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Abstract

Climate models for central United States predict increasing temperatures and greater variability in precipitation. Combined, these shifts in environmental conditions impact many ecosystem properties and services. Long-term climate change experiments, such as the Rainfall Manipulation Plots (RaMPs), can be used to address soil community responses to simultaneous manipulation of temperature and temporal variability in precipitation. The RaMPs experiment is located in a native tallgrass prairie at the Konza Prairie Biological Station and has been operational since 1998 providing the potential to address responses to long-term environmental manipulations. To test whether community composition, richness, or diversity respond to environmental change, more than 40,000 fungal amplicons were analyzed from soil samples collected in 2006. The data suggest that soil fungal communities are compositionally resilient to predicted environmental change. This is the case both for the community composition overall as inferred from ordination analyses as well as analyses of variance for each of the most common Operational Taxonomic Units (OTUs). However, while this study suggests compositional resilience, further studies are required to address functional attributes of these communities and their responses to environmental manipulations.

Introduction

Water and temperature are major abiotic factors that influence grassland carbon exchange on an ecosystem level both above- and belowground (Xu *et al.* 2004; Davidson & Jansens 2006). These factors are also fundamental drivers of the heterotrophic respiration that is estimated to account for 30-50% of the total soil respiration in most terrestrial systems (Raich & Schlesinger 1992; Bond-Lamberty *et al.* 2004), although estimates may vary widely depending on biome/ecosystem (compare Hanson *et al*, 2000; Wan & Luo 2003; Chen *et al.* 2009). The significance of the soil respiration and the large contribution of soil-inhabiting microbial communities to CO_2 flux emphasize their importance and underline the need to better understand the compositional and functional attributes of these communities, particularly so in the face of predicted future environmental conditions (Bargett *et al.* 2008; Drigo *et al.* 2008).

Grasslands are important biomes. They comprise approximately 32% of the natural vegetation of earth's surface (Adams *et al.* 1990) and store 28-37% of the terrestrial organic soil carbon (Lal 2004). The function and composition of the plant communities in grassland ecosystems have been proposed to be sensitive to variability in climatic conditions (Knapp & Smith 2001; Gao & Reynolds 2003). In grassland ecosystems, shifts in timing of precipitation – not in the total quantity – can be important controls of plant productivity (Knapp *et al.* 2002; Fay *et al.* 2011), soil respiration (Harper *et al.* 2005; Fay *et al.* 2011), as well as function and structure of soil communities (Zeglin *et al.* 2013). As soil moisture and its variability are major controls of the broad scale function and composition of the soil communities on various spatial (Brockett *et al.* 2012) and temporal scales (Zeglin *et al.* 2013), more detailed evaluation of community responses to long-term manipulations are timely.

IPCC (2007) predicted that effects of changes in extreme events – both increases in the duration of droughts and in the per event precipitation volumes – may exceed those expected for changes in average precipitation leading to more variable precipitation regime and soil moisture in mesic ecosystems (Knapp *et al.* 2008). While such changes in the per event volume of rainfall as well as in their frequency have been suggested to lead to rapid alterations in soil processes as well as aboveground community composition (Knapp *et al.* 2002; Fay *et al.* 2003; Harper *et al.* 2005; Swemmer *et al.* 2007; Fay *et al.* 2011), consequences of such alterations for belowground microbial communities have received comparatively less attention (Chou *et al.* 2008; Zeglin *et al.* 2013). This major gap prohibits our understanding of the soil community responses to changes in soil water that far exceed those witnessed for dominant plants (Huxman *et al.* 2004; Ogle & Reynolds 2004; Schwinning & Sala 2004), tend to be more dynamic, and responsive to pulse events (Austin *et al.* 2004; Ogle & Reynolds 2004; Carbone *et al.* 2011).

While both water and temperature are major abiotic controls of grassland ecosystem carbon exchange (Xu *et al.* 2004; Davidson & Jansens 2006; Fay *et al.* 2011), the effects of altered precipitation are not as well understood as those of elevated CO_2 or temperature (Weltzin *et al.* 2003). Studies focusing on the interactions of multiple global change drivers may be fewer yet (Bardgett *et al.* 2008). The responses to interacting climatic drivers of soil communities and their function tend not to be additive (Shen *et al.* 2009; Hayden *et al.* 2012; Matias *et al.* 2012). Accordingly, experimental manipulations that combine multiple global change factors are necessary to elucidate the potentially synergistic responses of soil systems. Increased soil temperatures tend to stimulate soil respiration directly through positive responses by acceleration of both autotrophic and heterotrophic metabolism (Rustad *et al.* 2001; Melillo *et al.* 2002; Shen *et al.* 2009). Alternatively, higher soil temperatures may shift soil function indirectly through increases in nitrogen mineralization, primary production, and litter production (Stromgren & Linder 2002; Pendall *et al.* 2004). Warming may also reduce overall microbial biomass (Frey *et al.* 2008) and increase (Castro *et al.* 2010) or decrease (Allison &

Treseder 2008; Hayden *et al.* 2012) fungal abundance. Overlaid with changing average temperatures, changes in soil water potential may directly and indirectly control soil community metabolic and physiological activities by affecting substrate availability via litter production and substrate diffusion (Skopp *et al.* 1990; Davidson & Janssens 2006; Bardgett *et al.* 2008). Warming may amplify these effects – potentially as a result of greater rate of water loss from the soil profile. Sheik *et al.* (2011) reported that with elevated temperatures, water budgets regulated microbial populations. More importantly, they concluded that timing of the precipitation events is critical for microbial populations.

In contrast to broad microbial community responses to environmental manipulations, few studies have explored the responses of general soil fungal communities – particularly to interacting environmental factors. Fungal community responses to warming would be expected, because fungal respiration tends to increase with temperature (Hacskaylo *et al.* 1965; Malcolm *et al.* 2008) although in the long-term the communities may adapt to the changing environmental conditions (Malcolm *et al.* 2008). Studies that have targeted the effects of elevated temperature have reported different and contrasting responses (Bardgett *et al.* 1999; Compant *et al.* 2010): experimental warming may increase diversity of soil fungi (Allison & Treseder 2008), decrease (Allison & Treseder 2008; Hayden *et al.* 2012) or increase (Castro *et al.* 2010; Ziegler *et al.* 2013) fungal abundance or biomass, change relative taxon abundances (Allison & Treseder 2008; Deslippe *et al.* 2011; Anderson *et al.* 2013), or have minimal effects on richness, diversity, and community composition (Allison *et al.* 2010; Papanikolaou *et al.* 2010) depending on the ecosystem. In addition to the ecosystem level context dependencies (Compant *et al.* 2010), interacting environmental drivers may further modulate the fungal community responses (Hayden *et al.* 2012). For example, Rygiewicz *et al.* (2000) showed that soil fungal community responses to elevated temperature differed between [CO_2] treatments. Heterotrophic fungal communities and their responses to shifts in environmental conditions are particularly important, because they are essential in

carbon and nutrient cycling (Dighton 2003), comprise a large recalcitrant belowground carbon sink (Treseder & Allen 2000), and may be affected directly by the shifts in the environmental drivers or indirectly through the responses in plant communities (Cregger *et al.* 2012). Anderson *et al.* (2013) observed that both elevated CO_2 and elevated temperature influenced the fungal community composition. These authors emphasized the importance of considering the community responses in the context of the plant host, as decoupling direct effects of environmental conditions and indirect effects modulated via plant communities are essential to understand the complexities of soil community dynamics (Bardgett *et al.* 2008). The plant community dependent responses are particularly important for root-colonizing mycorrhizal communities. Studies focusing on those fungal guilds have reported increases in ecto- and arbuscular mycorrhizal mycelium, species richness, as well as root colonization in response to experimental warming (Staddon *et al.* 2003; Clemmensen *et al.* 2006; Heinemyer *et al.* 2006; Hawkes *et al.* 2008; Deslippe *et al.* 2011; Büscher *et al.* 2012), although these responses are unlikely to be universal (Olsrud *et al.* 2010). Experimental warming may also lead to shifts in the relative abundances of soil inhabiting fungi and bacteria (Zhang *et al.* 2005; Rinnan *et al.* 2007; Frey *et al.* 2008).

Soil moisture also strongly influences fungal communities structurally and functionally (Toberman *et al.* 2008; Bell *et al.* 2009; Baldrian *et al.* 2010; Castro *et al.* 2010; Hawkes *et al.* 2011; Schmitt & Glaser 2011; Cregger *et al.* 2012). Variability in soil moisture may shift biomass or ratios of fungi and bacteria within a growing season (Clark *et al.* 2009; Gray *et al.* 2011; Cregger et al. 2012; Baldrian *et al.* 2013), in response to pulse events (Fierer *et al.* 2003; Zeglin *et al.* 2013), or in response to experimental manipulations of soil moisture (Nazih *et al.* 2001; Cregger *et al.* 2012; Zeglin *et al.* 2013). Some recent studies have suggested that soil fungal communities are highly responsive to changes in soil moisture within and across years (Hawkes *et al.* 2011). These responses translate to changes in fungal abundance, community composition, and further to function. Perhaps somewhat surprisingly, Hawkes *et al.*

(2011) concluded that fungal communities during low soil moisture were more diverse and abundant than during high soil moisture. Furthermore, these responses in fungal communities were rapid, reversible, and repeatable, thus highlighting the community plasticity in terms of possessed environmental or physiological tolerances (Cruz-Martinez *et al.* 2009). The community dynamics resulting from the rapid changes in soil moisture preceding and following rainfall pulse events may mask the long-term dynamics of fungal responses to available soil moisture (Hawkes *et al.* 2011; Cregger *et al.* 2012). Such short term dynamics on the scale of days are further overlaid by seasonal dynamics in soil moisture that further drive the soil fungal community structure and function (Hawkes *et al.* 2011; Cregger *et al.* 2012). Fundamentally, soil moisture is a pivotal driver of fungal biomass (Frey *et al.* 1999) and the communities respond rapidly to changes in available moisture.

Environmental change factors do not operate in isolation. Predicting the interactions among multiple drivers is problematic based on single driver experiments (Shaw *et al.* 2002; Larsen *et al.* 2011). Therefore, concerted manipulations of multiple environmental drivers are essential. The primary goal of this contribution was to utilize an existing experiment that has simultaneously manipulated temperature and precipitation timing in a tallgrass prairie ecosystem (Fay *et al.* 2000, 2002, 2003). Previous studies using this experimental infrastructure have observed responses in plant growth and aboveground annual net primary production (ANPP) (Knapp *et al.* 2002; Fay *et al.* 2011), leaf carbon assimilation (Fay *et al.* 2011), soil CO_2 efflux (Harper *et al.* 2005; Fay *et al.* 2011; Zeglin *et al.* 2013), as well as soil microbial community biomass and exoenzyme activities (Zeglin *et al.* 2013). Collectively, these studies have observed that the long-term manipulation of the rainfall frequency has increased the amplitude of the intra-annual soil moisture variability reducing the average soil water availability (Fay *et al.* 2011) leading to reduced rainfall use efficiency (Fay *et al.* 2011) and to reduced soil microbial community carbon use efficiency (Zeglin *et al.* 2013). The elevated temperature treatments in turn have advanced plant phenology in spring, increased winter soil CO_2 efflux, and reduced summer soil CO_2 efflux (Fay et al. 2011). These

treatments have also shifted the biomass allocation among the major plant functional groups via reduction of forb ANPP (Fay *et al.* 2011). In sum, the effects of elevated temperature seem to differ between cooler and warmer seasons and have major consequences on fundamental ecosystem processes. These studies also highlight the shifting relative importance of the temperature and rainfall manipulations: early in the growing season the ecosystem processes were most strongly driven by the elevated temperature, whereas the precipitation manipulations and the resultant variability in the soil moisture had more pronounced effects later in the season (Fay *et al.* 2011).

To elucidate compositional responses of the soil fungal communities, we analyzed fungal Internal Transcribed Spacer (ITS) regions from samples collected from this ongoing experiment via high throughput sequencing in combination with sample-specific DNA tagging. This allowed taking advantage of a well-replicated experiment and testing hypotheses on responses of fungal diversity and community composition to the environmental manipulations and their interactions. We hypothesized that fungal communities - their diversity, richness, and composition - will change due to alterations in the frequency - but not in the amount - of precipitation, changes in temperature, and/or the combination of these two environmental drivers. These studies were strongly motivated by our previous results (Zeglin *et al.* 2013) that suggest that the microbial communities are dynamic on multiple temporal scales functionally and compositionally, but did not specifically target specific compositional constituents of the fungal communities.

Materials and Methods

Site description

The study was conducted at the Konza Prairie Biological Station (KPBS, 39°05' N, 96°35' W), a Long–Term Ecological Research (LTER) site representative of native tallgrass prairie in the Flint Hills of eastern Kansas, USA. KPBS spans 3,487 ha, and the vast majority of the site remains undisturbed by agriculture. The vegetation is dominated by native C_4 grasses: big bluestem (*Andropogon gerardii*), indian grass (*Sorghastrum nutans*), little bluestem (*Schizachyrium scoparium*) and switch grass (*Panicum virgatum*) (for a complete list of vascular plants at the KPBS, see Towne 2002). The Flint Hills are generally characterized by shallow soils overlaying chert-bearing limestones and shales (Ransom *et al.* 1998) and our site is classified as typical chernozern according to the Food and Agriculture Organizations (FAO) soils classification used by the United Nations. Topographic relief divides the landscape into upland plateaus with shallow soils, slopes with outcrops of limestone, and lowlands with deeper alluvial and colluvial soils. January mean temperature is –3°C (range –9 to 3°C) and the July mean is 27°C (range 20 to 33°C). Annual precipitation averages 835 mm, 75% of which falls in the growing season between April and October.

Rainfall Manipulation Plots (RaMPs)

The RaMPs experiment was established in 1997 and has been operational since May of 1998. Twelve sheltered RaMPs and three non-sheltered reference plots are located on a gently sloping (1-2%) site with relatively deep (75-120cm to limestone) Irwin silty clay loams (fine, mixed, mesic Pachic Argiustolls). Because shelters reduce midday photon flux density by ca. 18% and may thus impact photosynthesis and carbon flow into the soil, the present study excluded the non-sheltered reference plots and the experimental design considers only the twelve sheltered RaMPs. The experimental site is managed by annual spring burning (typically in late March), a common management practice that controls woody vegetation and generally enhances productivity of the dominant perennial grasses.

Each RaMP comprises a fixed-location, 14 x 9 m rainout shelter over intact, native grassland (Fay *et al.* 2000, 2003). Each shelter consists of a clear (UV transparent) polyethylene roof to exclude natural rainfall, two 4 m^3 reservoirs to collect and store up to 10 cm of rainfall, and an overhead irrigation system to reapply (at 2.5 cm hr^{-1}) the stored rainfall. Thus, the shelter design allows for complete and efficient experimental control of the quantity, timing and variability in rainfall inputs (Fay *et al.* 2000). Rainfall is applied to each RaMP using 13 sprinkler heads to ensure uniform application over each plot. A subset of the RaMPs contains micro-meteorological stations for comparison with unsheltered plots. Each shelter covers a central 6 x 6 m sampling area, with a substantial surrounding buffer (0.8 m wide). A 1.2 m deep subsurface barrier limits lateral water movement into and out of each experimental plot. The large rainout shelter size relative to the plot size is effective at minimizing lateral (windblown) rainfall inputs to the central sampling area, and soil moisture in the plots vary independently from the surroundings (Fay *et al.* 2000).

Precipitation and Temperature Treatments
The RaMPs experiment has proceeded in phases outlined in detail in Fay *et al.* (2011). From 1998 to 2001, the RaMPs were assigned to one of four rainfall treatments in a completely randomized block design (4 RaMP treatments + one reference plot in each of three blocks). Treatments were factorial combinations of two within-season temporal rainfall distribution patterns (ambient or altered rainfall timing, detailed below) combined with two growing season rainfall quantities (100% or 70% of natural rainfall amounts). These manipulations occur only during the growing season, when > 75% of the annual precipitation falls. In 2002, the simple drought treatment (30% reduction in quantity) was eliminated (see Fay *et al.* 2002, 2003 for detailed analyses including the drought treatments) to increase sample size for the rainfall timing manipulations (n=6) and to prepare for the addition of the warming treatments. The former drought treatment plots retained their prior rainfall timing treatment (ambient or altered timing), but since

2002 after elimination of the drought treatment, all plots – controls as well as the altered interval plots – receive an amount equal to 100% of ambient rainfall quantities. In spring 2003, two 4 m^2 temperature manipulation plots were installed in each RaMP (and each non-sheltered reference plot), modifying the experiment to a split-plot design, with precipitation as the whole-plot treatment and warming as the subplot treatment. The final split-plot design comprises two precipitation treatments each with six replicates (2002). Each RaMPs plot includes four heat treatment plots (since 2003) – two heated plots, one ambient temperature plot with a non-heated lamp structure and one control plot where no structure has been installed. Thus, the experiment allows for assessing the impacts of increased temperature and altered rainfall as well as their interactive effects.

In the present study, a total of four treatment combinations were included; (1) *Ambient precipitation regime*: In six of the twelve RaMPs, collected rainfall is reapplied to the plots each time a natural rainfall event occurs. Rain gauges outside the RaMPs and an in-line flowmeter allow confirmation that precipitation amounts applied inside the RaMPs equal the amounts of rain falling outside. (2) *Altered precipitation regime*: The remaining six RaMPs receive a treatment that imposes a predicted rainfall regime of increased temporal variability in rainfall inputs and soil moisture relative to ambient precipitation patterns. This is achieved by lengthening the current ambient dry intervals between rainfall events by 50%. For example, a 2-week period between ambient rainfall events would be lengthened to 3 weeks. Any rainfall during these experimentally lengthened dry periods is stored and applied as a single larger event at the end of the dry interval. The total amount of growing season rainfall applied in this treatment is identical to ambient, only the event size and temporal distribution of inputs are altered. For the purposes of the experiment, an event is defined as precipitation >5 mm in a 24 hr period. Lesser amounts are almost entirely intercepted by the canopy (Seastedt 1985). A 50% increase in dry intervals was based on analyses of 20+ years of growing season rainfall patterns at Konza Prairie from which the average storm size and frequency was calculated and deviations assessed. This analysis ensured that the treatment selected resulted in a

rainfall regime fundamentally altered from typical ambient patterns. (3) and (4) *Increased air and soil temperatures with ambient or altered rainfall patterns*: Each RaMP is divided into four 2 x 2 m subplots. Overhead rectangular IR lamps (Kalglo MRM-1215 1500-W model) are suspended at ca. 1.5 m above the canopy in two subplots, whereas one subplot serves as a blank lamp control and another subplot as a no-treatment control. The lamps simulate climate warming increasing day (0.4-0.6°C) and night (1.0-1.5°C) canopy temperatures as well as soil temperatures (annual mean ~1°C at 5 cm soil depth) by enhancing downward IR flux (~70 W m^{-2}) (Fay *et al.* 2011). In contrast to rainfall treatments, warming occurs year round. While any experimental warming approach has its limitations, IR lamps are a widely used, and are a preferred option, for warming experiments under field conditions (Shaver *et al.* 2000). We raise the lamps during the growing season as vegetation height increases to maintain a constant IR flux at the canopy surface, and avoid unrealistic radiation loads on the canopy. For the analyses described here, we selected one elevated temperature treatment and its corresponding control in each of the twelve RaMPs canopies (total of 24 experimental units).

Soil sampling and DNA extraction
For each experimental unit (six replicates for each of the four experimental conditions), two 2.5 cm diameter 10 cm deep cores were collected in June 2006, pooled, and homogenized. From each composite sample, visible roots and large rocks were removed and genomic DNA was extracted from a 10 g subsample using a standard soil DNA extraction kit (UltraClean Mega Soil DNA Kit, MoBio, Carlsbad, CA). After extraction, the DNA was diluted to ~5 ng μl^{-1} and stored at -80°C.

Amplification and 454-sequencing of the Internal Transcribed Spacer
The extracted DNA was used to amplify soil fungal communities using primers with a bias towards fungi (ITS1 and ITS2; White *et al.* 1990) designed to amplify the highly variable Internal Transcribed Spacer 1 (ITS1). To allow direct 454-sequencing, we synthesized primer constructs which incorporated the 454-primers

(Margulies *et al.* 2005) similarly to Jumpponen & Jones (2009). Between the 454- and ITS1 primers, we included a 5-bp DNA-tag unique to each experimental unit (Supplemental Table S1).

To amplify the fungal ITS1, the PCR reactions [5 μl of Green GoTaq Flexi PCR buffer (Promega, Madison, Wisconsin), 2.5 mM $MgCl_2$, 200 μM dNTP, 0.2 μM each forward and reverse primers, one unit GoTaq Hot Start DNA polymerase, and 5ng soil extracted DNA] were run for 25 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 2 min, and a final 10 min extension at 72°C on MasterCyclers (Eppendorf, Hamburg, Germany). From each sample, the fungal ITS was amplified in three separate reactions, individual reactions pooled, and cleaned using an AmPure PCR cleanup kit (Agencourt Bioscience, Beverly, MA). One sample failed amplification and was omitted from further analyses. A total of 100 ng of each differentially DNA-tagged PCR product were pooled and sequenced on a GS20 genome sequencer at 454 Life Sciences (Branford, CT). The data are available through Sequence Read Archive (BioProject PRJNA219046; BioSamples SAMN02351567 – SAMN02351589).

Bioinformatics and OTU designation
The sequence data were analyzed as previously described (Jumpponen & Jones, 2009). Briefly, sequences were removed if they contained no valid primer sequence or DNA tag, contained ambiguous bases, or were outside of the set length threshold of 85 bp to 130 bp. The remaining reads were aligned with CAP3 (Huang & Madan, 1999) and assigned to operational taxonomic units (OTUs) at 97% similarity. The data were parsed by sample to calculate the OTU frequencies and counts for each sample. From this output, SAS (SAS Institute Inc., Cary, NC) was used to calculate richness and diversity indices as previously described (Jumpponen & Jones, 2009). We estimated a number of diversity indexes (Table 1; S_{Obs} – observed number of OTUs; Simpson's D; Shannon's H'; Fisher's α; and, evenness based on the ratio of Shannon's H' and the natural logarithm of the richness). To further explore organismal coverage, species accumulation (Mao Tau rarefaction) curves were

generated using EstimateS (Colwell 2006). Although we were tempted to estimate the extrapolative richness metrics, our analyses indicated that our sampling depth was inadequate for meaningful extrapolative estimators (see Simpson's D in Table 1).

OTU frequencies were analyzed based on OTUs assigned at 97% sequence similarity and the singletons omitted as recommended by Tedersoo et al. (2010). The non-singleton OTUs were analyzed as a proxy for species-level resolution for treatment effects in SAS (SAS Institute, Cary, NC). Student's t-test (α = 0.05 for hypothesis f(OTU) > 0) was used to identify OTUs whose frequencies were either too low or too variable to provide meaningful inferences on their responses to treatments.
The taxon affinities for fungal OTUs were based on BLAST queries (Zhang *et al*. 2000) against nt database (Supplemental Table S2). To minimize the number of environmental "unculturable fungus" matches to our queries with fungal sequences, we additionally applied an Entrez limit (Eukarya[ORGANISM] NOT environmental samples[FILTER] NOT unculturable[ALL FIELDS]) (Supplemental Table S3). To provide a broader taxonomic context, all non-singleton OTUs were assigned to classes based on the fungal lineage information for the top ranked matches among the ten that were retrieved from BLAST queries. The class-level resolution was chosen to increase classification recovery with the short (<130bp), partial ITS1 reads (see Porter and Golding 2011). For these analyses, the OTU frequencies were summed for each experimental unit and taxon, and analyzed for treatment effects.

Statistical analyses

Data were tested for normality and homogeneity of variance (Milliken and Johnson 1984). Observed variance heterogeneity was corrected through $\log_{10}$ transformation. The main (Warming, Precipitation) and interactive (Warming*Precipitation) effects were analyzed in JMP (SAS Institute Inc., Cary, NC) The effects "Block", "RaMP(Precipitation)" and "RaMP(Warming,Precipitation)"

were considered random effects in the mixed model analysis of variance (ANOVA). We also examined taxon (class, OTU) level responses. For the analyses on the level of a class, the OTU frequencies were summed for each experimental unit and taxon, and analyzed for treatment effects using mixed models ANOVA. To limit the number of OTUs considered in these analyses, we included only those OTUs that occurred on average at frequencies significantly greater than zero (Student's t-test; α = 0.05 for null hypothesis f(OTU) = 0); these analyses identified a total of 226 OTUs that were analyzed using the mixed models ANOVA as described above. To minimize the risk of Type I errors, we used False Discovery Rate (FDR) correction to identify classes and OTUs most likely to respond to the experimental manipulations. To examine community-level differences, the OTU frequencies were analyzed using Nonmetric Multidimensional Scaling (NMS) in PC-ORD (Version 4.1, McCune and Mefford 1999). Because the low frequency OTUs may have disproportionate effects on these ordination analyses, we omitted OTUs that did not occur in at least six (or 25%) of the experimental units. Pairwise community distances were estimated using Sørensen index and analyzed using Nonmetric Multidimensional Scaling (NMS, Mather 1976). The optimal number of dimensions (k) was selected based on Monte Carlo test of significance at each level of dimensionality comparing 40 runs with empirical data against 50 randomized runs with a step-down in dimensionality from 6 to 1 and a random seed starting value. Three-dimensional solution (k ≥ 3) yielded stress values smaller than those in randomized runs (P = 0.0196) and was selected for final configuration. To estimate the treatment level responses of the communities the axis scores were analyzed in JMP (SAS Institute Inc., Cary, NC) as described above.

Results

General data description

To characterize soil fungal communities, a total of 45,564 sequences that passed the quality control were analyzed. More than 20% of the total sequence yield was omitted because of missing primer or DNA-tag sequences, length (<85 or >130bp), or ambiguous nucleotides.

The sequences were assigned to a total of 1,642 fungal OTUs, 315 of which occurred only once in the dataset. Excluding groups that had no clear taxonomic affinities, these OTUs represented a total of 19 classes and 62 orders. The data were strongly dominated by Agaricomycetes (Basidiomycota; 56% of the sequences) followed by Eurotiomycetes (Ascomycota; 9.7%) and Leotiomycetes (Ascomycota; 6.1%); 7.4% of the sequences could not be assigned to a class (Fig. 1; Supplemental Table S4). The eleven most common classes that were present in an excess of 1% of the total sequencing yield constituted more than 99% of the acquired sequences with the exception of unknown sequences (7.4%). Although taxonomic information is desirable, it is necessary to exercise caution with these taxon assignments as the GS20-generated short reads presented a challenge for OTU taxon assignments, especially in less inclusive taxonomic ranks below order level (Porter and Golding 2011). Examination of the average bit scores (92.1 ± 40.8), e-values (9.1 x 10^{-8} ± 7.9 x 10^{-7}), and coverage (56.4 ± 26.3) indicate considerable uncertainty in the taxon placement based on these data. However, when the most common OTUs were examined in more detail, they were consistently placed in the same phylum and class suggesting that these assignments are likely to represent correct affinities. The inconsistencies that we observed were likely results from misidentified or erroneously annotated reads in the database (see Jumpponen & Jones 2010 for more detailed discussion).

Richness and diversity responses to environmental manipulations

The initial analyses indicated that the total number of sequences per experimental unit varied across the treatments: there was a significant interaction ($F_{1,10}$ = 5.56, P = 0.0421) between the warming and altered precipitation treatments (Tables 1 and 2). The sequence yields in the control treatment (ambient precipitation and ambient temperature) were higher than in the experimentally warmed treatments under ambient precipitation regime. Because differing sampling intensities may lead to bias and erroneous conclusions (Gihring *et al.* 2011), the data were analyzed with equalization of the sampling effort (1,095 random sequences were subsampled per experimental unit). Rarefaction analyses indicated that the sampling effort was inadequate to saturate the organismal richness in these samples (Fig 2). After subsampling, the observed OTU richness, diversity estimators and evenness were insensitive to the experimental manipulations and did not differ significantly among the treatments (Tables 1 and 2).

Taxon responses to the environmental manipulations

To explore the overall community responses, OTU frequencies were analyzed using NMS ordination after omission of the infrequent OTUs (OTUs that occurred in < 6 plots). This lead to a dataset with 211 OTUs that were best resolved in k ≥ 3 dimensions. Fungal communities were unresponsive to the experimental manipulations and the treatments were indistinguishable in these NMS analyses (Fig 3). To provide an estimate of organismal responses, those fungal OTUs that were abundant and invariable enough (based on *t*-tests at α = 0.05) were selected for further analyses. The *t*-tests identified a total of 226 among the 1,327 non-singleton OTUs that occurred on average at frequencies significantly greater than zero. Overall, 16 of these OTUs (7.1%) showed significant responses to the experimental treatments or their interaction. After correcting for multiple comparisons (FDR), none of these responses remained significant (Supplemental Table S5).

To provide a taxonomic framework for organismal responses to altered precipitation and experimental warming, the fungal OTUs were analyzed at a level of a class (Fig 4). These analyses provide broad scale information on the organismal responses to treatments. Among the nineteen fungal classes detected in our samples, only Lecanoromycetes were responsive and decreased in frequency in the warming treatment (Supplemental Tables S6 and S7). Similarly, Leotiomycetes seemed to be marginally responsive to precipitation manipulations and Tremellomycetes marginally responsive to warming and the interaction between warming and precipitation (Fig 4). However, none of these responses were significant after the FDR-correction for multiple comparisons.

Discussion

Our current study aimed to test hypotheses on fungal community richness, diversity, and compositional responses to manipulations of the precipitation frequency as well as temperature in a long-term experiment in a tallgrass prairie ecosystem. One of the greatest advantages of using the RaMPs study is its long duration – a minimum of four growing seasons of altered precipitation interval treatments and three growing seasons of warming treatments at the time of sampling for the current study. Studies reported here were motivated by previous research efforts that have reported plant community (Knapp *et al.* 2002), process level (Harper *et al.* 2005; Fay *et al.* 2011) shifts and changes in plant (Fay *et al.* 2011) or microbial community function (Fay *et al.* 2011; Zeglin *et al.* 2013) as a result of experimental manipulation of environmental conditions in the course of the RaMPs experiments. Those studies reported that the plant communities characteristic of the lowland deep soils had shifted towards communities that resemble the more xeric upland communities as a result of the precipitation manipulations and the resultant lower water availability (Knapp *et al.* 2002; Fay *et al.* 2002; 2003). Other ecosystem properties have also responded to these manipulations. Most importantly, C cycling as measured by CO_2 flux was observed to be tightly correlated with soil moisture (Harper *et al.* 2005; Zeglin *et al.* 2013). Those previous studies also suggest that soil CO_2 flux, plant CO_2 uptake, and the plant-utilized C (inferred from ANPP) are all reduced by altered timing or reduced quantity of rainfall (Fay *et al.* 2003; 2011; Harper *et al.* 2005). These ecosystem process level responses coincide with shifts in microbial biomass and activity (Evans & Wallenstein 2012; Zeglin *et al.* 2013) that seem reversible if the soil moisture conditions are homogenized across the long-term treatments (Evans & Wallenstein 2012). The observed above and belowground responses suggest that both the loss of C from soil as well as CO_2 capture by the plant community may be reduced by lower soil moisture, but partly compensated by the extension of metabolic activity by warming treatments (Fay *et al.* 2011). If ANPP reductions are not fully offset by the reduced soil CO_2 fluxes in the increased rainfall interval

treatments then this would lead to an increased net C sequestration over time. Zeglin *et al.* (2013) proposed that changes in microbial activities and biomass in the altered precipitation interval treatments could partly be reduced or compensated by more stress tolerant microbial communities that maintain growth at lower water potentials. Our current study specifically examined whether the soil inhabiting fungal communities would have changed compositionally and whether those shifts would coincide with the previously observed functional shifts.

Our data suggest that soil fungal communities remain largely unaltered and are resilient to experimental warming, manipulation of precipitation intervals, or their combination – leading thus to rejection of our original hypotheses. The fungal communities showed very few responses – particularly after accounting for multiple comparisons – regardless of whether the community composition was analyzed for responses of individual OTUs or for overall community distinction among treatments, or considered in terms of community summary statistics such as the richness or diversity. Our observations are consistent with some previous studies (Papanikolaou *et al.* 2010; Gutknecht *et al.* 2012; Anderson *et al.* 2013) but in contrast with others (Allison & Treseder 2008; Castro *et al.* 2010; Deslippe *et al.* 2011; Hayden *et al.* 2012). Our results suggest that: (1) soil communities are unresponsive to environmental manipulations; (2) the resident communities may adapt to the changing environmental conditions (Malcolm *et al.* 2008); or (3) the soil communities are adequately plastic to withstand environmental extremes (Cruz-Martinez *et al.* 2009; Hawkes *et al.* 2011) exaggerated by the experimental manipulations. It remains unclear whether our studies suffer from poor statistical power as high replication is difficult to achieve in large-scale experimental manipulations. However, previous studies have observed clear responses to these manipulations (Fay *et al.* 2003; Harper *et al.* 2005; Zeglin *et al.* 2013), suggesting therefore that the fungal community responses are likely to be lesser in scale.

Both soil temperature (Hacskaylo *et al.* 1965; Malcolm *et al.* 2008) and moisture (Frey *et al.* 1999) are important drivers of fungal growth, metabolism and

physiology. Yet, the effects of these drivers on fungal communities remain unclear and studies often yield contrasting results (Bardgett *et al.* 1999; Compant *et al.* 2010). Interpretation of soil community responses may be further complicated by indirect effects of plant communities (Cregger *et al.* 2012; Anderson *et al.* 2013), temporal dynamics that may take place on multiple time scales (Hawkes *et al.* 2011; Cregger *et al.* 2012; Zeglin *et al.* 2013), ecosystem level differences (Compant *et al.* 2010), or interacting environmental drivers (Hayden *et al.* 2012). Our data provide no support for strong soil fungal community responses to environmental manipulations in the RaMPs experiment, despite a relatively long manipulation of the environmental conditions. In comparison, many warming studies take place for a few months in growth chambers (*e.g.,* Büscher *et al.* 2012; Anderson et al. 2013) and precipitation manipulations are limited to few growing seasons (*e.g.*, Cregger *et al.* 2012; Krashevska *et al.* 2012). More extensive manipulations (*e.g.,* manipulation of precipitation over 5 years in Hawkes *et al.* 2011 and manipulation of CO_2 and temperature for 5 years in Hayden *et al.* 2012) provide an experimental platform to address environmental change effects in more relevant time scales. However, community turnover may mask fungal responses to environmental manipulations (Cregger *et al.* 2012), as fungal community responses may be rapid, reversible and repeatable (Hawkes *et al*. 2011). Taken together, it is important to tie fungal community analyses to a short-term temporal context that permit assessment of dynamics suggesting plastic environmental tolerances (Cruz-Martinez *et al.* 2009). Our experiments targeted long-term responses to environmental manipulations and did not address short-term temporal dynamics.

Studies that aim to address interacting environmental change drivers are few and complex to interpret; partly because responses tend not to be additive (Shen *et al.* 2009; Hayden *et al.* 2012; Matias *et al.* 2012), partly because interacting environmental drivers may exaggerate the environmental extremes. Hayden *et al*. (2012) analyzed soil bacterial, archaeal and fungal responses to elevated CO_2, elevated temperature and their interaction. They found that the soil organisms responded differently to the two environmental factors and their interaction. Fungi

were divided into three distinct groups based on ambient CO_2, elevated CO_2, or when elevated CO_2 was combined with experimental warming. These authors concluded that taxa responded differently under elevated CO_2 or warming and that response under any one condition could not be used to predict responses to interacting environmental drivers. In our study, we observed no obvious responses to increased precipitation intervals, elevated temperature, or their combination. This is unexpected as previous studies show that the altered precipitation intervals lower the average water potential (Harper *et al.* 2005; Fay *et al.* 2011), thus controlling soil function (Harper *et al.* 2005; Zeglin *et al.* 2013) as well as above ground productivity (Fay *et al.* 2011). Given the responses in function, we would have expected concomitant community responses, because elevated temperature may accelerate microbial metabolism (Rustad *et al.* 2001; Melillo *et al.* 2002; Shen *et al.* 2009) or increase substrate availability in soil (Stromgren & Linder 2002; Pendall *et al.* 2004). Simultaneously, available soil moisture – or lack thereof – sets inhibitory boundaries (Sheik *et al.*, 2011) even if the temperature might still be within the optimal range for the resident soil organisms. Such conditions may lead to communities that are more resilient or stress tolerant (Zeglin *et al.* 2013). Based on our data, soil fungal communities in a seasonally dynamic mesic grassland system that undergoes frequent periodic low water potentials and high soil temperatures are well buffered and incorporate adequate environmental plasticity to withstand the extremes created by interacting environmental drivers.

We also assessed potential taxon (class, OTU) responses to experimental manipulations. The taxon assignments of the short GS20 reads should be considered with caution (Nilsson *et al.* 2009; Vilgalys 2003). However, we compared the ranking of classes in this study to those reported previously in the same tallgrass prairie site (Jumpponen *et al.* 2010). Consistent with the previous studies, the soil fungal communities are dominated strongly by Agaricomycetes and Eurotiomycetes. In contrast to the relatively high proportion of Leotiomycetes in the present study, Leotiomycetes were relatively infrequent in the previous study and outranked by Dothideomycetes, Sordariomycetes and Lecanoromycetes. On the coarse class-level

resolution, we saw minimal responses to our experimental manipulations. Notwithstanding, while the database assigned taxonomic affinities even to the coarsest levels of taxonomic hierarchy may be inaccurate, OTUs represent mathematical creatures and differences – or lack there of – among them are independent of taxonomic hierarchy. Consistent with the diversity and richness estimators, the OTU level responses in our dataset were few and none were supported after correction for multiple comparisons. We also specifically examined OTUs assigned to Glomeromycota, the phylum known to form arbuscular mycorrhizas. Despite the removal of most roots prior to DNA extraction, approximately 3% of the sequences represented Glomeromycota. These data likely represent extramatrical hyphae and spores, or small root fragments that could not be removed from the clayey soil matrix. Our analyses suggest that despite the reported responses in the above (Fay *et al.* 2002; 2003) or below ground processes (Harper *et al.* 2005; Zeglin *et al.* 2013), there were no strong responses to the experimental manipulations among the arbuscular mycorrhizal symbionts. Only one Glomeromycotan OTU (OTU717) seemed responsive (P = 0.0308) to the interaction between warming and precipitation prior to, but not after, FDR-corrections (Supplemental Table S5).

Our GS20 data are burdened by some potential problems. First, pooling of amplicons across the experimental treatments was inaccurate and necessitated corrections for sampling effort (see also Ghiring *et al*. 2011). Second, the short GS20-generated 454-sequences provided a poor taxonomic framework. However, even if the taxonomic classification of the acquired sequence data may prove inadequate, the OTU assignments based on sequence similarities provide an estimate of organismal responses (Liu *et al.* 2008). Similarly, since community analyses based on the relative OTU frequencies neither require nor rely on approximation of taxon affinities, the estimates of the community turnover are likely reliable. These issues are important when considering adoption of ultra-high-throughput next-generation sequencing technologies that provide enormous data volumes but relatively short reads (Porter & Golding 2011; Caporaso *et al*. 2012; Brown *et al*. 2013). Finally, the

sequencing depth did not approach saturation of the organismal richness as suggested by our rarefaction analyses. However, successful saturation of the organismal richness in an analysis of soil microbial communities may be unattainable in studies seeking statistical power instead of most accurate species richness estimate (see Buée *et al.* 2009; Roesch *et al.* 2007). Furthermore, we chose to use the relative frequencies of common and abundant OTUs in our compositional analyses as reliance on the infrequent taxa may exaggerate observed community richness or differences therein. The use of frequent – or core (Magurran & Henderson 2003) – taxa provides reasonable estimates of the community compositional shifts. Sequencing data may also have a greater resolution or somewhat different view of the resident communities than DNA fingerprinting (*e.g.* Hayden *et al.* 2012) or phospholipid fatty acid (*e.g.* Gutknecht *et al.* 2012) tools employed in other recent studies.

The stability of the fungal communities may be explained by the microbial persistence in soil even at low water potentials as well as by their ability to respond and gain function rapidly even after small precipitation events (Schwinning & Sala 2004). Overall, across a variety of ecosystems, soil function has been shown to respond rapidly to wetting events as indicated by the soil CO_2 efflux (Liu *et al.* 2002; Fierer & Schimel 2003; Smart & Penuelas 2005; Sponseller 2007; Chou *et al.* 2008; Chen *et al.* 2009; Zeglin *et al.* 2013) suggesting that resident, metabolically inactive organisms rapidly gain their metabolic and physiological activities after unfavorable environmental conditions. Combining warming treatments with manipulation of precipitation may further exaggerate loss of water from the soil profile (Sheik *et al.* 2011) and therefore reduce the periods of metabolic activity. However, we have no evidence for fungal community responses to combined manipulation of precipitation intervals and temperature in this study. It is possible that repeated sampling or sampling targeting pulse events would prove more informative for identifying community responses to environmental manipulations.

The stability of communities in a mesic tallgrass prairie ecosystem is perhaps not

completely unexpected. Ecosystems and environments with large seasonal variability in precipitation can buffer the effects of climate change because a broad range of environmental and physiological tolerances likely exist in the resident microbial communities (Cruz-Martinez *et al.* 2009; Hawkes *et al.* 2011). Alternatively, in such environments, environmental change may exaggerate the severity of variation or increase the frequency of extreme events, leading to distinct communities under the predicted future environmental conditions (Hawkes *et al.* 2011). Our results support the former view. However, we emphasize that these soil community responses to precipitation may be strongly influenced by seasonal and pulse dynamics. For example, Chou *et al*. (2008) reported that soil CO_2 fluxes did not respond to rainfall during the wet season, whereas the fluxes increased rapidly and strongly in the early, drier season wet-up events. These findings are consistent with Zeglin *et al.* (2013), who observed rapid community responses to pulsed wetting events in the RaMPs experiment. The short-term and seasonal dynamics likely mask community responses to experimental manipulations. For example, Cregger *et al.* (2012) reported that seasonal community dynamics far exceeded the responses to experimental precipitation treatments in a piñon-juniper woodland highlighting the importance of including either multiple sampling occasions or controlling for the available soil moisture at the time of sampling. Additionally, use of a more labile marker such as rRNA may be suitable for targeting the community dynamics in temporally more appropriate context.

It is likely that in a mesic tallgrass prairie ecosystem, where most of the precipitation falls during the growing season, responses of microbial communities fueled by changes in long-term shifts in soil water potentials remain small. This is not to say that the function of those communities remains unaltered. Previous results from this study system indicate that alterations in precipitation lead to reduced soil respiration (Harper *et al.* 2005; Fay *et al.* 2011) raising the possibility of functional changes that remain uncorroborated in our compositional analyses of the soil communities. A portion of the CO_2 response observed in those studies may

also be an autotrophic response, as the fluxes were measured under field conditions with intact plant root systems.

What are the functional consequences of environmental change in the long term based on our current study? Bardgett *et al.* (2008) reviewed soil community contributions and their importance as a carbon interface between terrestrial and atmospheric carbon pools. While studies on ANPP, many ecosystem functions, and soil community function or activity (Fay *et al.* 2011; Zeglin *et al.* 2013) have highlighted the shifts resulting from the environmental manipulations in the RaMPs experiment, our data suggest that the soil fungal communities are largely unresponsive to manipulations of precipitation frequency and elevated temperature. Perhaps the most important conclusion emerging from these studies is that the soil communities may be structurally buffered against interacting environmental change drivers, whereas their function is determined by environmental constraints – available soil moisture in particular. Our concurrent studies on soil community carbon dynamics suggest that the measured activities decline with reduced soil moisture, leading to greater carbon storage at least in short-term (Zeglin *et al.* 2013). The tight coupling of above and below ground processes (Bardgett *et al.* 2008) necessitate estimating the whole ecosystem carbon budget that establish whether the tallgrass systems serve as net sinks or sources in the face of changing environments.

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FIGURE LEGENDS

Fig 1 – Distribution of the GS20-derived sequences across fungal classes for all samples in the RaMPs study. The chart shows the reads assigned to most common classes (> 1%) as a proportion of all acquired sequences. Unknown sequences did not return a phylum level classification in our BLAST queries. Group "Other" includes Agaricostilbomycetes (0.01% of all sequences), Chytridiomycetes (0.03%), Entomophthoromycotina (0.04%), Microbotryomycotina (1%), Neocallimastigomycetes (0.06%), Orbiliomycetes (0.09%), Pucciniomycetes (0.04%), Tremellomycetes (0.11%), Ustilaginomycetes (0.11%).

Fig 2 – Rarefaction (Mao Tau) analysis of fungal communities in the RaMPs experiment manipulating the precipitation intervals and temperatures. P = extended precipitation interval, W = experimental warming, W x P = W and P treatments combined. Horizontal lines show the treatment means. Text box on the upper left corner lists treatment means ± standard deviations. Vertical line shows the subsampling level for the richness and diversity estimators.

Fig 3 – Nonmetric Multidimensional Scaling (NMS) ordination of the fungal community data in the RaMPs experiment manipulating the precipitation intervals and temperatures (mean axis score ± standard deviation). Percentages on axes show the proportion of the variance represented. Inserts provide the mixed model ANOVA tables testing treatment differences for each of the axes: P = extended precipitation interval, W = experimental warming, W x P = W and P treatments combined. *P*-values for the *F*-test values: ns – $P > 0.05$.

Fig 4 – Rank ordered responses of fungi on a class level to manipulation of precipitation intervals and experimental warming (mean ± standard deviation). While most fungal classes show no significant response, Lecanoromycetes increase in frequency in response to experimental warming. This response is not significant after correction for multiple comparisons.