitted model estimates, and all values are standard normalized. Interactions showed that encroachment by woody plants lead to increased, decreased fungal richness in sites with lower, higher precipitation and increased, decreased bacterial richness in sites with lower, higher temperature as compared to herbaceous plant communities. All values are standard normalized as was done prior to modeling

Fig 4. Diagram of impacts of woody plant leaf traits on bacterial and fungal richness via changes in soil abiotic conditions based on the Bayesian SEM. Red lines show significant negative relationships and blue lines show significant positive relationships. Slope coefficients (standardized) show the magnitude and line thickness reflects the associated credible interval of each relationship (85%, 90%, 95%). Leaf traits shown in each corner reflect loadings on each Principal coordinates (PC) axis. Parameter estimates and credible intervals are listed in table S2 and trait loadings are shown in Fig S3.

Fig 5. NMDS plots of community dissimilarity using Bray-Curtis and Weighted Unifrac distance for soil fungi (a-c) and bacteria (d-f) respectively. Colored ovals represent 95% confidence intervals of sample ordination grouped by vegetation and root symbiont type. The strongest abiotic predictor of each

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microbial group (MAP-Fungi and soil pH-Bacteria) is plotted on the right with a color ramp for continuous values. Model parameter estimates are listed in Table S3.

Tables and Figures

Table 1. Woody Encroachment study sites included in this synthesis and corresponding information. Symbiont type refers to root microbial symbionts of woody plant species Arbuscular mycorrhizal (AMF), Ecto- or Ericoid mycorrhizal (ECM.ERM) and N_2-fixing bacterial (Nfix). Reference manuscripts describe woody encroachment patterns at each site.

Site	Latitude	Longitude	Elevation (m)	Symbiont type	Woody species	Reference
China	33.66536	101.8663515	3506.000	AMF	*Potentilla fruticosa*	Klein et al. 2007
Colombia	4.792977	-75.4254868	4024.000	AMF	*Hesperomeles obtusifolia*	Matson and Bart 2013
Czech Rep	50.768887	15.5398797	1343.749	ECM.ERM	*Pinus mugo*	Soukupová et al. 1995
France	45.421500	6.1780400	1797.946	Nfix	*Alnus alnobetula*	Anthelme et al. 2007
Italy	46.673611	10.5919444	2357.600	ECM.ERM	*Rhododendron ferrugineum*	Cannone et al. 2007
Japan	43.563258	142.9011030	1771.600	AMF	*Sasa kurilensis*	Kudo et al 2011

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Mexico	19.064165	-97.2669115	4110.500	AMF	*Chionolaena lavandulifolia*	Ramírez-Amezcua et al. 2016
Spain	42.575821	1.3667150	2100.000	AMF	*Juniperus communis*	Montané et al. 2007
Spain Ordesa	42.602807	0.0332073	1942.007	Nfix	*Echinospartum horridum*	Komac et al. 2011
Sweden	68.360658	18.7368890	740.000	ECM.ERM	*Salix lapponum*	Rundqvist et al. 2011
Switzerland	46.621100	8.6349430	1598.800	Nfix	*Alnus alnobetula*	Caviezel et al. 2014
US CA	37.576447	-118.240913	3750.000	AMF	*Artemisia rothrockii*	Kopp and Cleland 2014
US CO	40.153600	-105.670750	3530.000	ECM.ERM	*Salix glauca*	Bueno de Mesquita et al. 2018

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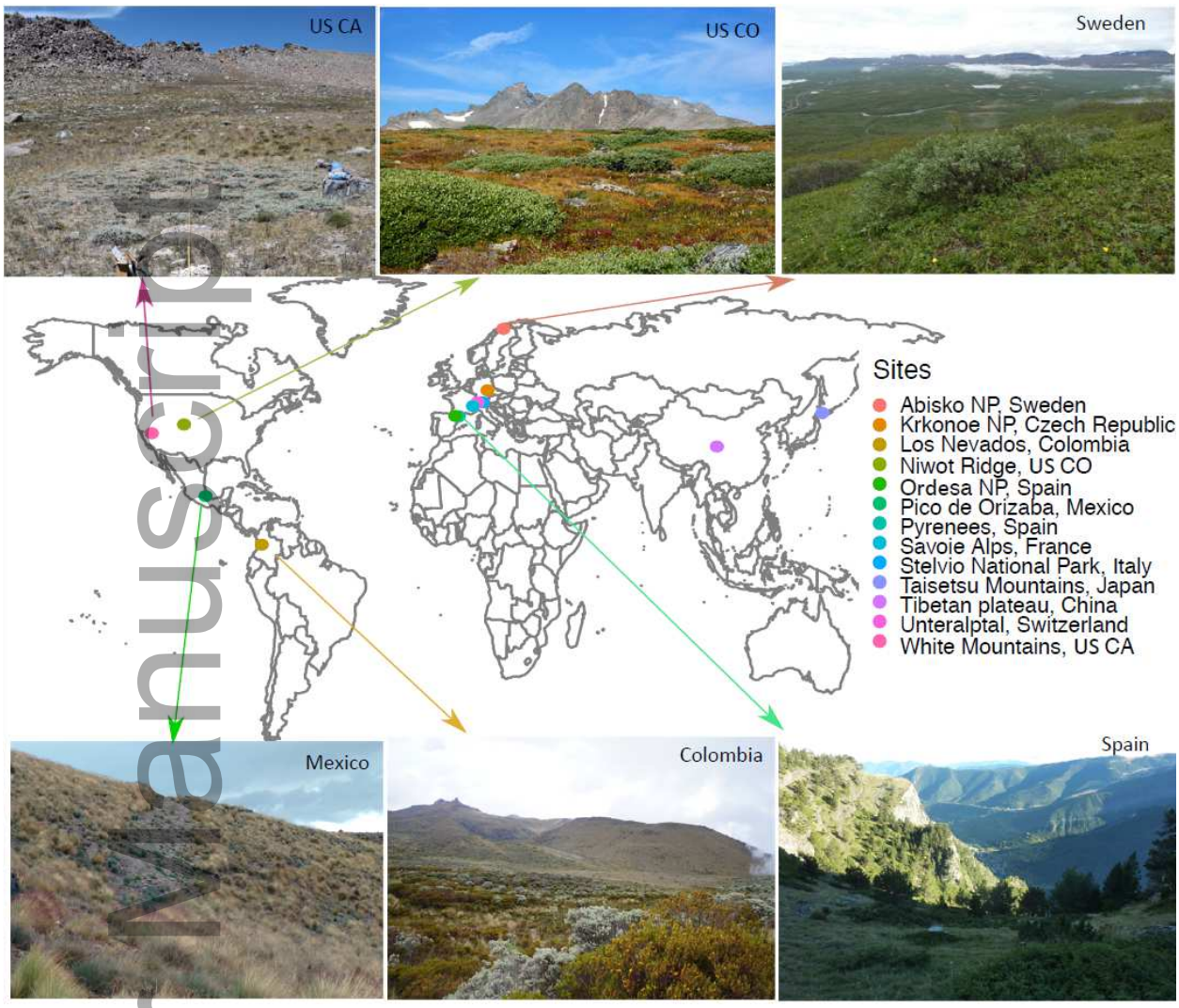

Fig 1.

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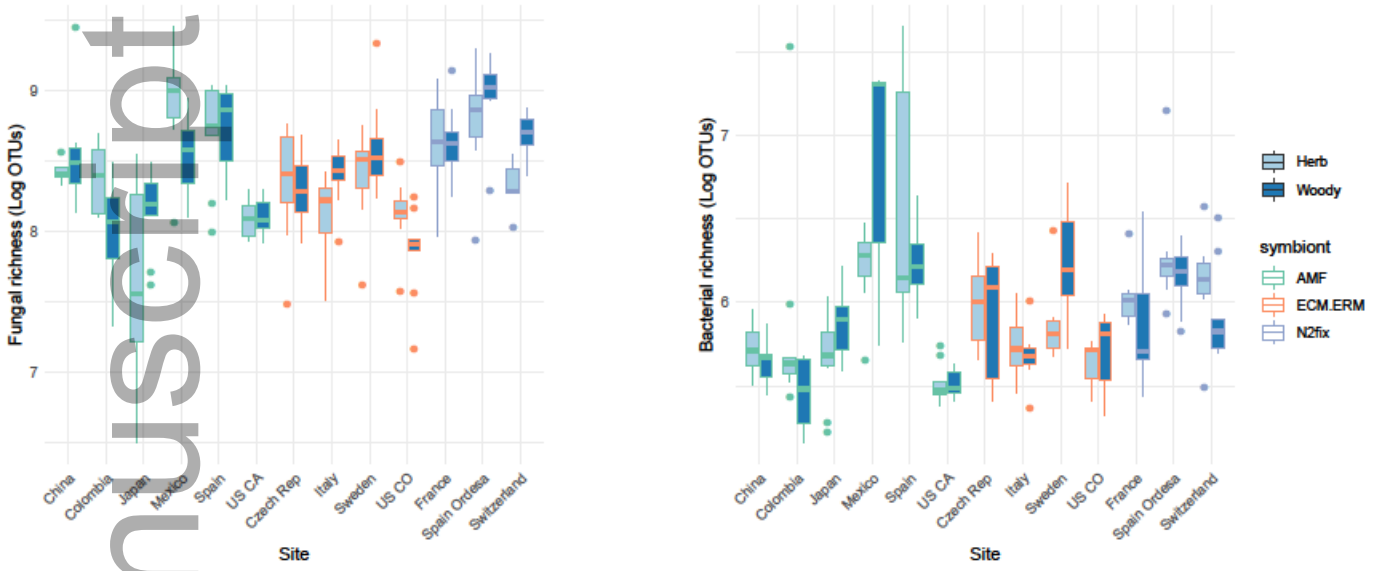

(c) (a) (d)

(b)

Fig 2.

(a) **(b)** **(c)**

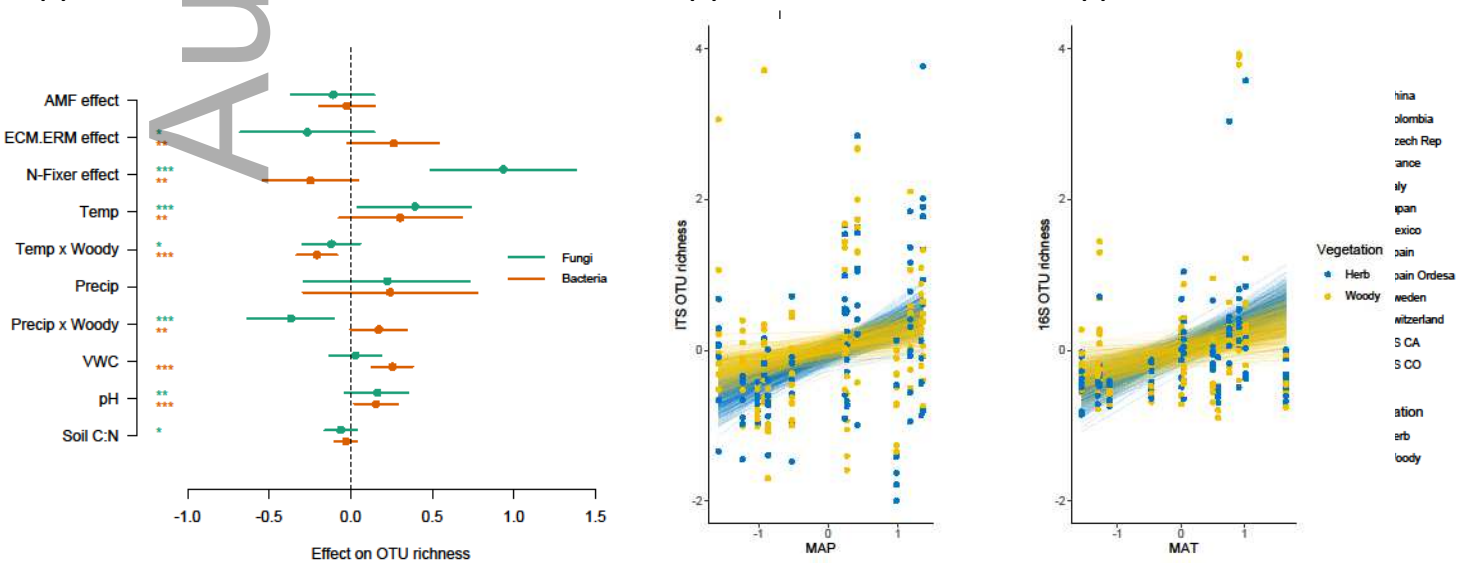

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Fig 3.

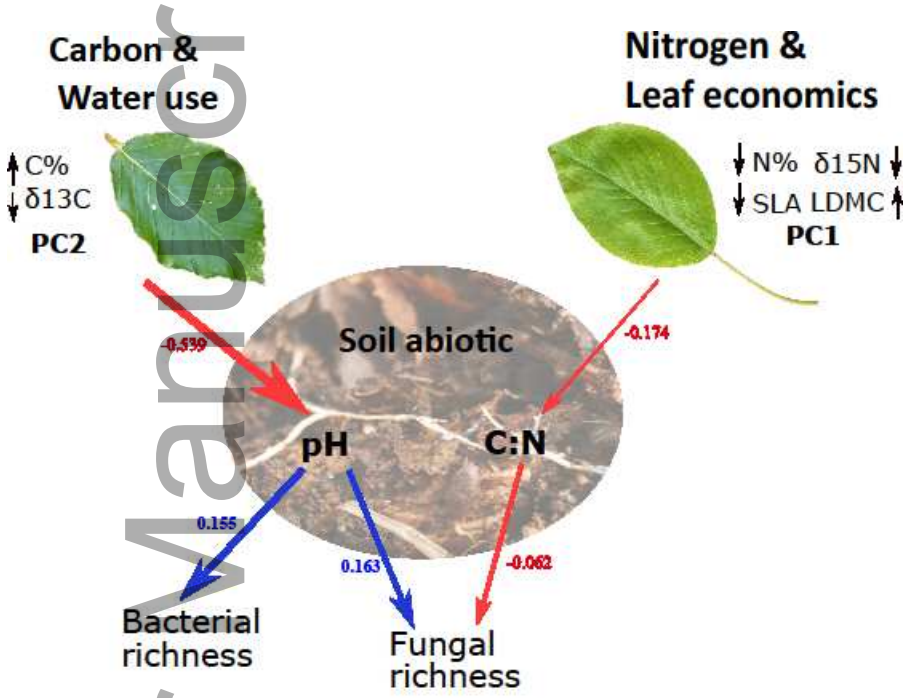

Fig 4.

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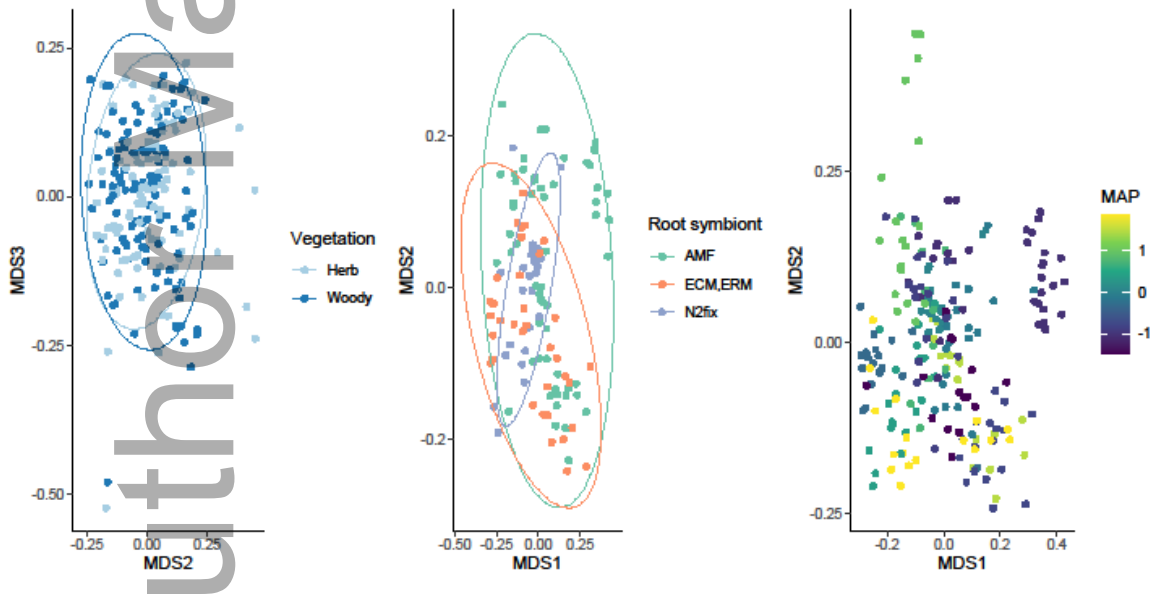
1062 **Fungi**

1063 **(a)** **(b)** **(c)**



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1065 **Bacteria**

1066 **(d)** **(e)** **(f)**

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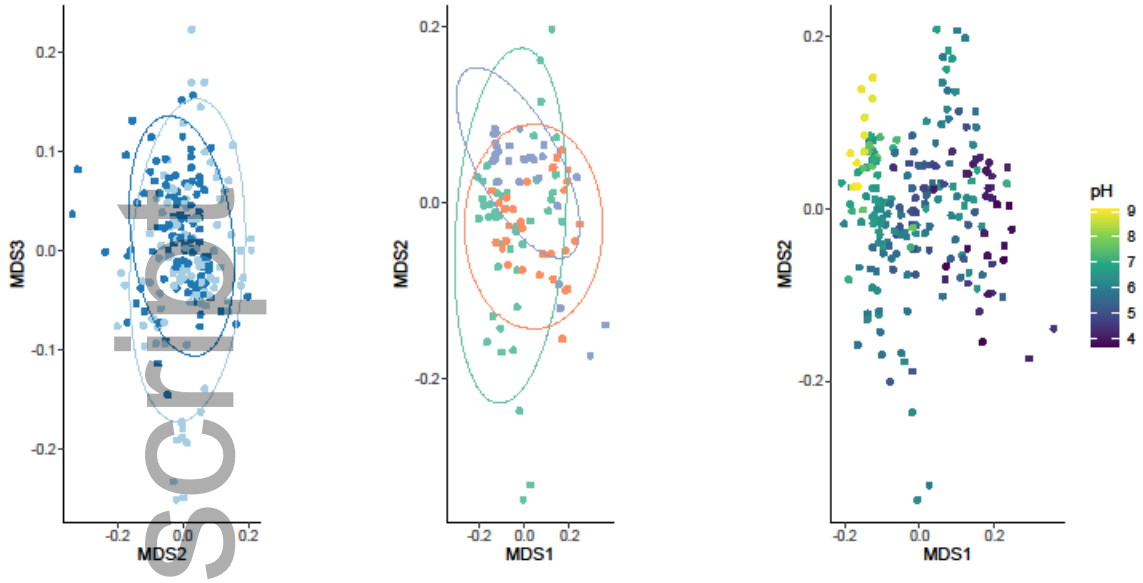

Fig 5.

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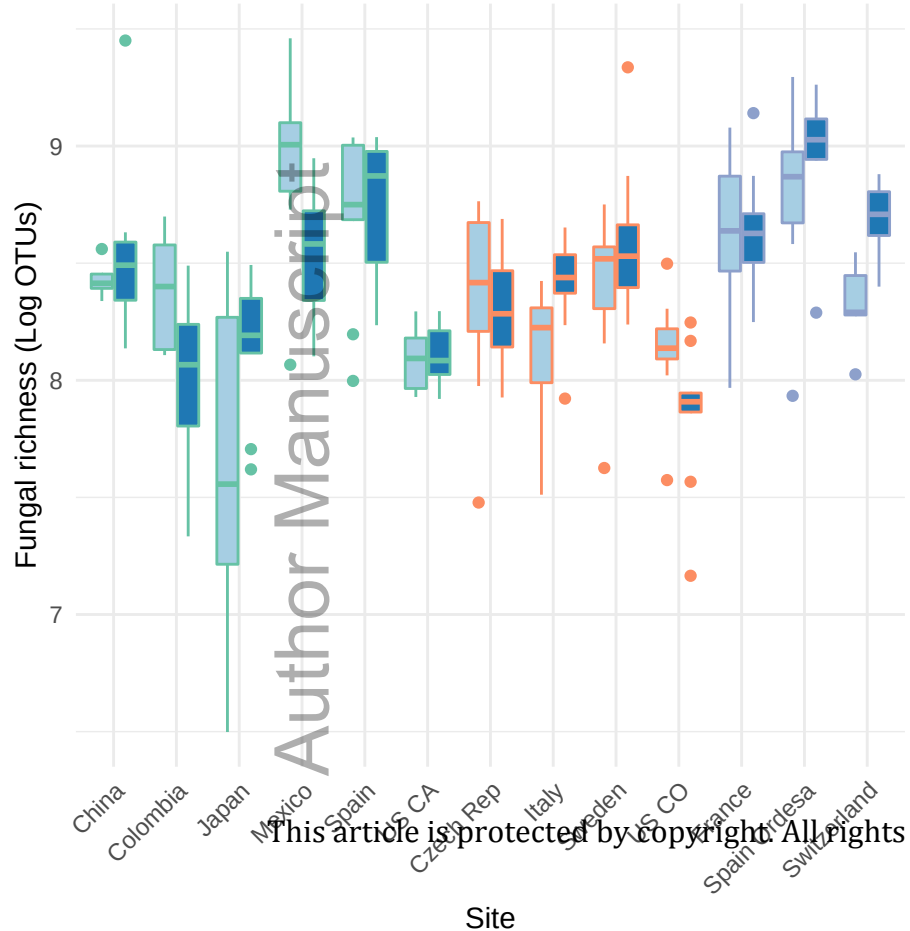

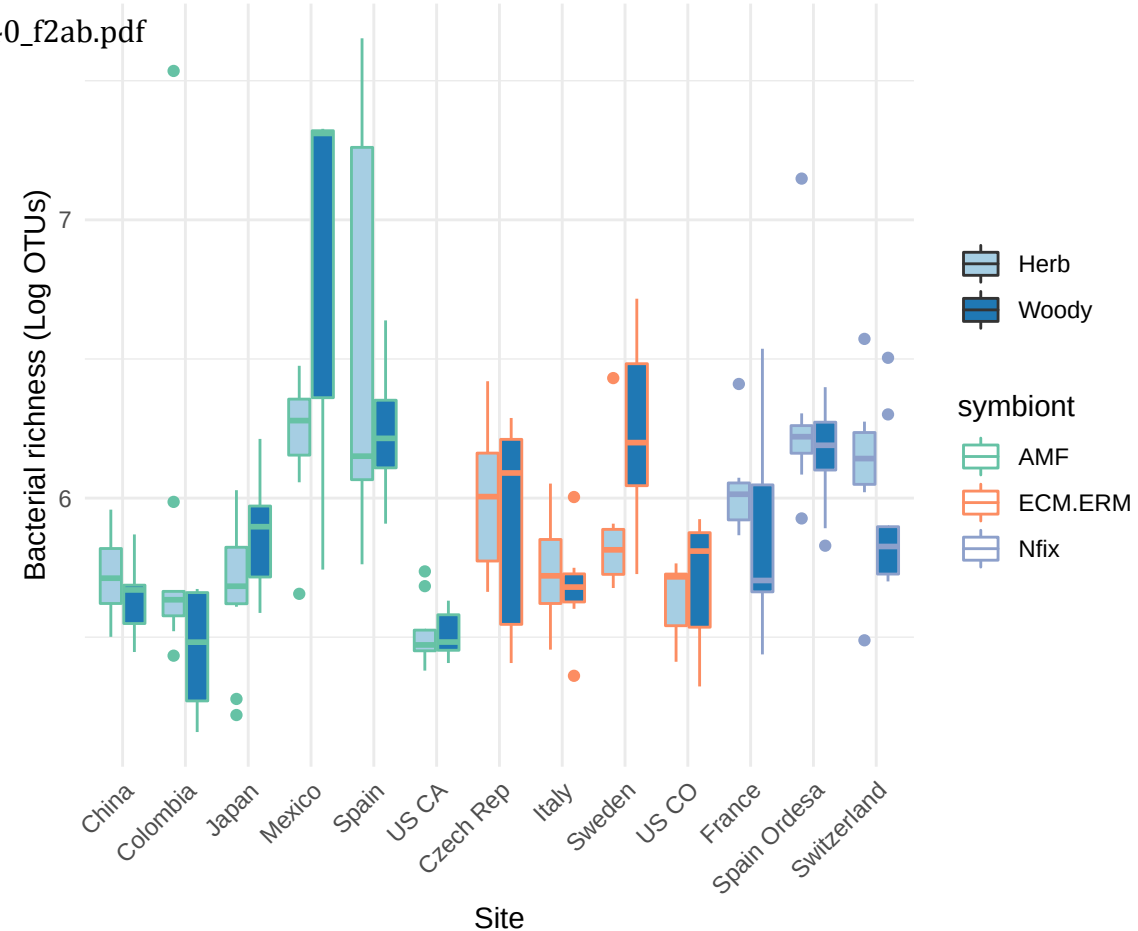

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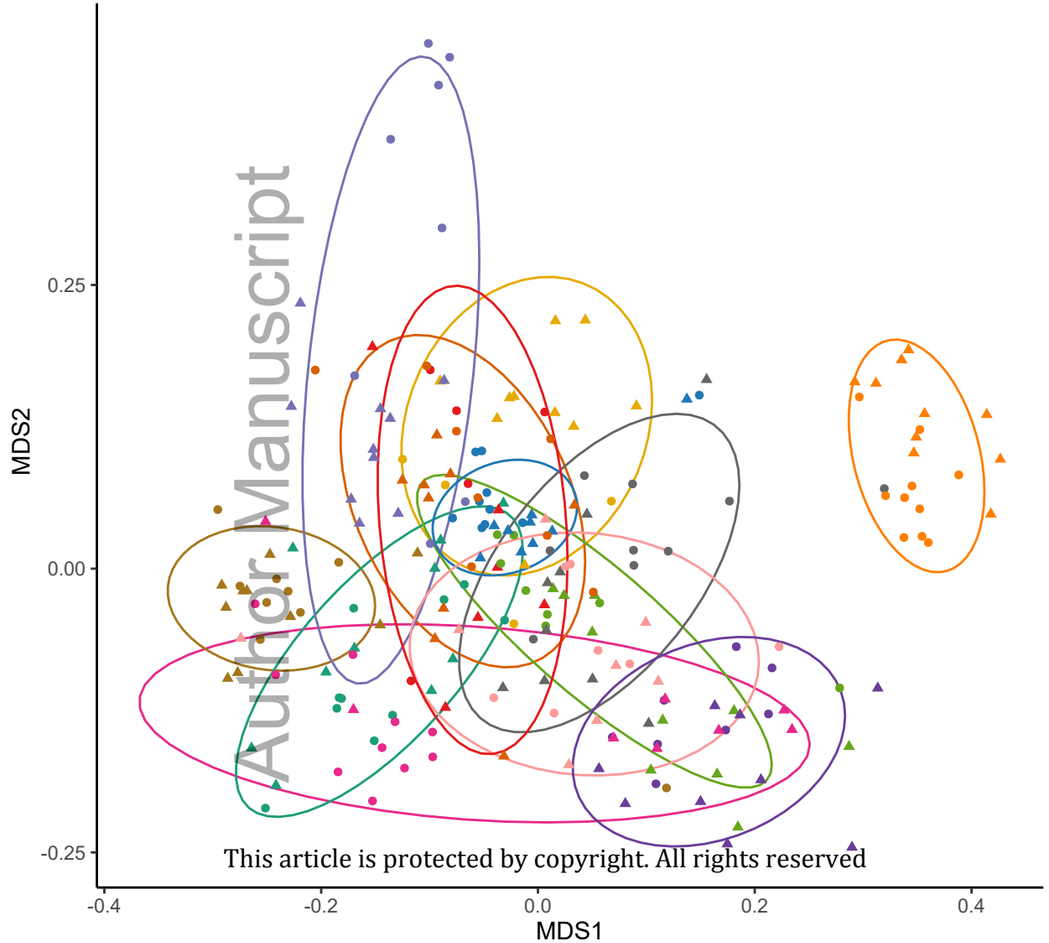

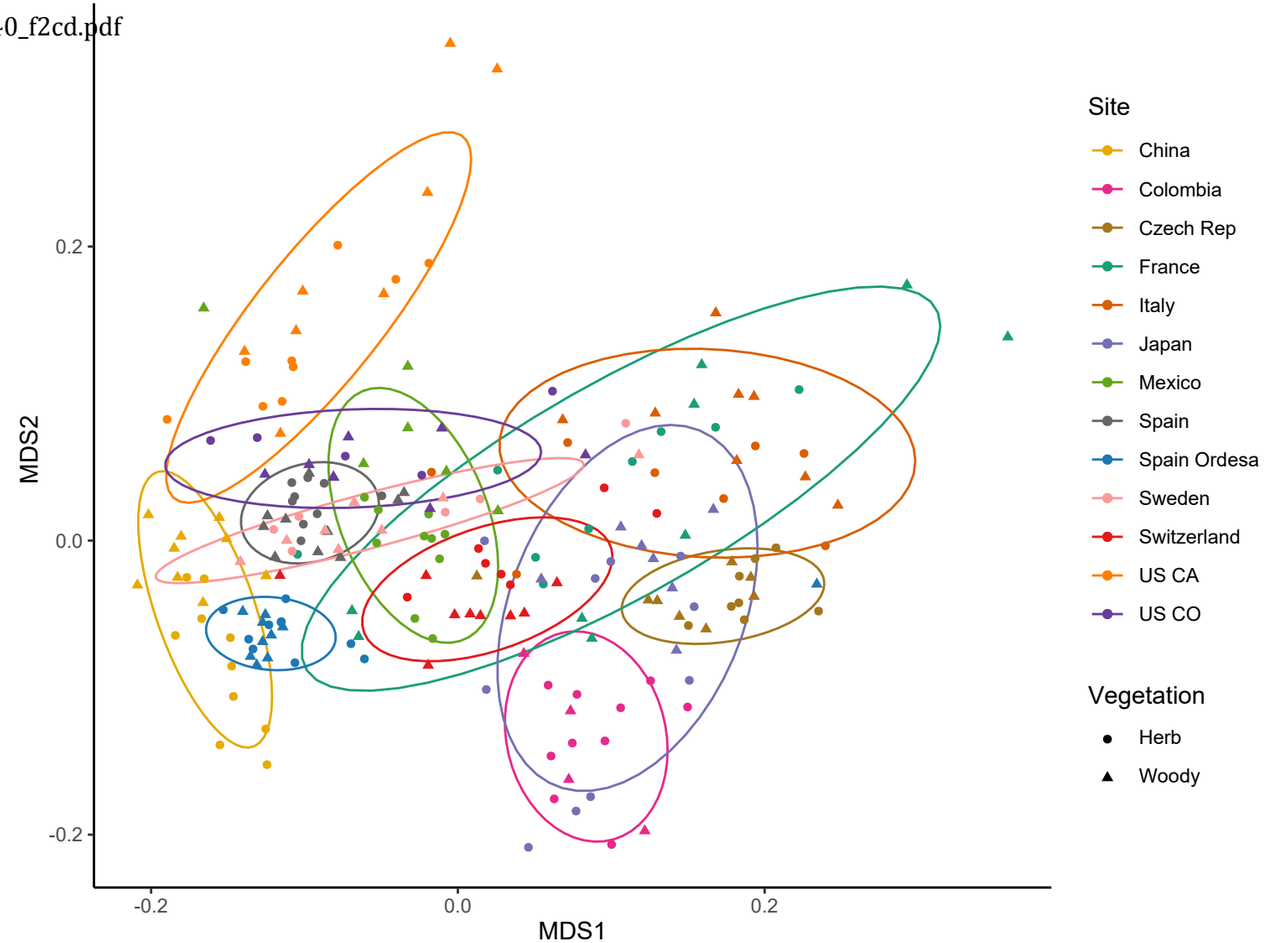

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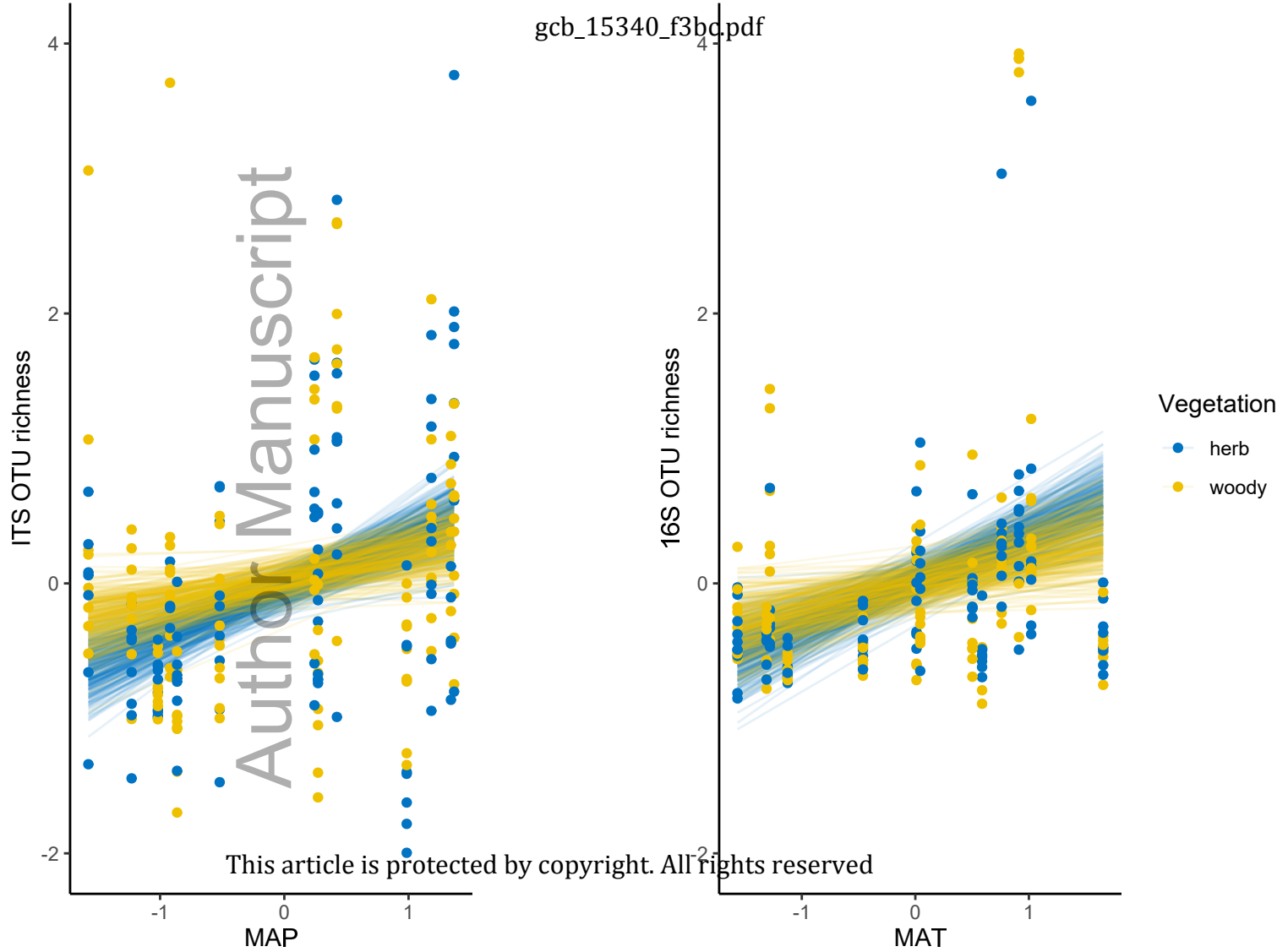

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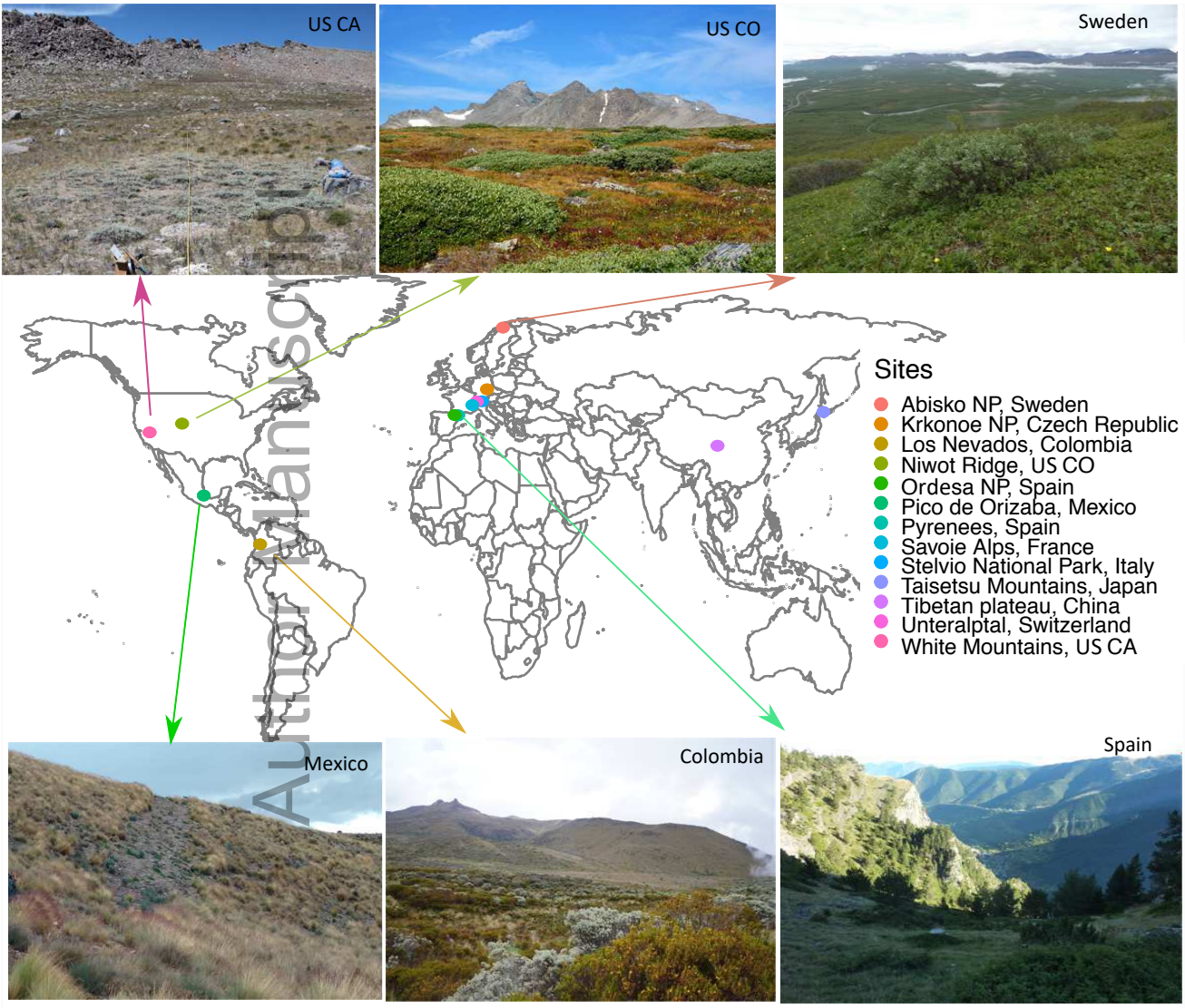

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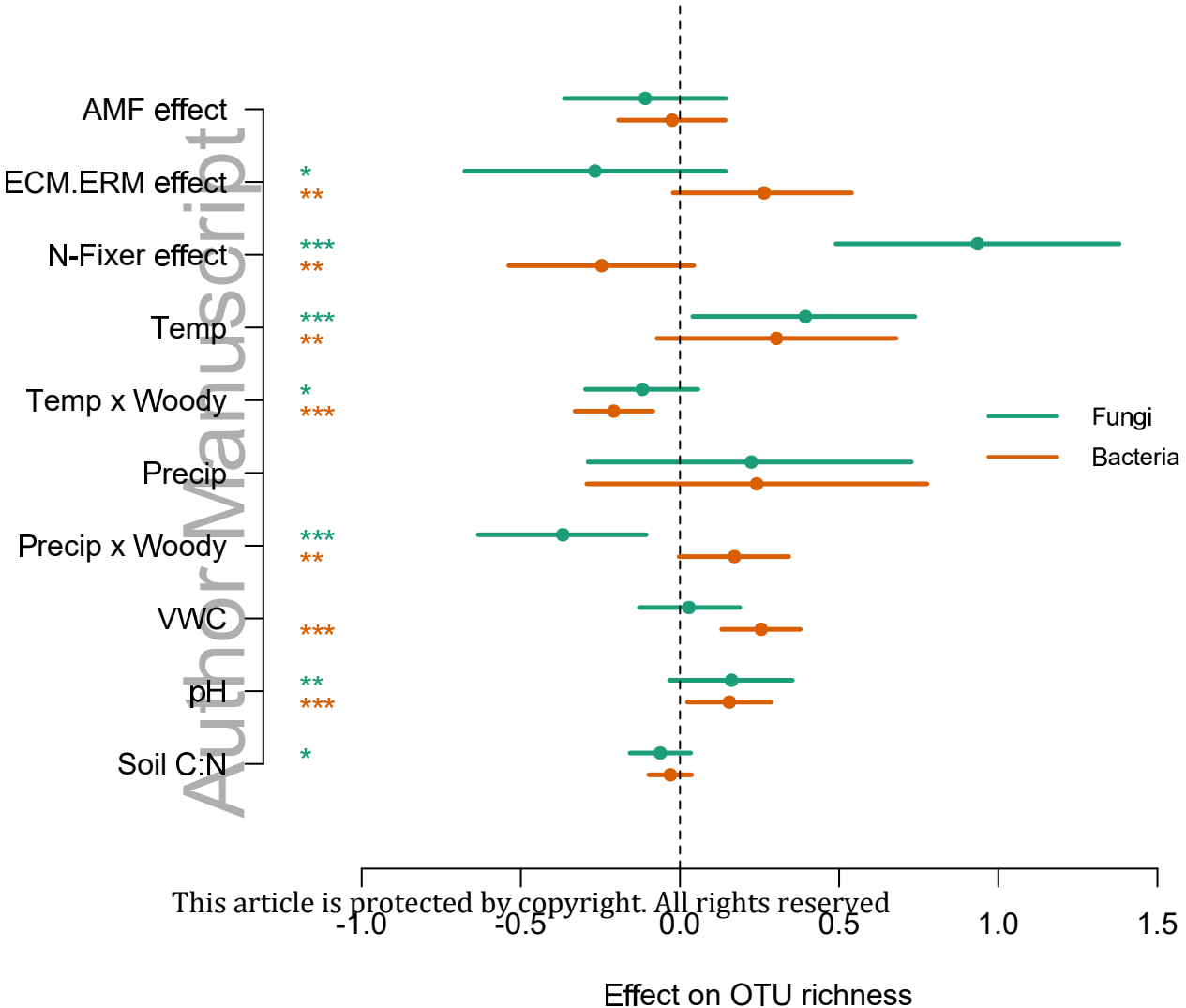

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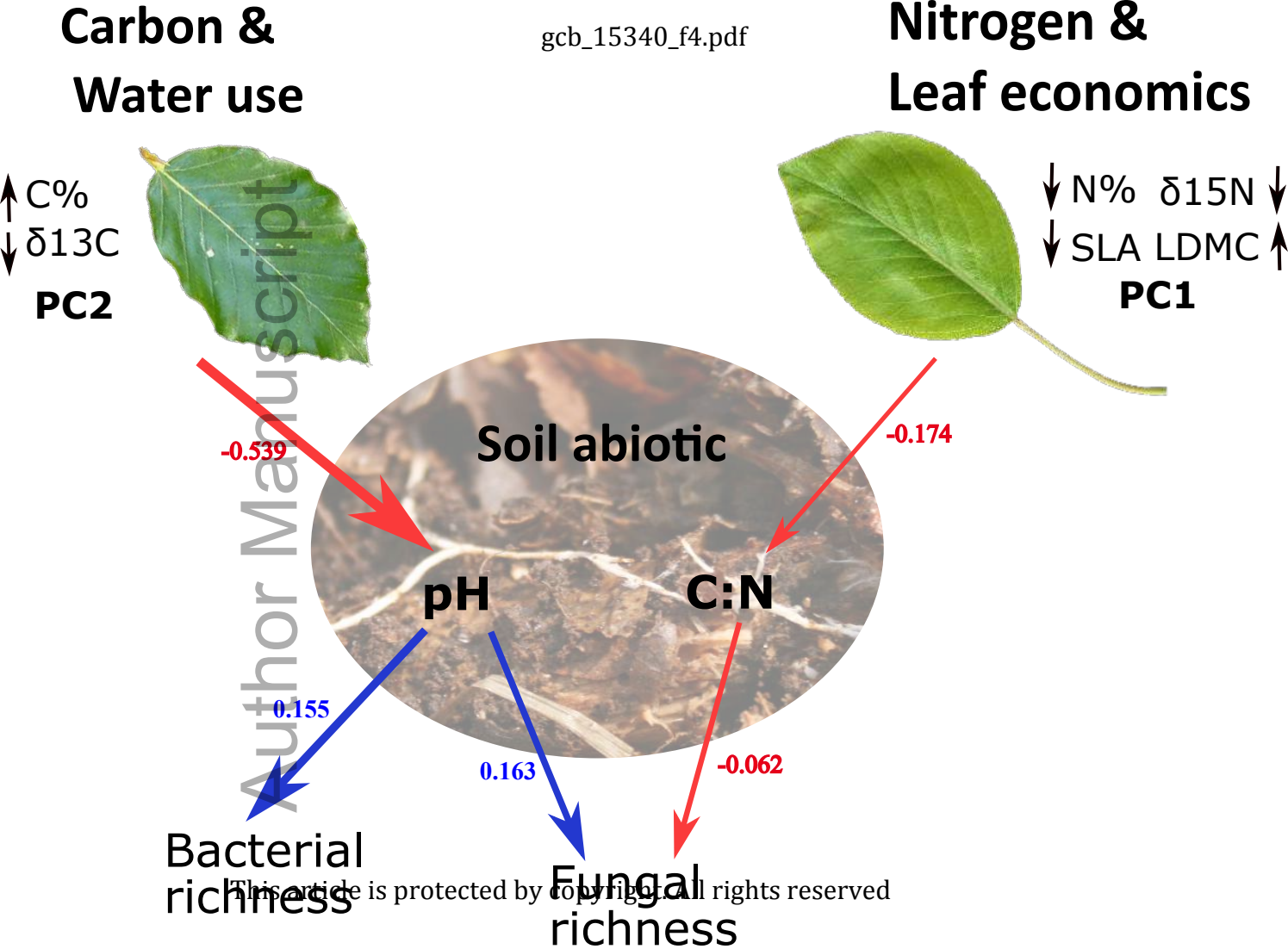

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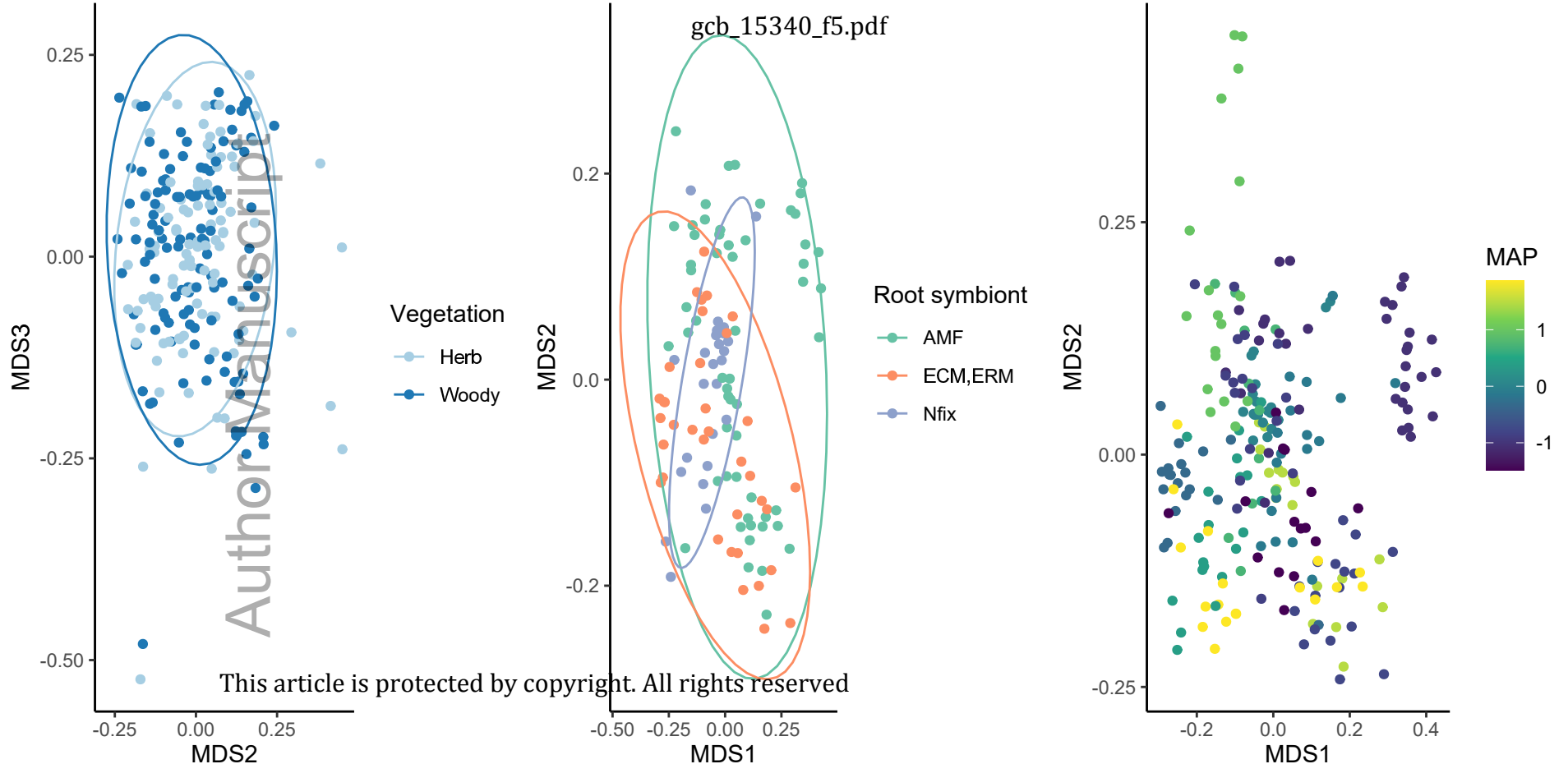

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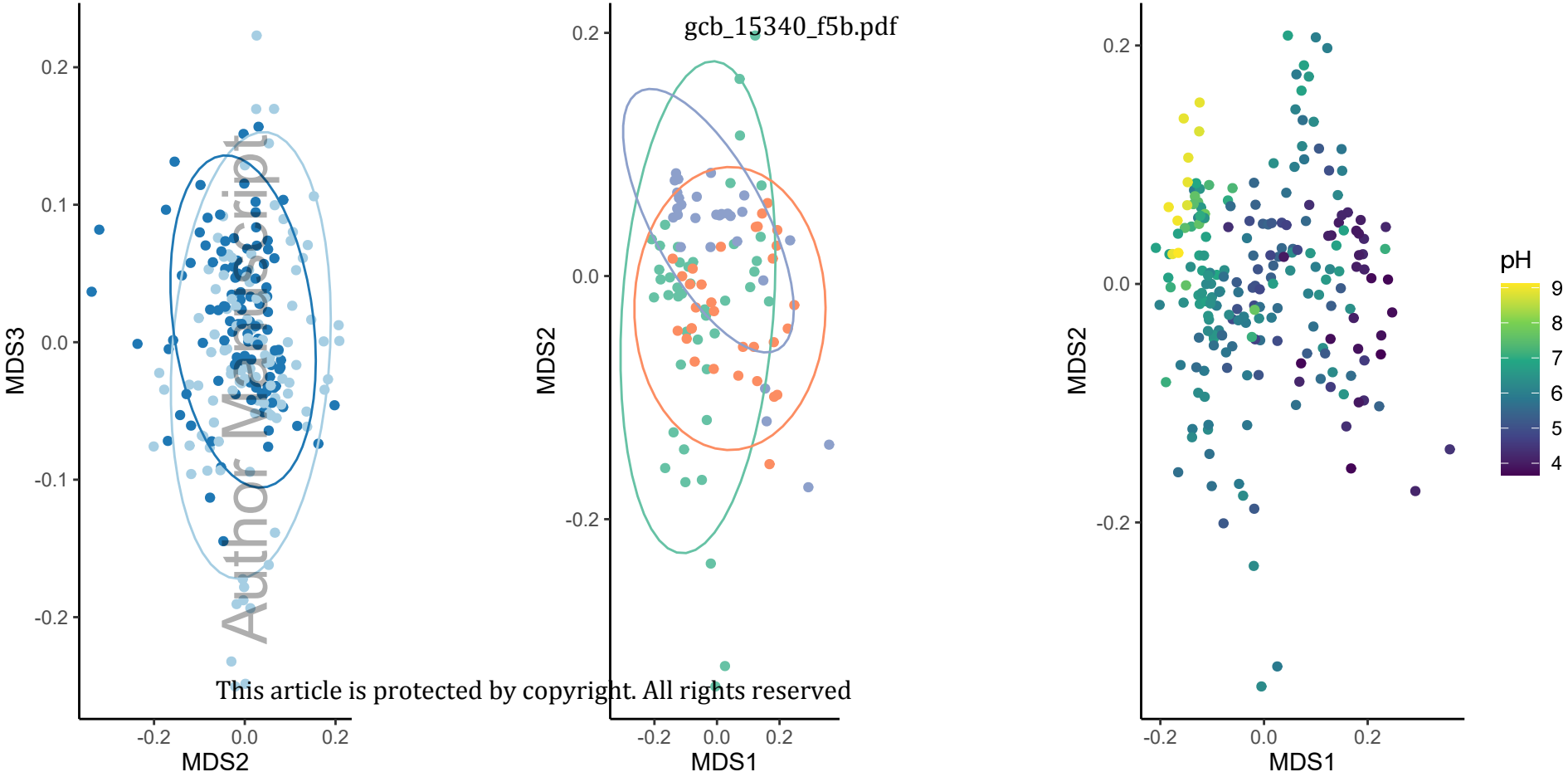

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