7-1-2016

Experimental drought and soil depth interactively influence fungal community composition in piñon-juniper woodland

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Bachelor of Arts in Chemistry
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THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master of Science

Biology

The University of New Mexico
Albuquerque, New Mexico

July, 2016

Acknowledgements

This manuscript would not be possible without the support and extensive guidance provided by Dr. Lee Taylor, my advisor and committee chair. He provided invaluable counsel and coaching in developing this piece of scientific prose, but more importantly in developing my scientific skillset. Dr. Taylor forced me to think and talk like a professional scientist, while using my own voice and exploring my own interests. The lessons I have learned from him will shape me for many years to come.

I also wish to thank Dr. William Pockman and Dr. Catherine Gehring for valuable edits to this manuscript and advise in designing and implementing this project. Dr. Robert Pangle was invaluable in providing information on the study site. Dr. Irina Lagutina provided extensive laboratory support and training throughout. Dr. Jennifer Rudgers provided access to statistical software and help with statistical methods.

I thank the Sevilleta National Wildlife Refuge for permitting sampling at our study site. Also I thank the UNM Department of Biology and the Taylor Lab for funding this project.

A special thanks to Paul Olivas, Marc Olivas, Renne Olivas, Jim Clinch, Gabriel Zambello, Savannah Shoup, Dr. Lee Taylor, Dr. Irina Lagutina, Samantha Fox, Raquel Williams, and Sammy Yousif for help with field work. Without their time and sweat this project would not have been possible.

Lastly I wish to thank my beautiful fiancé Savannah and my parents Paul and Renne for all of their love and support throughout my graduate career.

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ABSTRACT

Drought can flip ecosystems between states and in the process alter key systems such as plant biomass and carbon balance. Many drought-related studies have focused on plant responses or charismatic megafauna. Less work has addressed drought in the context of aboveground-belowground feedbacks. Impacts of drought are increasing, particularly in arid environments such as the southwestern United States. Major tree species of these regions, such as piñon pine, are adapted to a wetter and more predictable climate than the projected future climate. The effects of drought on piñon and its ectomycorrhizae have been studied observationally and in laboratories. Soil depth has also been established in other systems to be an important driver of soil processes, but has thus far been ignored in the piñon-juniper system. Our study is the first to address the effects of long-term experimental moisture manipulation and associated piñon mortality on the soil fungal community of the piñon-juniper woodland as well as that of piñon's obligate ectomycorrhizal partners across soil depth.

We used Illumina sequencing to profile the fungal community of piñon pine at two soil depths along an experimentally imposed moisture-stress gradient from 2008-2013 including drought, ambient, and irrigated treatments. We used field collected soil samples expected to include roots, spores, and free-living mycelia to address fungal change community-wide. We found significant effects of moisture treatment and depth in structuring the overall fungal community of piñon-juniper soils. The drought treatment reduced richness by 20% and 38% in surface and deep soils, respectively. We used FUNGUILD to assign functional roles and found a significant reduction in the proportion of ectomycorrhizal fungi in drought plots, particularly on plots with high piñon mortality.

The change in guild dominance suggests significant impacts of plant mortality coupled with environmental pressures on ectomycorrhizal fungi. Shifts in climate and plant mortality are likely to alter the distribution of members of the belowground community, particularly ectomycorrhizal fungi, which in turn may limit the establishment and/or recovery of plant species. Based on our findings, we predict greater patchiness of fungi, particularly ectomycorrhizal taxa, in drought-impacted habitats and/or significant retractions in plant and fungal geographic ranges.

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Chapter 1

Introduction

Piñon pine (*Pinus edulis* Engelm.) may vanish from much of its present range over the next 50 years due to the combined effects of increasingly severe droughts (McDowell and Allen 2015) coupled with drought-induced changes in its microbial community, i.e. belowground legacies of disturbance. Feedbacks between microorganisms and megafauna are increasingly recognized as critical to understanding living systems in a holistic context. For example, work on the human micro-biome has shown the species composition of the gut flora to influence disease susceptibility and weight status (Turnbaugh et al. 2007; Cho and Blasser 2012). Such work demonstrates the importance of profiling and understanding the contributions of microbes such as soil fungi to eukaryotic organisms and ecosystems at large. A key question is: how do feedbacks between the aboveground plant community and belowground microbial community influence the successional trajectory of an ecosystem after a major disturbance event?

Drought is a major environmental disturbance affecting regions across the globe. While drought may be most common in arid land environments, it is by no means limited to these regions, as recent droughts in places such as Western Europe and the northwestern United States have shown (Cook et al. 1999; Ciais et al. 2003). Drought in the arid southwestern United States is a recurring environmental stressor (Milne et al. 2003) that is predicted to increase in duration and intensity as climate change progresses (Houghton et al. 1996; Seager et al. 2007; Pachauri 2014). Severe drought in 2002-2003 greatly affected the performance and survival of piñon pine across the western United

States (Shaw et al. 2005 McDowell et al. 2008; Adams et al. 2009). Mortality rates of mature piñon trees as high as 100% have been recorded in some stands. (Breshears et al. 2005; Mueller et al. 2005; Royer et al. 2011; Redmond and Barger 2013).

Absence or loss of key soil organisms such as mycorrhizal fungi can lead to poor establishment or low vigor of associated plant species, while lack of plant species can constrain the mycorrhizae present in a system (Molina et al. 1992; Parker 2001; Haskins and Gehring 2005; Nunez et al. 2009). Over 80% of land plants associate with some form of mycorrhizal fungi (Allen 1991; Smith and Read 1997). Mycorrhizal fungi provide plants with increased access to limiting resources such as nutrients and water (Lambert et al. 1979; John and Coleman 1983; Allen 1991). Such aboveground-belowground interactions and their feedbacks are increasingly recognized as critical to understanding ecosystems in a holistic context (Wardle et al. 2004). In agricultural systems, soil organisms such as *Rhizobia* bacteria and arbuscular mycorrhizal fungi are often used to boost crop yields (Biswas et al. 2000; Johansson et al. 2004; Gosling et al. 2006). Manipulation of mycorrhizal fungi and other microbial symbionts has been used by land managers for reforestation efforts in disturbed landscapes (Perry et al. 1987; Cordell et al. 2002; Urgiles et al. 2009). Likewise, lack of a traditional plant host is often found to lead to local extinctions of once dominant microbial soil symbionts (Warner and Mosse 1980; Oehl et al. 2004; Nguyen et al. 2012). Most mycorrhizal fungi, both ectomycorrhizal fungi (EMF) and arbuscular mycorrhizal fungi (AMF), cannot complete their lifecycle without a plant partner to provide carbon (Janos 1980; Allen 1991; Tinker et al. 1994; Smith and Read 1997).

Mutualistic aboveground-belowground feedbacks such as mycorrhizal symbiosis are just only type of soil community change that can occur following a disturbance event. Soil pathogens, decomposers, and other soil guilds may be affected by a disturbance and in turn alterations in these communities may feed back to the aboveground community. For example, fire has been established to lead to a pulse of decomposer activity 1-4 months after burn (Arianoutsou-Faraggitaki and Margaris 1982) which leads to renewed access to soil resources for later plant colonists. Other work has found pathogenic soil microorganisms to proliferate in water-stressed environments (Crist and Schoeneweiss 1975). These pathogens can then infect plants and cause changes to the overall landscape due to plant mortality and associated successional series. Hence to gain a full picture of the feedbacks between the aboveground community and soil fungi, studies should include mycorrhizal fungi and other functional guilds, such as pathogens (e.g. Hewitt et al. 2016).

As all members of the genus *Pinus* that have been directly tested are obligately ectomycorrhizal (ECM)(Molina et al. 1992; Smith et al. 2009; Karst et al. 2014), we expect the same is true of piñon pine across its range throughout the nearly 15 million acres of piñon-juniper (PJ) woodland in the United States (Little 1965; Grabherr et al. 1995; Breshears et al. 2005). Over most of its range, regardless of the presence or absence of other ECM plants, piñon is the dominant ECM host plant. This system thus confronts us with several key questions about belowground legacies of disturbance: What effects, if any, do drought and associated piñon mortality have on the total fungal community of PJ soils? What happens to the obligate ECM fungal partners of the piñon pine when the system experiences severe drought coupled with piñon mortality? What are

the implications for the successional trajectory of the PJ system following tree mortality, i.e. piñon regeneration, given drought impacts on belowground communities?

A significant amount of work has been done to characterize the EMF community of piñon pine at sites in northern Arizona through observational studies and greenhouse manipulations (Gehring and Whitham 1994_a; Gehring and Whitham 1994_b; Gehring et al. 1998; Swaty et al. 1998; Swaty et al. 2004; Haskins and Gehring 2005; Mueller et al. 2005; Gehring et al. 2014). In general, piñon have been demonstrated to benefit from their association with ECM, particularly in stressed environments (Gehring and Whitham 1994). Sites affected by drought and other abiotic stressors such as soil type have been demonstrated to have different EMF communities than lower stress sites. Observations included a shift from Basidomycota to Ascomycota along a gradient of increasing stress and reduced diversity of ECM at the most stressful sites (Gehring et al. 1998; Swaty et al. 2004; Mueller et al. 2005; Gordon and Gehring 2011). A marked shift of the EMF community of piñon pine has been demonstrated to occur in natural settings and seedling bioassays as drought and associated piñon mortality differentially affected certain genotypes of piñon (Gehring et al. 2014). Hence it was shown that natural variation in the aboveground community and its biotic interactions can cause feedbacks to the belowground community. The filtering of the fungal community by stress likely reduces its functional diversity through selection for fungal species that can tolerate the harsh conditions; this filtering may reduce the ability of the EMF community to confer certain benefits (Gordon and Gehring 2011).

Less work has profiled the overall fungal community of piñon pine (i.e. all guilds, not just EMF), particularly with respect to fungi. Kruske et al. (2003) offers one of the

only studies of the non-ECM microbial community of piñon pine. The Kruske study evaluated the differences in the piñon rhizosphere community between a high stress cinder site and a lower stress sandy-loam site. The study used culture-based techniques, and focused primarily on bacterial members of the microbial soil community. The study did examine fungal heterotrophs and finds little correlation with heterotroph abundance and site/stress. However, the fungal heterotrophs were closely associated with the piñon rhizosphere and declined with distance from piñon. In general, there is little information on how the non-ECM community may respond to drought and piñon mortality despite the importance of these groups in nutrient cycling and other ecosystem functions (VanDerPutten et al. 2001; Wardle et al. 2004).

In profiling the mycorrhizal community and the non-mycorrhizal community, some methods are biased against different types of fungal material capable of forming an ecologically relevant living fungus, i.e. inoculum. Certain types of fungal inoculum, especially spores, can possess traits that allow them to persist in harsh conditions for long periods of time (Gallo et al. 1996; Hong et al. 1997; Taylor and Bruns, 1999; Bruns et al. 2009). Most spores, particularly those of basidiomycete EMF taxa (Galante et al. 2011), are dispersed locally, though some taxa have been found to produce spores particularly well suited for long distance dispersal (Peay et al. 2012). Quantity and frequency of spore production is also highly species specific (Peay et al. 2012), but is generally thought to be low and infrequent in arid land systems due to the low availability of water throughout most of the growing season (Sato et al. 2012), though no studies have specifically measured spore production of EMF in arid lands. Work in *Pinus muricata* forests has shown resistant propagules, such as spores, to be the primary agents of mycorrhizal

fungal inoculum after wildfire disturbance (Baar et al. 1999). Even in situations where resistant propagules are available as an inoculum source, poor establishment of ECM seedlings has been linked to the lack of an existing ECM mycelial network (Dickie et al. 2002; Onguene et al. 2002; Nara 2006; Booth and Hoeksema 2010). Live mycelia are likely to survive a much shorter period of time in the absence of a mycorrhizal plant (Jasper et al. 1993). Previous work has shown that piñon seedlings had little inoculum available in juniper-dominated woodland where there were few existing ECM plant species (Haskins and Gehring 2005). Considering the harsh conditions fungi may experience under prolonged drought and other disturbance events it is more informative to use methods that can profile changes in both resistant propagules and live mycelia.

Fungal inoculum potential may also be related to soil depth. Deeper soil horizons present unique challenges for dispersal and colonization, but are sheltered from UV pressure, freeze/thaw cycles, aeolian disturbance, and intense desiccation of surface soils, all of which are particularly strong in arid lands. Other abiotic factors which typically vary with depth include: O_2 concentration, CO_2 concentration, percent organic matter, and pH. These factors, along with biotic factors such as root density, have been found to lead to a different mycorrhizal fungal community in deeper soil horizons relative to the organic horizon community (Taylor and Bruns 1999; Dickie et al. 2002; Landeweert et al. 2003; Rosling et al. 2003). A key study by Lindahl et al. (2007) found a strong spatial segregation of decomposers and EMF with decomposers generally higher in the soil profile. Deep soil mycorrhizal species distributions are also expected to be more patchy, given the difficulty of dispersing to deeper layers of the soil profile (Ferrier and Alexander 1985; Grogan et al. 2000; Tedersoo et al. 2003).

In the PJ system, juniper (*Juniperus Monosperma,* Engelm.) fine roots often dominate the top layers of soil along with their obligate AMF, which are incompatible with piñon (Haskins and Gehring 2005). The dominance of juniper roots at the surface likely causes a segregation of actively growing ECM mycelia deeper in the soil profile (Haskins and Gehring 2005). These findings along with the rapid elongation of the piñon taproot (Harrington 1987) suggest that the deep soil species pool of EMF may be particularly important to piñon. However, the largest and oldest piñon trees, which are most likely to provide ECM networks, are also the most vulnerable to drought-induced mortality (Redmond and Barger 2013). With regard to non-EMF fungi, we might expect their distribution to be more cosmopolitan in accordance with their diverse functional roles and particular substrate preferences, but little is known about this community in the PJ system.

In the piñon-juniper biome within the Sevilleta LTER site, a unique long-run precipitation manipulation experiment presented an opportunity to test the effects of drought and intimately associated piñon mortality on the soil fungal community. We also evaluated the interaction between moisture treatments and soil depth. Together with previous findings in this system, our study stands to fill several gaps. Though general trends of ECM community response to drought have been documented, this experimental manipulation provided the opportunity to explicitly test the effects of moisture-stress while avoiding some of the biases and confounding factors encountered in greenhouse and observational studies. Furthermore, to our knowledge, our study is the first to examine depth as a potential factor in structuring the fungal community in this aridland system. The methods we used can detect a broad range of fungal inoculum from resting

spores to live root tips, thus revealing community-wide changes. Lastly, while focusing on EMF due to their close association with piñon pine, we also profile the soil fungal community at large. Based on the results from our study, we attempt to draw inferences about the ability of piñon to regenerate under permissive climatic conditions.

Drought and associated plant-stress/mortality is predicted to cause shifts in the soil fungal community due to increases in litter from piñon mortality and decreases in soil carbon deposition due to tree carbon starvation during drought. In the absence of healthy, mature piñon trees to provide fungal associates with carbon, together with the direct effects of water deficit, we predict that the EMF community will be strongly filtered. Only a subset of species that can tolerate the more hostile conditions and lack of a plant partner will remain relative to controls. In addition to the elimination of a direct sugar supply by the trees, extreme piñon mortality, and prolonged drought exposes soil fungal communities to greater UV pressure, increased desiccation, and increased freeze-thaw cycles. The major soil community co-inhabitants of the piñon system, particularly pathogens and decomposers, are predicted to increase in relative proportion due to a likely increase in favorable niche space for these guilds, such as dead wood and stressed-plants.

Surface soils are subject to near constant homogenization by wind and water while deep soils are subject to rare and stochastic fungal colonization. Surface soils also see high inputs of organic matter, while the main source of organic matter in deep soils is roots. Hence we predict a strong distinction in fungal community composition between surface horizon soils and deeper soils. Due to inaccessibility of deeper soil, we predict lower fungal species richness compared to the surface horizon. On the other hand, we

predict that the communities of the deeper horizons are somewhat buffered from the effects of drought and piñon die-off because the abiotic stresses are less intense. Hence deep soils are likely to see less alteration in community composition across treatments. We predict that drought will decrease dominance of EMF across all soil types, but more so in the surface soils. Decomposers and other fungal guilds are likely to proliferate as ECM niche space opens, but richness is likely to decline overall as inputs to the system decline. Lastly we predict that soils receiving additional water, which saw similar levels of piñon mortality as controls, will not see greatly altered fungal communities resulting from a weak release from ambient moisture availability (Pangle et al. 2015). We test the following hypotheses:

1. Drought reduces the total soil fungal community richness of piñon pine
2. Drought alters the overall composition of the soil fungal community
3. Deep soil harbors a more patchy and altered fungal community relative to surface soils.
4. Drought alters the ECM community composition
5. Deep soil EMF taxa show less decline than surface soil taxa exposed to severe drought
6. Soil decomposers and soil pathogens increase with moisture-stress

Chapter 2

Materials and Methods

Site Description

Our study was conducted within a long-term precipitation manipulation experiment located on the Sevilleta Long Term Ecological Research site (LTER) at the Sevilleta National Wildlife Refuge near Socorro, NM, USA. Detailed descriptions of the plots, setup, and rationale for the manipulation can be found in Pangle et al. (2012) and Plaut et al. (2012). The study site is located on the eastern slopes of the Los Piños mountains (34°23'11" N, 106°31'46'' W) at an elevation of approximately 1911 m. Mean annual precipitation in the area over a 20 year period (1991 – 2011) was 362.7 mm/yr. Temperatures ranged from a daily minimum of -3.3°C to a daily maximum of 31.0°C.

Piñon pine and juniper dominate this mature (50+ years of ungrazed growth) piñon-juniper woodland. The understory includes several species of shrubs (*Fallugia paradoxa, Rhus* spp., *Mahonia* spp., and *Quercus* spp), grasses (*Bouteloua* spp.), and cacti (*Cylindropuntia* spp., *Opuntia* spp., and *Yucca* spp.). The manipulation experiment was comprised of twelve 40 x 40 m plots, which were grouped into 3 blocks and assigned to 4 treatments. The treatments were an ambient control, a cover control, drought, and irrigation (see Supplemental Figure 1 for site map). Blocks varied in slope and aspect with one north facing, one south facing, and one flat block. Both sloped blocks had rocky, shallow soils, while the flat block soils were a sandy loam in texture and comparatively deep. Ambient control plots were not manipulated other than instrumentation to measure target tree physiology and environmental variables. Drought

plots were equipped with upward-facing plastic troughs, reducing natural precipitation delivered to plots by 45.5% (Pangle et al. 2012). Cover control plots used identical infrastructure as the drought plots, but with downward-facing troughs to test the effect of the plastic infrastructure while allowing full precipitation to reach the plot. Cover controls and drought plots had air and soil temperatures that were consistently elevated 1°-3°C above controls and irrigation plots (Pangle et al. 2012). We opted not to sample cover control plots because of the relatively minor effect of infrastructure observed. The warming provided by the drought infrastructure also provided a proxy for climate-change-induced drought where warming is coupled with decreased precipitation. Irrigation plots were equipped with overhead sprinklers to add an amount of water approximately 73mm above ambient rainfall over a season, roughly a 20% increase from the 20 year average (Pangle 2012). Irrigation events coincided with several pulse events during the growing season (April-October).

Infrastructure was installed in the summer of 2007, the drought treatment began in August 2007 and the irrigation treatment began in the summer of 2008. Drought treatments were maintained through the duration of this study. Irrigation treatments were decommissioned in October of 2013. We sampled soils during February of 2015. The experiment also included instrumentation of target trees to measure physiological parameters and soil instrumentation to measure abiotic factors (Pangle et al. 2012). Abiotic conditions were recorded under piñon canopy, juniper canopy, and inter-canopy areas, including soil temperature at 5cm depth, air temperature at 10cm above soil, and volumetric water content at 5cm depth. All measurement sites were located inside a 10m buffer zone to avoid plot edge effects.

Supplemental Figure 1:

Supplemental Figure 1: Layout of original PJREx manipulation as described in Pangle et al. (2012) and others. Flat block plots are numbered 1-4. North block plots are numbered 5-8. South block plots numbered 9-12. We did not sample cover control plots (3, 7, and 11), which are not shown. Irrigation infrastructure was dismantled in October 2013.

During much of the experiment there was a moderate to severe natural drought ongoing in the region, which affected all plots simultaneously (US Drought Monitor June 2007 – October 2013, accessed online 4/01/2016). Most plots experienced some level of piñon mortality, including irrigation plots. Mature piñon mortality was 100% in drought plots on hill slopes (both North and South facing) within approximately 11months of the initiation of the drought treatment (Plaut et al 2012). Some juvenile (<10 years of age) piñon trees persisted on the sloped drought plots. Heavy juniper canopy loss also occurred in drought plots on hill slopes, although the juniper decline occurred over a longer time period than piñon mortality, and appears to consistent of partial canopy dieback rather than death of the entire tree. Many mature piñon trees persisted on the flat drought plot and there was little juniper canopy loss.

Piñon canopy cover prior to experimental setup as measured by basal area at 30cm stem height (m^2/ha) was 5.2 for irrigated plots, 1.9 for drought plots, and 2.4 for ambient control plots (Pangle et al. 2012). Juniper canopy cover prior to experimental setup was 19.1 for irrigated plots, 15.7 for drought plots, and 17.7 for ambient control plots (Pangle et al. 2012). In the 2012 basal area census, piñon basal area revealed a 10% reduction for irrigated plots, 68% reduction for drought plots, and a 9% reduction for ambient control plots (Pangle et al. 2015). The 2012 juniper basal area census revealed no change for irrigated plots, a 26% reduction for drought plots, and a <1% reduction for ambient control plots (Pangle et al 2015).

Plot Data

Each soil sampling point was designated as juniper canopy, piñon canopy, or inter canopy. These designations were used to weight the number of each type of

moisture/temperature sensor used in statistical analysis. Data were removed from analysis if more than 10% of the data range was not available. Of the remaining probes available, data were trimmed by date to span the start of each treatment through the sampling date, in order to account for the cumulative effects environmental variables on a given treatment. Measurements were recorded every 15 minutes. In the case of the irrigation treatment this allowed us to capture the effect of added moisture during the treatment, but also account for the ~2 years between the termination of the irrigation treatment and our sampling.

Average soil temperature (°C), average air temperature (°C), and average soil moisture (%VWC) were analyzed as covariates along with soil nutrient data. Approximately 237 ml of homogenized plot x depth soil combination was sent for manure/compost nutrient analysis (Soil, Water and Plant Testing Laboratory, Colorado State University, Fort Collins, Colorado, USA). Nutrient and soil properties in the analysis included: percent organic matter, pH (1:5), total N (ammonium + nitrate + organic), total P (mineral + ortho + organic), total K (mineral + organic + soluble), and C:N ratio.

Sampling Design

We sampled soils from all irrigation, ambient control, and drought plots between 2/21/2015 and 3/1/2015. We assigned 20 sampling points within each plot using a haphazard stratified-random spatial design, as follows. Four people, each with five disks, chose a plot corner and then moved toward the plot center roughly 15m. From this position, each person threw his or her disks in random directions. Disks were re-thrown if they fell within the 10m edge-buffer or if they landed in a spot that was inaccessible, i.e.

bole of a tree, center of a cactus, or on a large rock. Acceptable points were then flagged and numbered 1-20 on each plot for sampling.

At each point, a soil pit approximately 20cm long by 20cm wide by 25cm deep was excavated. Pits were offset from the flag by roughly 5cm. All instruments were sterilized with 70% ethanol between plots. A new "face" was added to each pit at the time of sampling with a sterile trowel. The top face was added and the surface soil was sampled before facing and sampling the deep soil. Instruments used for surface sampling were never used for deep sampling and vice versa to avoid cross contamination between soil depths. Surface soil samples were taken from the surface to approximately 5cm deep. Deep samples were collected at approximately 18-23cm deep. We used homemade PVC and galvanized pipe corers that were easily field sterilized and durable enough to withstand the hard, rocky soils at our site. Roughly 120ml of soil/rock mix was taken from each depth at each sampling point for a total of approximately 2.4 liters of soil/rock mix per depth per plot. All soil from each plot by depth combination was combined in sterile plastic containers.

Plastic containers were vigorously shaken and mixed by hand with a sterile trowel to homogenize the soil. Sterile pots were filled with homogenized soil in the field for a parallel greenhouse experiment. Two sterile 50ml falcon tubes (VWR International, Radnor, Pennsylvania, USA) were filled in the field with sieved soil. Each tube was considered a technical replicate of its plot by depth combination. Sieves were constructed from ¾ inch PVC and window screen with 2mm diameter mesh openings. Sieves were easily sterilized in the field. Falcon tubes were immediately put on ice and stored at -80˚C

no more than 6 hours after collection. Remaining soil/rock mixture was bagged and stored at -80˚C for soil nutrient analysis.

Bench Methods

Genomic DNA was extracted from approximately 5 grams of soil per technical replicate using the Mo Bio Powersoil kit (MO BIO Laboratories, Carlsbad, California, USA) according to the manufacturer's instructions. DNA extracts were checked visually by gel electrophoresis and via nano drop (NanoDrop-2000, Thermo Scientific, Waltham, Massachusetts, USA) to approximate DNA concentration. We utilized a two-step polymerase chain reaction (PCR) approach to amplify the ITS2 region of the nuclear internal transcribed spacer and then add indexes and Illumina sequencing adaptors (Craig et al. 2008). The first-step reactions contained 5ul 5x HFbuffer (Thermo Scientific, Waltham, Massachusetts, USA), 0.5ul 10mM dNTPs (New England Biolabs, Ipswich, Massachusetts, USA), 0.125ul of each 50mM primer, 0.25ul Phusion DNA polymerase (Thermo Scientific, Waltham, Massachusetts, USA), 14ul sterile PCR water, and 5ul of 1:10 diluted template DNA in a total volume of 25ul. The core primers (in bold) were ITS4_Fun (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGCCTCCGCTTATTGATATGCTTAART**) and 5.8S_Fun (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**AACTTTYRRCAAYGGATCWCT**) with universal Nextera adaptor sequences (underlined) (Taylor et al. 2016-under review). The following thermocycling protocol was used in the first-step PCR: 30 seconds at 98°C, followed by 24 cycles of 10 seconds 98°C, 10 seconds 58°C, and 4 minutes 60°C, lastly 1 round of 20 minutes at 60°C and incubation at 4°C. All PCR

reactions were carried out using a C1000 touch thermal cycler (Bio-Rad, Hercules, California, USA) or a SimpliAmp thermal cycler (Applied Biosystems, Foster City, California, USA). Amplified fragments were visualized on a 1.5% agarose gel. Positive PCR controls, a mock community control (described in detail in Taylor et al. 2016, under-review), and negative controls were included in all PCR runs (Lindahl et al. 2013; Nguyen et al. 2015). Positive controls were discarded after confirming successful PCR amplification. We used the lowest possible number of cycles that still amplified a visible fragment to reduce PCR bias and potential chimera formation (Polz and Cavanaugh 1998; Sipos et al. 2007; Gibson et al. 2009; Lahr and Katz et al. 2009; Lindahl et al. 2013). For all samples that did not yield visible fragments, we increased the number of PCR cycles to 26 (i9m1, i9m2, d2m1, d2m2, d6m1, d6m2, d10o2, c4m1, and c4m2). D10o2 required a 1:100 dilution of template DNA at 26 cycles to yield a visible amplified fragment.

Each sample was amplified in four PCR replicates, which were pooled following the PCR. All negative controls were also pooled. By pooling multiple PCR replicates we sought to average across stochastic events in any one reaction that might have contributed to bias (Lindahl et al. 2013, but see also Smith and Peay, 2014). All pooled samples were run through the Zymo Clean and Concentrator-5 kit according to the manufacturer instructions (Zymo Research Corporation, Irvine, California, USA). Samples were eluted in 50-ul TE elution buffer. After cleaning and pooling all samples were arrayed on a 1.5% agarose gel for approximate quantification of DNA. Dilutions were calculated based on visual analysis in Microsoft Paint (Microsoft Corporation, Redmond, Washington, USA) to dilute all samples to the approximate strength of the weakest samples. Each sample was replicated for sequencing by placing 22-ul of each normalized

sample into two separate sterile 1.5-ml micro centrifuge tubes (USA Scientific Inc., Ocala, Florida, USA). Hence each sample had two replicates sent for sequencing e.g. i1o1a and i1o1b, each of which utilized a different pair of forward and reverse indexes (see below).

All samples were sent to the Genome Sequencing and Analysis Facility (The University of Texas, Austin, Texas, USA) for Illumina MiSeq paired-end sequencing. The second round PCR at UT was prepared in 96 well plates with each reaction containing 15 uL NEBNext 2x master mix, 5 uL Hyb_Barcoded Primers, and 10 uL amplified DNA for a total of 30 uL per PCR reaction. The second round thermal cycler conditions were as follows: 98 °C for 30 seconds 1x, 98 °C for 30 seconds followed by 62 °C for 30 seconds, followed by 72 °C for 30 seconds with these 3 steps repeated 7 times, then 72 °C for 5 minutes, and lastly incubate at 4 °C. Following thermal cycling, samples were cleaned using AMPure bead XP purification. qPCR was used to determine sample concentrations. Products were pooled following qPCR and pooled samples were then run on a BioAnalyzer DNA chip to check for successful second round amplification and purity. The pooled samples were then run for 500 cycles on an Illumina MiSeq instrument using an Illumina kit (MS-102-3003-MiSeq® Reagent Kit v3).

Bioinformatics

Most bioinformatics processing was performed using QIIME version 1.9.1-20150604 (Caporaso et al. 2010). For each sample, the sequencing center provided a forward and reverse read in fastq format along with corresponding index files. Overall sequence quality was initially examined with FASTQC (Andrews 2010). Our mock community contained eight species of fungi (one chytrid, one zygomycete, one

ascomycete and five basidiomycetes) combined in known proportional abundances spanning three orders of magnitude (Taylor et al. 2016 -in review). All QIIME settings were established using our mock community dataset with the goal of clustering to obtain one OTU corresponding to each community member in approximately the correct proportions (Nguyen et al. 2015).

We first performed the split_libraries_fastq.py command using lax quality settings (q=1, p=.01, r=5, n=5) to filter out sequences with barcode mismatches without greatly reducing sequence length through quality trimming. The convert_fastaqual_fastq.py script was used to convert split_libraries_fastq.py output into fastq format prior to joining paired-end reads. The resulting sequences were then run through the join_paired_ends.py script (j=40 p=10); the forward un-joined reads and joined reads were then combined for further processing. We did not want to bias against taxa that have longer ITS2 regions and would therefore be less likely to successfully join at this step, so we elected to use both joined and forward reads (Nguyen et al. 2015). The forward read is from the ITS4 end of the amplicon. Forward and reverse primers were removed using the extract_barcodes.py script. The resulting sequences were run through split_librarties.py again using strict quality filtering parameters tuned to the mock community (q=19, p=.23, n=0).

Sequences were clustered using the pick_open_reference.py command. Pre-filtering of sequences was enabled to remove the vast majority of non-fungal sequences by requiring a match to a sequence in the UNITE 97 database version 7 updated 1/8/2015 (Kõljalg et al. 2005) at the 50% identity threshold. USEARCH 6.1, a complete-linkage clustering algorithm, (Edgar 2010) with s=94 was used as the clustering algorithm. The

UNITE 97 database was also used as the reference database for clustering. Representative sequences were assigned based on the most abundant read present in an OTU. The assign_taxonomy.py script with the blast method was used to match the representative sequences for each OTU to the UNITE 97 database. OTUs that did not return a UNITE 97 blast hit, with more than 100 sequences were manually blasted against the GenBank database. For OTUs returning a GenBank blast hit, taxonomy was assigned according to GenBank with a preference against uncultured submissions. This manual assignment was only used for the Funguild analysis described below. The final OTU table was created with the make_otu_table.py script. Biom_convert was used to change the format of the OTU table from standard QIIME .biom format to .csv format for further analysis.

Statistical Methods

Species richness was compared among treatments and depths using EstimateS software (Colwell 2006). Estimate S was also used to generate OTU accumulation curves for each sample to provide an estimate of how thoroughly the fungal community was sampled in each treatment by depth combination.

All further statistical analyses were performed in PRIMER 6 unless otherwise noted (Anderson et al. 2008). Data were standardized by sample totals (McCune et al. 2002; Nguyen et al. 2015). A Bray-Curtis similarity matrix was calculated to compare community composition across samples (Bray and Curtis 1957; McCune et al. 2002). Non-metric multi-dimensional scaling (ordination) was used to visualize the similarity of different samples using a kruskal fit scheme of 1, a minimum stress of 0.01, and 9,999 restarts (Carroll and Chang 1970; McCune et al. 2002). Various factors, sample groupings, and covariate vectors were overlaid on these plots with specific

methods/parameters described below. Grouping by similarity was performed using the group average cluster mode with groups overlaid at various similarity levels. The PERMDISP function in PRIMER was used to test for heterogeneity in groups defined by a given factor i.e. depth or treatment. Distances were tested relative to centroid mean and p-values were calculated from 9,999 permutations.

A nested, hierarchical permutational MANOVA, or perMANOVA, was used to test for differences among samples in relation to predefined factors (Anderson et al. 2005). PerMANOVA is a statistical test for the simultaneous response of one or more variables to one or more factors in an ANOVA experimental design on the basis of a chosen resemblance method (Anderson et al. 2005). Factors included depth (fixed, n=9), treatment (fixed, n=3), plot (random-nested in treatment, n=1), and aspect (fixed, n=3). We used the Bray-Curtis similarity matrix as our resemblance method with a type III (partial) sum of squares, permutation of residuals under a reduced model, and 9,999 permutations.

Continuous covariates were normalized and a Euclidian distance similarity matrix was calculated between samples (McCune et al. 2002). The covariate similarity matrix was compared to the OTU x site similarity matrix using a Mantel test under the BEST function in Primer with the spearman rank correlation method (Smouse et al 1986). Covariates were also analyzed with Pearson correlation vectors overlaid on ordination plots (Lawrence and Lin 1989). Further analysis of environmental covariates was not performed due to low replication inherent in the original experimental design and issues of co-linearity associated with many of our environmental variables (Belsley et al. 2005).

We used a SIMPER analysis in Primer to examine the contribution of individual species (OTUs) to average similarity among treatment groups. We used a one-way design with groups defined by treatment factor and a cut off for low contributions of 90%.

Phylum level taxonomy was assigned in QIIME and analyzed and plotted by treatment and depth combination. Guild assignments were made in Funguild (Nguyen et al. 2015). Guilds were combined as follows: other = ericoid mycorrhizal + lichenized + arbuscular mycorrhizal + endophyte; ectomycorrhizal = ectomycorrhizal; pathogen = plant pathogen + animal pathogen + mycoparasite; saprotroph = soil saprotroph + litter saprotroph + undefined saprotroph + wood saprotroph. These combinations were made to reflect our key questions about fungal community change resulting from drought that might result in feedbacks relating to piñon pine regeneration.

Differences in proportions of EMF, saprotrophs and pathogens were calculated between pairs of blocked controls and drought treatments within a soil depth. In order to compare guild changes between treatments we subtracted percent of a given guild in the drought treatment from percent of a given guild in the control treatment within a given block/aspect. These differences were tested against a null hypothesis of no difference between pairs using a Wilcoxon signed-rank test with a 95% confidence interval, $p<0.05$ (Wilcoxon and Wilcox 1964). All graphs and figures were created in PRIMER, Microsoft Excel, or R (R Core Team 2014).

Chapter 3

Results

Quality Control

Raw read quality, as visualized with FastQC, was generally best between base 25 and 200. Number of raw reads per sequencing replicate ranged from 205 reads to 27,308 reads. We obtained a total of 889,032 raw reads for all non-control samples combined. The quality filtering parameters above resulted in removal of 25% of the raw reads. The median per sample number of reads after filtering was 9,509 with a minimum of 26 reads and a maximum of 20,653 reads.

Negative control A and negative control B, had 1,087 raw reads and 788 raw reads respectively. After quality filtering, negative control A had 11 total reads and negative control B had 6 reads. These reads fell into 5 and 2 OTUs, respectively. Due to the small number of reads and OTUs in our negative controls, indicating very little contamination, we felt it was unnecessary to correct species abundances in our samples based on the negative controls (see Nguyen et al. 2015).

Mock Community Analysis

The "mock b" sample had 7,979 reads prior to bioinformatics processing. After barcode filtering, paired-end joining, and quality truncation 82% of reads were retained. The final parameters resulted in recovery of 7 of the 8 mock community members as distinct OTUs. We did not recover an OTU corresponding to the least abundant fungal genus of the mock community (*Mortierella*) under any parameters. Only one sequence from this taxon occurred in the original raw reads, but was removed during quality filtering. Except for the least abundant taxon, our final parameters reflected the known

community in both composition and relative abundance. A total of 14 fungal OTUs were recovered from the mock b community dataset, with 7 of these OTUs being the expected OTUs in correct proportional abundance. Of the "extra" OTUs, two returned blast hits similar to two of the expected OTUs, but having only 4 reads combined. Repeated attempts to correct this over-splitting by changing parameters led to a loss of expected OTUs, so we proceeded with the parameters above. The remaining fungal OTUs were likely contaminants, but occurred at such low proportional abundances that they were not considered further.

The "mock a" dataset, a sequencing replicate of mock b, had just 586 reads before quality filtering. Using the parameters tuned to the mock b dataset, the number of reads was reduced to 194 through read joining and quality trimming. We elected to not use this sample for parameter tuning, as this dataset was too small. However, using the parameters from mock b we recovered 6 of the 8 mock community members in correct proportions. Reads corresponding to the 2 missing known community members were not found in the mock a dataset. The two missing community members were the lowest and second lowest abundance community members.

Clustering and Taxonomy

After bioinformatics processing in QIIME using a 94% species identity, we found 5,147 OTUs across all samples. The complete-linkage clustering method of the USEARCH algorithm we used generally creates more OTUs than the more widely used single-linkage method at a given percent identity (Lindahl et al 2013), supporting our decision to use a lower absolute species identity than is commonly applied in studies utilizing single-linkage methods. Sequences occurring only once across all samples were

represented by 1,525 OTUs (29.6% global singletons). Global singletons were removed from further analysis. Non-singleton OTUs that did not match a sequence in the UNITE 97 database or blast to a sequence in the database accounted for 19% sequences. OTUs which did not return a UNITE 97 match or blast hit in QIIME accounted for 8.59% of clustered OTUs. The median number of reads per unassigned OTU was 8. The maximum number of reads for an unassigned OTU was 4,560. 82 unassigned OTUs had 100 or more reads.

Preliminary 2d and 3d ordination showed no observable effect of sequencing replicate on the fungal community observed. We therefore combined sequencing replicates for all analyses (minimum number of reads per sequencing replicate was 26 with a maximum number of reads of 20,653). A major benefit of this approach was that sequencing depth per sample was more even and much higher with a minimum number of sequences per technical replicate of 9,096 and a maximum of 130,808 sequences per sample. Technical replicates also showed considerable similarity in the location of samples within ordination space; we elected to not combine these replicates as they were derived from discrete subsamples of soil.

Ascomycota was the dominant phylum in all treatments and soil horizons except for irrigated deep soils where Basidiomycota was dominant with 47.27% of sequences (Figure 1). Ascomycota was dominant in the drought surface horizon soils with 74.54% of reads compared to 10.93% of reads for Basidiomycota. Basidiomycota reads generally accounted for a larger percentage of sequences in deep soils than in surface soils. The “other” phyla category accounted for 4% of sequences across both soil horizons, this

category includes sequences assigned to Glomeromycota, Zygomycota, Chytridiomycota, and Rozellomycota.

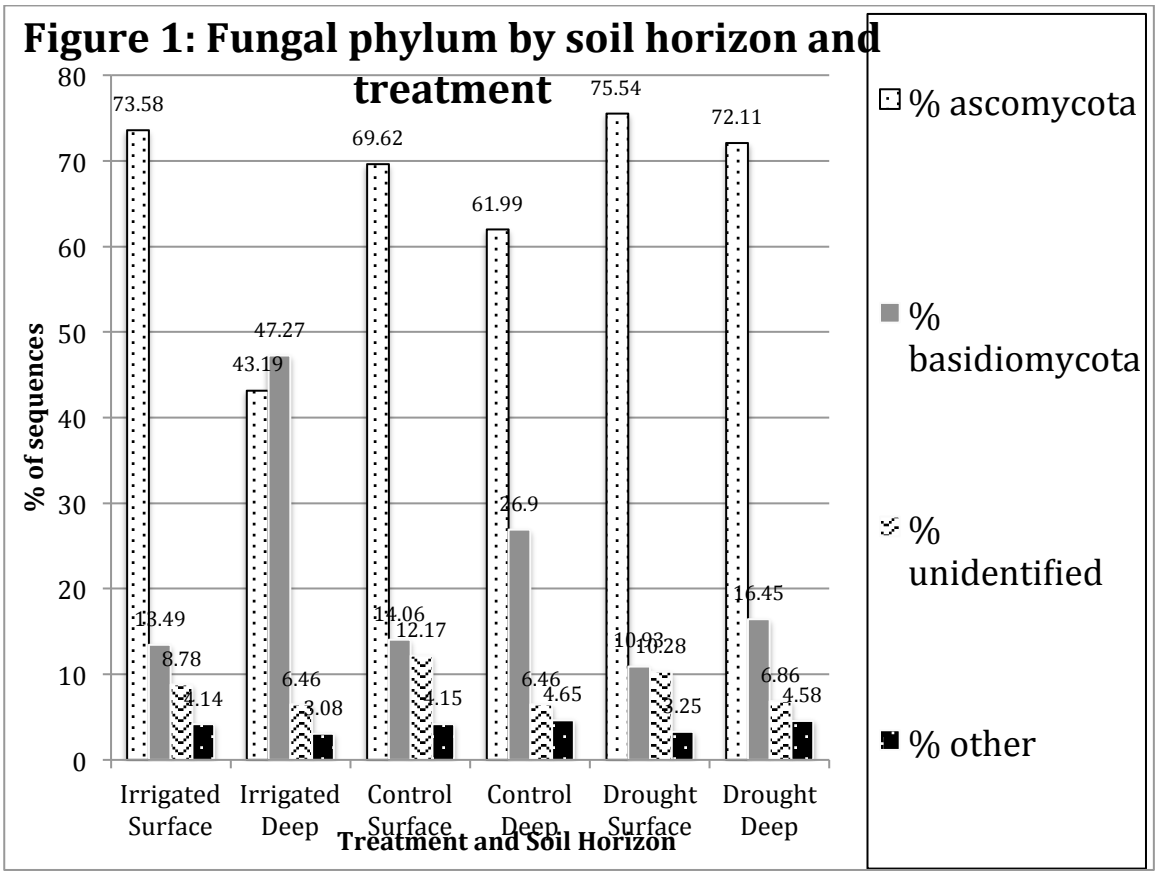

Figure 1: Phylum level taxonomic composition of pooled treatment and soil horizon combinations by percent of reads assigned to a given fungal phylum according to UNITE 97 blast taxonomy assignment in QIIME. Ascomycota were the dominant phylum across treatment and soil horizon except in irrigated deep soils where Basidiomycota were dominant (47.27%). Note that Basidiomycota percent abundance generally declined across the experimental moisture gradient and that Basidiomycota are generally higher in percent abundance in deep soils relative to surface soils. "Other" phyla included sequences assigned to: Glomeromycota, Zygomycota, Chytridiomycota, and Rozellomycota. Unidentified sequences were sequences that did not return a blast hit, which accounted for 8.6% of post-QC, non-singleton reads.

OTU Accumulation Curves

The surface drought treatment had an average reduction in richness of 36% relative to the control treatment in that soil layer and an average reduction of 19% relative to the irrigated treatment (Figure 2). Richness of the deep drought treatment was reduced an average of 18% relative to the deep soil control treatment and an average of

14% relative to the deep soil irrigated treatment. Richness is 50% lower in deep soils compared to surface soils, averaged across treatments. The surface soil control community had the highest level of richness, with an average richness 21% greater than the irrigated treatment. In deep soils, the control and irrigated communities had smaller differences in richness, with an average 5% reduction in richness in irrigated plots. The deep soil OUT accumulation curves approach saturation, indicating that our sampling of the fungal community in this horizon was fairly thorough. The surface soil curves indicate a less thorough sampling of the community, with a positive slope at the maximum sequencing depth.

Precipitation Manipulation Effects

Drought treatment samples generally group together, while controls and irrigated samples cluster away from drought plots along the X-axis of ordinations at both soil depths (Figure 3 and Figure 4). The drought plots in the deep soil horizon have an even stronger separation relative to surface soils along the X-axis from other treatments (Figure 3). The block effect is evident along the Y-axis of the deep soil horizon ordination, where the flat block/aspect plots d2, c4, and i1 are all aligned towards the top of the plot away from the other blocks (Figure 4). In addition to ordination, the full perMANOVA model including depth

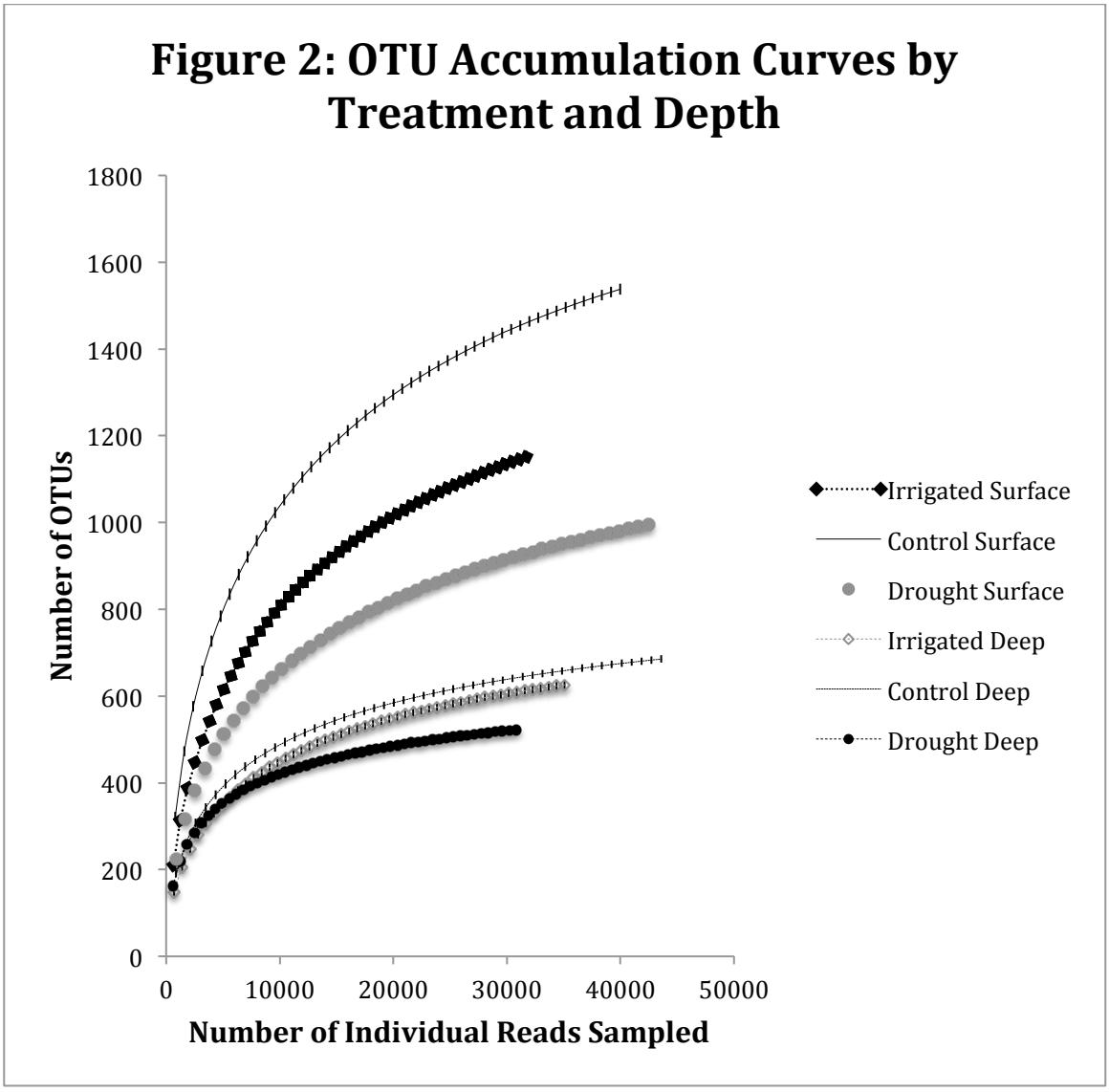

Figure 2: OTU accumulation curves by treatment and soil horizon. Vertical lines indicate 95% confidence intervals. Fungal richness is greater in surface soils than in deep soils. Drought soils have reduced fungal richness relative to control/irrigation treatments across depths.

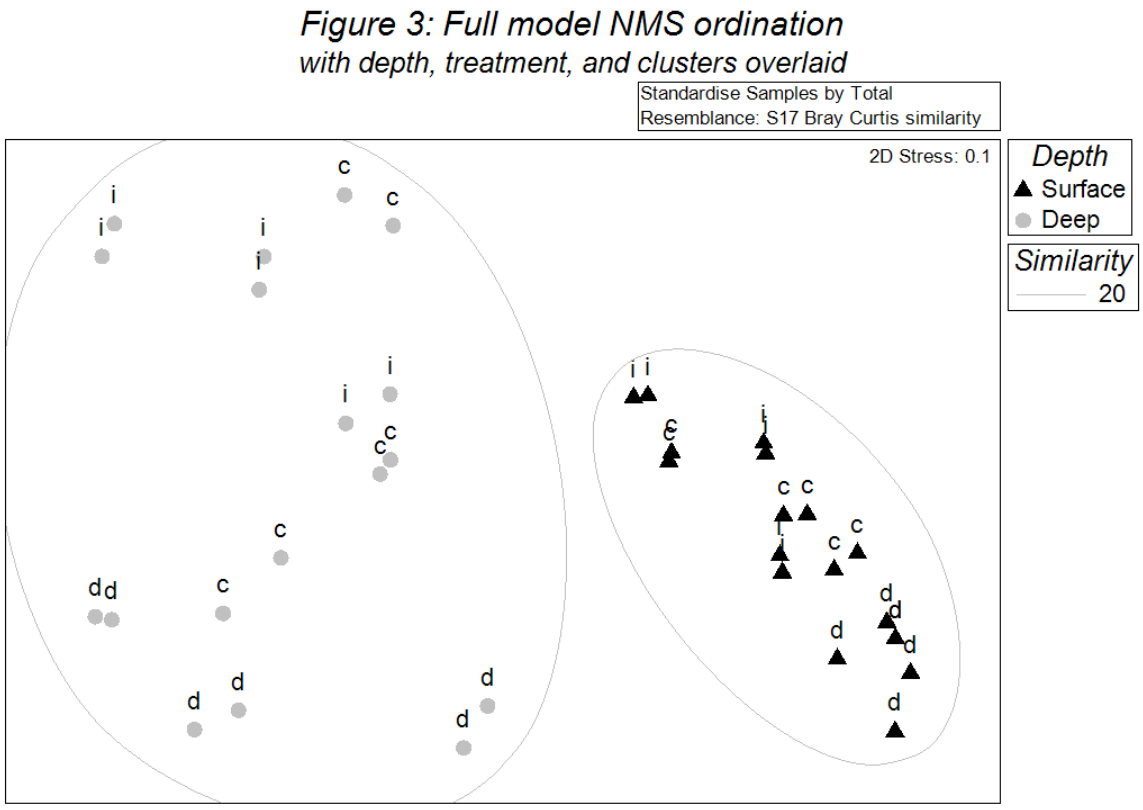

Figure 3: Non-metric multi-dimensional scaling (ordination) with depth and treatment overlaid. The ordination is based on a Bray-Curtis similarity matrix with samples standardized by total. Spheres represent group average clusters using a 15% similarity threshold. Highly similar points with the same label are technical replicates. Sample labels are as follows: d=drought, i=irrigated, c=control. Note the strength of the depth effect on the structure of the fungal community as revealed by the two distinct clusters that separate along the x-axis. The y-axis appears is related to treatment wherein drought soils cluster away from other treatments near the bottom of the plot. Surface soils were much more similar to each other regardless of treatment while deeper soils were more heterogeneous in composition.

Figure 4:

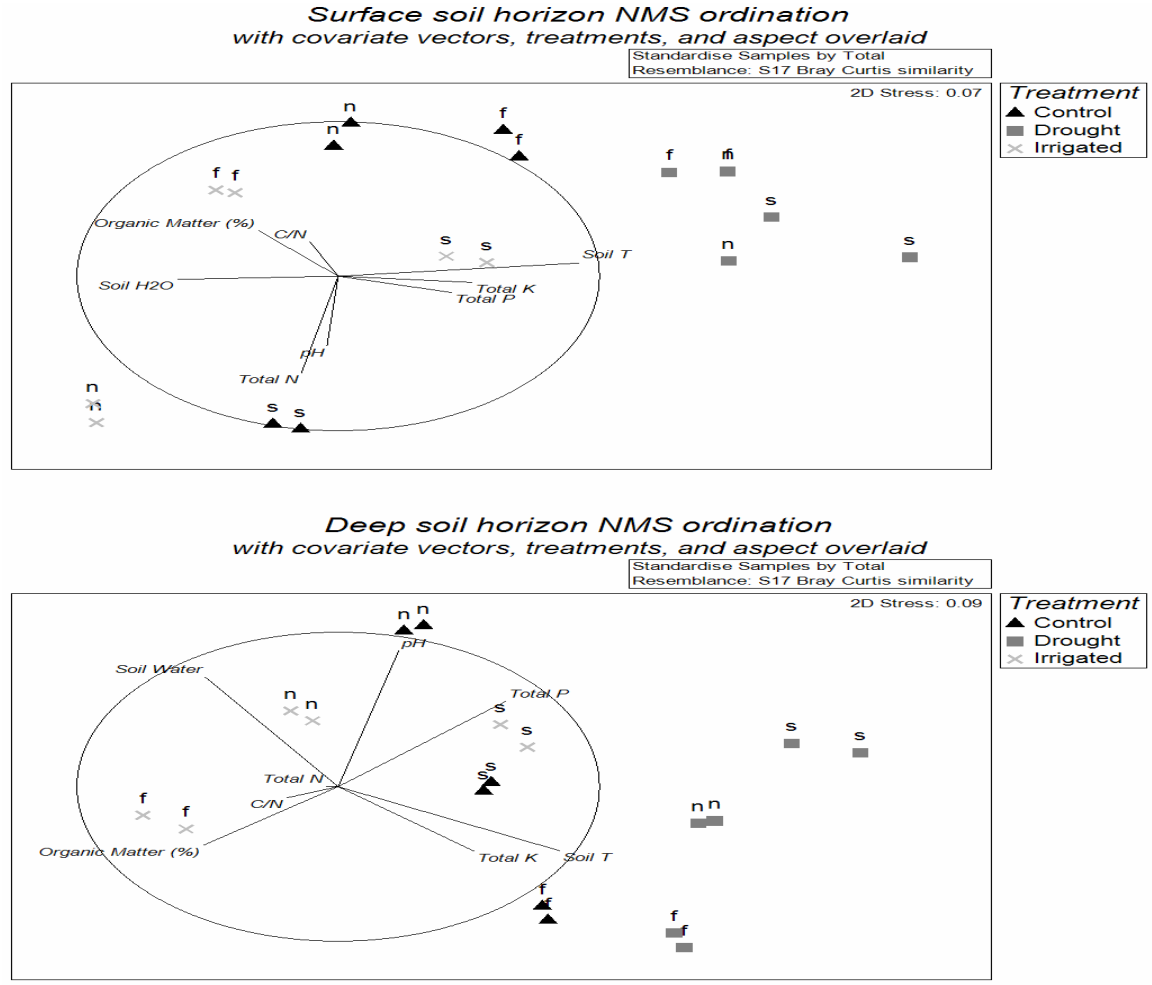

Figure 4: NMDS (ordination) with separate plots for each soil depth. The ordinations are based on Bray-Curtis similarity matrices with OTU abundances standardized by sample totals to account for variation in sequencing depth. Symbols represent treatment groups with letter labels corresponding to aspect (f=flat, n=north, s=south). Highly similar points with the same label are technical replicates. Pearson correlation vectors with correlation circle are overlaid for major environmental variables. Length and direction of line indicate strength and direction of correlation. **Surface Soil Horizon:** The effect of the drought treatment on the fungal community is evident from the independent drought grouping. This group also correlates strongly with increased soil temperature, decreased soil water content, increased K, and increased P. Control and irrigation treatment plots do not cluster by treatment, but sample locations in species space appear correlated with several environmental variables (pH, N, C/N, and organic matter). Visual differences in the dispersion patterns of the drought treatment and others were corroborated by PERMDISP test (P=0.0006, F=13.376, df1:2, df2: 15). **Deep Soil Horizon:** A strong drought treatment effect on the fungal community is reflected along the x-axis and in the grouping of all drought plots together. The drought treatment also correlates strongly with increased soil T, decreased soil water, and increased potassium. The statistically significant aspect effect in this horizon (P=0.0004, F=2.6781) is reflected along the y-axis where the flat block plots aggregate towards the top of the graph. These flat plots are also correlated with increased soil organic matter, and greater C/N. PERMDISP dispersion tests did not indicate a significant difference between treatments (P=0.3181, F=1.1139, df1=2, df2= 15)

indicates a significant treatment effect (P=0.0001, F=5.734). In our reduced model, examining the surface soil horizon community only, we found a significant treatment effect (P=0.001, F=14.28). We did not find a significant block/aspect effect in the surface horizon (P=0.0849, F=1.6752). Our perMANOVA model for the deep soil indicated a significant treatment effect (p=0.001, F=11.832). This model also indicated a significant effect of block/aspect on fungal community composition (P=0.001, F=10.631).

Drought plots were grouped much more closely together in sample space than the controls or irrigated treatments, i.e. there was less dispersion in drought treatments (P=0.0006, F=13.376, df1: 2, df2: 15). In pairwise comparisons we found significant differences in patterns of dispersion between drought and control plots (P=0.003, t=4.069) and drought and irrigated plots (P=0.0024, t=3.9687). No significant difference was found in the patterns of dispersion between control and irrigated plots (P=0.378, t=0.63558). In the deep soil horizon community, there was also no significant difference in dispersion between treatment groups (P=0.3181, F=1.1139, df1: 2, df2: 15).

Depth Effects

In tandem with a strong depth effect on richness (Figure 2), perMANOVA and the ordination of all samples and depths (Figure 3) also indicated a highly significant depth effect on the composition of the fungal community (P=0.0001, F=25.363). In fact, depth appeared to have a stronger effect on the fungal community than treatment (Figure 3). Due to the strength of the depth effect, with little overlap between shallow and deep soil taxa, we elected to run separate analyses for each soil depth. The visible differences in dispersion (Figure 3) indicate that the inter-sample distances in community space are greater in deep soils. PERMDISP tests in PRIMER supported the contention that

dispersion patterns were significantly different between soil depths (P=0.001, F=39.59, df1: 1, df2: 34).

Indicator species

Between soil horizons, higher species dissimilarity values are generally associated with deep soil communities than surface soil communities, corroborating the dispersion patterns reported above. The largest dissimilarity in the surface horizon community is between drought and irrigated treatments (dissimilarity=65.14). Within the surface soil horizon, control and irrigated treatments are the most similar in species composition (dissimilarity= 55.09). In the deep soil horizon, the largest dissimilarity is again between drought and irrigated treatments (dissimilarity= 81.06). In the deep soil horizon the smallest overall dissimilarity was between control and irrigated treatments (dissimilarity=70.65). More ECM species contributed to overall dissimilarity in the deep horizon than in the surface horizon (Table 1). Average abundance was generally higher for dominant species in the surface community than dominant species in the deep soil community. In the surface horizon, *Geopora* spp. and *Pleosporales* spp. are major contributors to dissimilarity and are also highly abundant taxa in this soil community. In the deep soil horizon *Geopora* spp. and *Inocybe* spp. played a major role in structuring dissimilarity between treatments and also tended to be highly abundant in this horizon.

Table 1: Fungal species percent abundance between treatments in the surface soil and deep soil horizons. Only species with percent contributions to average dissimilarity >1.5% are shown. Starred taxa are EMF. **Surface soil:** Dissimilarity values were generally lower for a given pairwise comparison in the surface horizon than in the deep soil horizon, indicating a more homogeneous surface community. Changes in the EMF taxa *Geopora sp.* and *Tuber sp.* and the non-MF *Pleosporales sp.* drove the differences between treatments in surface soil**. Deep Soil:** In general, dissimilarity values were higher for the deep soil horizon comparisons than the same pairwise treatment comparison for a surface soil horizon, indicating a more patchy deep soil community. Changes in the average abundance of ECM species dominate the major contributions to dissimilarity in this soil horizon. Changes in *Geopora sp.*, *Inocybe sp.,* and other ECM taxa abundance drive major differences in community composition in this soil horizon.

Table 1: Indicator Species: Species % Abundance By Treatment and Depth

	Species	Irrigated % Abundance	Control % Abundance	Drought % Abundance
Shallow Soils (5cm)				
	Chaetothyriales sp.	4.67	4.89	4.75
	*Clavulina sp.**	1.85	2.78	3.66
	Curvularia sp.	1.52	0.82	1.07
	*Geopora sp1.**	12.43	10.45	0.23
	*Geopora sp2.**	6.25	2.29	0
	Knufia sp.	3.06	4.33	3.21
	Pleosporales sp.	2.31	3.24	17.2
	*Tuber rufum f. nitidum**	0.14	0.27	2.68
Deep Soils (20cm)				
	*Clavulina sp. 1**	5.47	2.71	0.12
	*Clavulina sp. 2**	3.44	2.01	0.05
	*Eremiomyces echinulatis**	0.62	2.91	4.08
	*Geopora sp. 1**	9.43	8.34	0.82
	*Geopora sp. 2**	0.99	4.93	0.01
	*Inocybe cf. rimosa**	3.58	6.28	0.96
	*Inocybe niveivelata**	7.61	0.28	0
	Pleospora sp.	0.56	1.09	4.37
	*Rhizopogon guzmanii**	3.9	1.37	0.05
	*Russula cessans**	5.3	0.16	0
	*Russula sp.**	0.65	3.44	0
	*Tricholoma myomyces**	1.8	0.82	5.45
	*Tuber rufum f. nitidum**	2.9	0.51	0

Guild Changes

OTUs for which we were able to assign taxonomy in QIIME were run through the Funguild database for guild assignment. Of the OTUs run through Funguild, 49% returned hits to the Funguild database. The OTUs not assigned to a guild accounted for 53% of all sequences with taxonomy assigned in QIIME. In surface soils, reads not assigned a guild accounted for 60.5 ± 0.6% of total reads assigned taxonomy, while deep soil reads lacking guild designation accounted for 44.0 ± 2.0% of reads assigned taxonomy. Overall there was no significant difference between any of the treatments in the percentage of reads not assigned a guild, but a 2 sample t-test indicated that surface soils had significantly more unassigned guild reads than deep soils ($p=0.0078$, $n=9$). Considering the relative dominance of EMF in structuring the deep soil community (Table 1), the difference in proportions of assigned reads between surface and deep soils may be due to a bias of the Funguild database towards ectomycorrhizal taxa. Due to the significant percentage of reads not assigned a guild, we opted to not examine richness at the guild level.

Sequences belonging to the "other" guild class accounted for an average of 3.7% of total reads across all treatments, while sequences belonging to the pathogen guild accounted for an average of 21% of assigned reads. In general, pathogen guild members were more abundant in surface horizon soils (26.6%) than in deep horizon soils (11.6%). The dominant guild in surface soil was the saprotroph guild. In the deep soil horizon, the dominant guild was generally the EMF fungi, except in drought soil, where saprotrophs were dominant (44.2%). However, none of these differences in guild relative abundance

were statistically significant (described below), likely due to high variance between treatments caused by the strong block effect.

The Wilcoxon signed-rank test, however, demonstrated a significant change in guild percent abundance between drought and control plots paired by block/aspect. There were no significant differences between control and drought plots in the percent of fungi assigned to the pathogen or saptroph guilds (p=.1291, W=9; p=.9999, W=18, respectively). In contrast, all drought plots had a lower percent ECM than the paired control plot except for the deep flat drought plot (p=0.04934, W=30; Figure 5). The average shallow soil drought reduction in ECM was 6.7%, or 9.5% if we exclude the flat aspect. We believe it is justifiable to exclude the flat aspect due to its status as a drought outlier with little piñon mortality and a net increase in proportion EMF. The average deep soil drought reduction in EMF was 23.3%, or 49% with exclusion of the flat aspect pair.

Environmental Covariates

In both soil horizons, increased soil temperature and decreased volumetric soil water content were strongly correlated with drought treatment community composition (Figure 4). In the surface horizon, larger quantities of total potassium and total phosphorous were correlated with drought communities. In the deep soil horizon, only increased total potassium was correlated with the drought communities. The surface soil horizon irrigation and control treatment fungal communities exhibited correlations with higher total nitrogen, pH, C/N, percent organic matter, soil water content, and lower soil temperature. The deep soil horizon irrigation and control treatment fungal communities were weakly correlated with higher total nitrogen and higher C/N. The block effect of the

flat plot community composition in the deep soil horizon was strongly correlated with higher percent soil organic matter.

Figure 5:

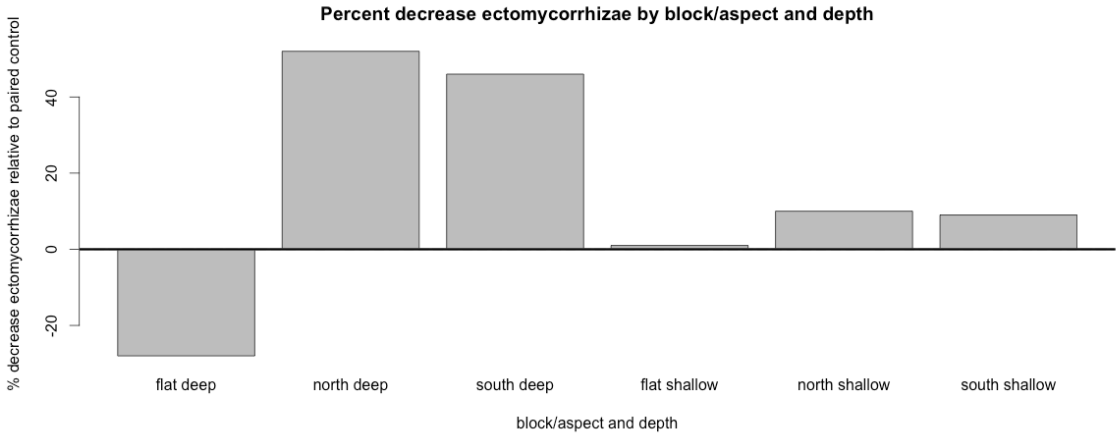

Figure 5: The percent change (paired control minus treatment) in proportion of the fungal community composed of EMF by block/aspect and soil horizon. Plots on the sloped blocks exhibited a large decrease in EMF relative to controls, while the plots in the flat block exhibited little change at the surface and an increase in EMF in the deep horizon. Overall, shallow soils revealed less dramatic swings in EMF relative abundance, while deep soils displayed the largest changes in percent EMF. Note that the flat drought plot experienced little piñon mortality, while sloped plots suffered nearly 100% mortality.

Chapter 4

Discussion

With climate change predicted to increase the proportion of land under moisture stress globally, addressing the effects of drought on fungal communities is an urgent priority. Substantial prior work in the PJ system has addressed the relationships between drought and fungal community composition through the use of observational studies and greenhouse bioassays (Gehring and Whitham 1994 AJB; Gehring and Whitham 1994 Trends; Gehring et al. 1998; Swaty et al. 1998; Swaty et al. 2004; Haskins and Gehring 2005; Mueller et al. 2005; Gehring et al. 2014). Our study contributes to this growing body of knowledge through our use of next-generation sequencing, which allowed us to profile the total soil fungal community of piñon pine, including both mycorrhizal and non-mycorrhizal taxa and both resting structures and growing mycelia. In addition, by utilizing an experimentally imposed moisture-stress gradient and examining soil depth, we were able to minimize confounding environmental variation and definitively pinpoint drivers of community change. Our study also expands the geographic range of our knowledge on the microbial community of piñon pine, as most prior work has been conducted in northern Arizona.

Hypothesis 1: Drought reduces total fungal richness

Drought treatment reduced fungal richness regardless of soil depth (Figure 2). This finding is consistent with our predictions that the pressures of plant mortality coupled with more stressful environmental conditions would reduce overall niche space in the system for fungi. The lower richness in the irrigated plots relative to control plots is notable.There are numerous potential explanations for this observation. First, the

decreased richness may also be a result of a fungal "crash" resulting from the cessation of irrigation two years prior, but this seems unlikely in light of the next point. Second, the increase in basidiomycetes found in irrigated plots (Figure 1), particularly in deep soils, suggests that the irrigated piñon trees allocated more energy/carbon to their fungal mutualists, as basidiomycetes tend to be more costly symbionts. We can infer that this increase is due to primarily EMF taxa based on our indicator species analysis showing a proliferation of basidiomycete EMF taxa in irrigated plots. Many of these basidiomycete mutualists may be better competitors that excluded diverse ascomycetes under these conditions (Bruns 1995). Third, the regional drought that occurred over most of the experiment likely offset most benefits of the irrigation treatment, so the differences may be stochastic. In support of this reasoning, irrigated plots did not exhibit any major "relief" from drought stress reflected in piñon mortality (9% in controls 10% in irrigated plots). Lastly, the irrigated plots began with a higher piñon density, which might explain some of the dominance of basidiomycetes relative to controls.

Hypothesis 2: Drought alters the overall composition of the soil fungal community

As predicted, the experimental drought treatment, which reduced ambient precipitation, increased soil temperature, and led to high piñon mortality, had a significant effect on the fungal community of piñon-juniper soils across soil depth. A strong drought effect was evident in the full model including both soil depths as well as in the reduced models for individual soil depths. The fungal communities of drought treatment plots clustered independently from other treatments in the surface soil and were also distinct in the deep soil. Visually, the surface soil drought treatment communities were very similar in "community space" indicating that the drought treatment applied an

intense, homogenizing filter to this community regardless of aspect. Dispersion testing also indicated that the drought treatment drove these communities toward a more homogenous composition in the surface soils. The lack of a strong drought-driven dispersion pattern in the deep soil community is consistent with a strong depth effect and concomitant distinctiveness of each plot at depth. Depth likely buffers deeper microbial communities from homogenizing factors such as wind and rainfall.

Hypothesis 3: Deep soil harbors a more patchy and altered fungal community composition relative to surface soils

Depth had a strong effect on the fungal community found in piñon-juniper soils, as illustrated by differences in species accumulation curves (richness), NMS ordination, perMANOVA testing, and dispersion patterns. The deep horizon samples had lower richness than the surface samples. The ~38% reduction in richness of drought treatments relative to ambient controls in the surface soil horizon was greater than the ~20% reduction in fungal species richness observed in the deep soil horizon. The greater richness reduction in surface soils is consistent with previous findings that deeper soils are more buffered from environmental changes (Brady and Weil 1996). The greater species turnover across plots in the deep horizon relative to the surface horizon (Figure 3) further indicates a strong depth effect on the fungal community of PJ soils. We speculate that this may be due to the lower probability of dispersal to deeper soil, leading to greater spatial patchiness of the community in deep soil (Ferrier and Alexander 1985; Grogan et al. 2000; Tedersoo et al. 2003). The richness effect, coupled with the differences in the dispersion patterns between soil horizon communities, indicates that depth structures the fungal community of PJ soils, as has been shown previously in other ECM systems

(Taylor and Bruns 1999; Dickie et al. 2002; Rosling et al. 2003; Izzo et al. 2005). However, our study is the first, to our knowledge, to directly address the effects of depth on the fungal community of an aridland forest system.

The contrasting effects of aspect also illustrate the significance of depth in structuring the fungal community. In the surface soils, there was no block/aspect effect. In the deep soil horizon, the flat block stands out from the other blocks. This effect is correlated with higher organic matter in the flat block soils, likely due to the deep sandy/loam in this block. North and south blocks had shallow soils that quickly transitioned to partially weathered bedrock, which likely explains the higher tree mortality on these plots (Plaut et al. 2012; Gaylord et al. 2013). Soil organic matter and soil texture have been previously established as strong drivers of fungal community composition in other systems (Saksena 1955; Harvey et al. 1987; Lauber et al. 2008; Tedersoo et al., 2012) and in piñon pine (Gehring et al. 1998). While we cannot tease apart the indirect effects of tree mortality from the direct effects of drought and soil structure, the combined effects of these three factors strongly influenced the fungal communities of these soils.

Hypothesis 4: Drought alters the ECM community composition

The reduction of percent EMF in drought treatments relative to paired controls indicated that the direct effects of drought compounded with extensive piñon mortality on most plots had a strong negative impact on the ECM community. While nearly all piñon trees suffered rapid mortality on the sloped plots, the flat drought plot experienced little piñon mortality (Pangle 2012). The surface soil of the flat drought plot had low ECM inoculum, but the deep soil of this plot was visually more similar in community

composition to its flat block control in NMS ordination than to the other drought plots. This was also the only drought plot that had a higher proportion of EMF relative to its paired flat block control. Though greater replication would be needed to validate this trend, this observation suggests that indirect effects of drought on EMF due to host tree mortality are more important than direct effects due to lowered soil moisture. The proliferation of ascomycete fungi and decline of basidiomycete fungi across depth under experimental drought mirrors the change we found in guild relative abundances. This trend of increasing ascomycete dominance under moisture stress has been previously reported in the piñon system (Gordon and Gehring 2011).

While the percent abundance of Basidiomycota did not vary greatly between treatments in surface soils (Figure 1), the percent abundance of EMF does not decline as much in drought surface soils as in in deep soils (Figure 5). With the high piñon mortality on the drought plots it seems unlikely that these sources of EMF inoculum in the drought plots are live mycelia, particularly since piñon roots have been generally found to occupy deeper soils than co-occurring juniper (Haskins and Gehring 2005). While we did not expect active ECM root tips to proliferate in this soil horizon under drought conditions, some fungal spores have the ability to resist disturbance, including fire and soil compaction, for a prolonged period of time (Taylor and Bruns 1999; Peay et al. 2009). Considering our methods should detect (but do not distinguish between) both resting spores and actively growing mycelia, we speculate that this EMF inoculum represents resistant propagules that have increased in *relative* abundance due to an overall decline in *absolute* fungal abundance.

The changes seen in guild percent abundance, particularly that of EMF, also correlate well with general changes in percent abundance of EMF indicator species (Table 1). There was a steep decline in the percent abundance of *Geopora sp.* in the drought treatment soils. *Geopora* is a genus of ECM ascomycete fungi previously demonstrated to be highly drought tolerant and common associates of piñon pine (Gordon and Gehring 2011; Flores-Renteria et al. 2014 Gehring et al. 2014). The ECM ascomycete genus *Tuber* vanished completely in the deep drought soils, suggesting a loss of live mycelia. The functional implications of the sharp increase in order *Pleosporales* are less obvious, because the ecological roles of members of this order are diverse. In general, this order is not considered to include ECM species (Bates et al; 2010; Marquez et al. 2012). The slight increase in the genera *Clavulina* and *Tuber* along the moisture-stress gradient in surface soils is notable in that many members of these genera are ECM, particularly considering the sharp decline of these genera in the deep soil community. The increase of these taxa in surface soils is in agreement with our theory that these sequences originated from resistant propagules that survived in the surface soil in the face of a decline in overall fungal abundance.

Hypothesis 5: Deep soil EMF taxa show less declinethan surface soil taxa exposed to severe drought

In general, we did not find evidence to support deep soil acting as a reservoir of ECM species, since many ECM species were absent in deep drought soils while present in control and irrigated soils. In the deep soil horizon, there was a gradient of declining basidiomycete relative abundance as moisture stress increased (Figure 1). This decline is consistent with previous research indicating that some ascomycetes do well in disturbed

areas (Danielson and Pruden 1989; Danielson 1991; Finlay 2006; Gordon and Gehring 2011; Treseder et al. 2014). The decline in basidiomycete abundance mirrors the decline in EMF abundance along the same moisture-stress gradient shown in Figure 5. While these guild designations are broad and are by no means unambiguous, the general trend that EMF declined under the drought treatment across soil depth in our piñon pine study site is clear.

Indicator species analyses also demonstrated a decline in members of the EMF guild in the deep soil horizons with increasing moisture stress. Much as in the surface soil, important EMF genera such as *Geopora* declined or were eliminated from the species pool by the drought treatment. Several other EMF genera also declined significantly along the deep soil moisture-stress gradient, including *Inocybe, Russula, Rhizopogon,* and *Clavulina.* Interestingly, all of these are basidiomycete genera. The decline of *Clavulina* in the deep soil horizon was in contrast to the increase of this genus in the surface horizon. The relative increases in the EMF genera *Eremiomyces* and *Tricholoma* are also notable in drought treated soils. *Eremiomyces* is a drought-tolerant ascomycete truffle found in harsh desert environments such as the Kalahari (Trappe et al. 2008; Trappe et al. 2014). Hence it is not surprising that this genus would perform comparatively well under extreme drought and associated competitive release from less drought tolerant competitors. *Tricholoma myomyces* is a common ECM basidiomycete of many conifers (Shanks 1996*;* Kernaghan and Harper 2001) also found to expand its relative influence in our drought soils. The occurrence of *Tricholoma myomyces* (also identified as *Tricholoma terreum*) under extreme drought is not surprising considering its widespread identification on root tips in stressed cinder soils of the PJ woodland in

northern Arizona (Gehring et al. 1998; Haskins and Gehring 2004; Swaty et al. 2004; Sthultz et al. 2009; Gehring et al. 2014). However, as mentioned above, we have no means of discriminating live mycelia from resistant propagules, or of discerning whether this result reflects an absolute increase in these species or merely a relative increase; due to the high tree mortality, we suspect the latter.

Hypothesis 6: Soil decomposers and soil pathogens increase with moisture-stress as plant-stress and plant mortality increase

A general shift to saprotrophic and pathogenic fungal species and away from EMF was correlated with the decline of living piñon along the moisture-stress gradient, though this shift was not statistically significant. Other trophic guilds may have expanded to occupy the fundamentally different niche comprised of dead piñon roots and dead ectomycorrhizal hyphae. It is notable that the "other" guild classification, which included arbuscular mycorrhizal fungi associated with juniper and many other plant taxa of this system, did not appear to change significantly across the moisture gradient. The only guild that changed significantly was EMF, which declined under drought, aside from the flat block. The flat block underwent little piñon mortality. Pathogens and saprotrophs generally increased with drought, but the increase was not uniform in magnitude or direction, and thus was statistically non-significant. Again we note the confounding effect of the flat drought block, which exhibited opposite trends in guild changes due to a limited response to the drought treatment. This lack of significant differences in abundances of other guilds corresponds with indicator species analysis (Table 1), which showed most of the OTUs driving community change were EMF taxa.

Experimental Limitations

To our knowledge this is the first study to explicitly test the effects of drought on the whole fungal community using a long-term drought experiment. Our approach allowed us to detect changes in both live and resting fungal tissues across depths. While the use of high-throughput sequencing for community level analysis has exploded in recent years, the availability of suitable bioinformatics tools and data analysis tools has lagged behind (Pop and Salzberg, 2008; Gonzalez and Knight 2012; Preheim et al. 2013; Nguyen et al. 2015). We recognize inherent limitations in several of our methodologies, as discussed in the following sections.

The drought experiment was originally designed to address plant ecophysiology. Due to logistical constraints, the design did not include the high level of replication that would be desirable for addressing alterations in patchy, hyper-diverse soil fungal communities. As a result, our analyses were limited in statistical power and may have failed to detect some important drivers of fungal community composition. Nevertheless, replication was sufficient to reveal strong drought and soil horizon effects.

Most previous work using paired-end sequencing has utilized only the joined reads (Bartram et al. 2011; Glassman et al. 2015; Oliver et al. 2015). However, in our case, the forward reads were generally of sufficiently high quality that leaving them out would exclude a large amount of putatively legitimate data (Nguyen et al. 2015). Using only joined reads may also exert a bias in favor of species with a shorter ITS2 region; hence using forward reads when they did not pair with the reverse read was an attempt to circumvent this bias (Nguyen et al. 2015). While many of our QIIME parameters were strict (i.e. q=19, r=0, n=0), some, such as p=.23 (minimum proportion of consecutive high quality base calls to include a read) and our clustering species identity of 94% are

relatively low. However, our use of a mock community to tune settings justified these parameters (Lindahl et al. 2013; Nguyen et al. 2015). Furthermore, different clustering methods produce different sizes of clusters and numbers of OTUs, even at the same identity threshold. USEARCH is a complete-linkage clustering method, which makes our 94% threshold roughly comparable to a 97% threshold with a single-linkage method.

The FUNGUILD database is a relatively new tool for assigning functional roles to taxonomic output of high-throughput sequence processing. This database is constrained by both the limited literature available on fungal guilds and the uncertainty of guild assignments at various taxonomic levels, but considering the large quantity of sequences generated with Illumina sequencing, we found it to be a helpful tool in assigning ecological meaning to observed patterns. The fact that only 49% of our OTUs (53% of reads total) submitted to FUNGUILD returned a guild assignment demonstrates current limitations of this tool, but the strong correlation between changes seen in guild structure and changes in phylum level taxonomy and indicator species analysis suggests that the guild assignment process was relatively non-biased, even if it was incomplete. In addition to missing taxa, the assignment of guilds to ‘known’ taxa is challenging. Incorrect taxonomy assignment may occur due to GenBank and/or UNITE errors. In addition, errors in the FUNGUILD database may also inflate this issue as taxa submitted might be incorrectly named and/or trophic status may be incorrectly assigned. Hence we acknowledge that the guild/functional role assignment is nebulous at best, but the general trend we see in our data aligns with other metrics of fungal community change in our system, particularly the indicator species analyses which definitively delineate impacts on EMF taxa.

Future directions

Some systems, such as the North American boreal forest and Arctic tundra have been reasonably well profiled (Brockett et al. 2012; Taylor et al. 2014; Timling et al. 2014), but most systems around the world have not (Hawkswork 2001; Tedersoo et al. 2012). For example, little work has been carried out to profile fungal communities in locations with outsized ecological roles such as urban environments and areas of the developing world (Newbound et al. 2010). Profiling communities is only the first step toward understanding the broader implications of changes in fungal community structure. Time series and spatial series are also important to understanding how fungal communities diverge and turn over. While our long-running drought and infrastructure associated soil warming experiment was costly and difficult to set up, man-made global climate change provides an opportunity to test many of our findings across different systems and time scales over the next century. Hence, profiling an array of fungal communities across the globe now should be a major priority to establish a baseline before the major impacts of climate change are manifest.

The limitations we encountered in assigning functional/guild roles to fungal taxa indicated a need to improve and further develop guild assignment tools. The functional role of many fungi is poorly understood, and most functional classifications either come from direct observation, i.e. a fungal hypha penetrating a root cell, or from laboratory isolation and manipulation. Both of these methods have certain biases and should be carried out in conjunction with enzymatic or metagenomics profiling of natural systems (Courty et al. 2005; Cravat et al. 2008). Only with a thorough understanding of a species

functional role(s) in a system can we understand the consequences of shifts in community structure.

In general, our findings further underscore the need to incorporate plant-fungal interactions into climate change predictions (see Kivlin et al 2013). In the piñon-juniper system specifically, we need to better understand the traits and roles of the fungi surviving prolonged drought, particularly the EMF. For arbuscular mycorrhizal fungi associated with juniper and most other plants in this system, there are a number plant partner options in the event of plant mortality; for EMF there is generally only piñon pine and the occasional scrub oak (*Quercus spp*). How long the EMF survive, and by what mechanisms, may be of great importance to understanding to ability of piñon to regenerate following drought. While some work has been done on piñon regeneration following drought-induced mortality (see Redmond and Barger 2013), explicit tests of the germination and survival of piñon seedlings following prolonged drought are also necessary in order to draw explicit conclusions about the ecological roles of fungi in piñon regeneration.

Conclusions

Our results indicate that drought can significantly alter fungal communities. Such alterations are likely to cause feedbacks to the aboveground plant community, in our system resulting in changes in the distribution of piñon pine, and globally altering the distribution of ECM plants. Such alterations are likely to lead to further shifts in other plant and fungal taxa filling former ECM niche space. The steep decline of EMF in deep soils (Figure 5) and surface soils experiencing prolonged experimental drought does not

bode well for the reestablishment of the obligately ECM piñon pine (Haskins and Gehring 2005).

Our findings confirm previously demonstrated differences in ECM colonization of piñon roots at sites differing in soil texture, among other characteristics (Swaty et al. 1998). We demonstrate the importance of soil characteristics in controlling whether piñon seedlings experience favorable conditions if they are able to disperse to a drought-disturbed site. For example, if a seedling germinates on a site that has deep soil with high organic matter, it stands a better chance of reaching suitable EMF inoculum as its taproot elongates into the soil profile. On the other hand, it may be increasingly difficult for piñon to recolonize soils that are shallow, where surface inoculum has been dramatically reduced by drought. Hence we might expect that as climate change progresses, piñon habitat will become increasingly fragmented, only persisting in areas where soil characteristics provide deep refuge for suitable ECM inoculum. More generally our findings reinforce the importance of aboveground-belowground feedbacks when modeling change resulting from disturbance factors.

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