DISSERTATION

DROUGHT IMPACTS ON THE MICROBIOME IN GRASSLANDS ACROSS THE GREAT PLAINS: A STORY OF LEGACY EFFECTS, RESISTANCE, AND RESILIENCE

Submitted by

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ABSTRACT

DROUGHT IMPACTS ON THE MICROBIOME IN GRASSLANDS ACROSS THE GREAT PLAINS: A STORY OF LEGACY EFFECTS, RESISTANCE, AND RESILIENCE

Drought is increasing in frequency and severity across the US Great Plains as a direct result of climate change and if nothing is done to remedy climate change, drought will only continue to get worse over the next century. Thus, understanding how drought impacts natural and rangeland systems in the US will be vital to protecting these systems from negative impacts due to drought. Further, there has been a great deal of research on the aboveground response to drought, but little research on how the belowground soil community responds to drought. Lastly, some research exists on how drought impacts systems during the drought, but even less research exists on what happens after the drought. To further complicate this, the terms used to describe the period after drought are variable and inconsistent, leading to difficulty in synthesizing this literature. This dissertation aimed to re-define and make the terms used to describe the post-drought period consistent, understand how belowground communities respond after the drought has ended at one field site, and understand how microbial communities in the greenhouse respond to drought both during and after across several sites in the US Great Plains. The first chapter of this dissertation was a literature review that examined how researchers define the terms used after a drought ends and attempted to synthesize definitions for future use. The second chapter of this dissertation examined whether there were impacts leftover after a four-year drought on nutrient cycling in a mesic grassland. The third chapter examined whether there were leftover impacts from the same drought as chapter two on the microbial community. Lastly,

the fourth chapter examined how microbial communities respond during and after the drought across four Great Plains sites when the microbial community was isolated from the plant community.

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TABLE OF CONTENTS

INTRODUCTION

Climate models predict an intensification of drought throughout the US Great Plains with semi-arid regions forecast to experience more frequent and intense drought throughout the next century (IPCC, 2014; Guinard et al. 2015; Asadieh and Krakauer, 2015; Rahmani and Harrington, 2019). Increases in greenhouse gases and subsequent rises in temperature are likely responsible for this intensification (IPCC 2014), and if nothing is done to mitigate the rise in global temperatures (below the 1.5°C mark), drought will only become more frequent, widespread, severe, and long-lasting over time (Cook et al. 2015; Lehner 2017). This predicted increased extremity of drought has the potential to catastrophically impact plant production (Cook et al., 2015; Lesk et al., 2016). For example, the widespread, extreme drought of 2012 affected 65% of the continental US, cost the US $30 billion in agricultural and rangeland losses (Rippey, 2015), and drastically reduced aboveground productivity (Knapp et al., 2015, Knapp et al., 2020). Thus, there is a pressing ecological, economic, and societal imperative to understand the impacts of intensifying drought on grassland ecosystems. Additionally, these effects may persist after drought has ended and affect ecosystem responses to future drought events (Schwalm et al. 2017). Thus, understanding the potential lasting effects of drought will be vital in developing Earth system models that can predict the true impact of drought both during and after these events.

In the past decade, we have increased our understanding of how grassland ecosystems respond to drought, particularly regarding aboveground production (Hoover et al. 2014; Knapp et al. 2015; Hoover et al. 2016; Kreyling et al. 2017; Knapp et al. 2020) and shifts in plant species composition (El Haddi & Tilman 1992; Fry et al. 2014; Hoover et al. 2014). However, many of the key impacts of intensifying drought are mediated by microbial processes (Monohon et al. 2021;

Ngumbi and Kloepper 2016), critical aspects of the grassland system that have been largely overlooked. Drought may strongly affect the soil microbiome community (Naylor et al. 2017; de Vries et al. 2018; Naylor and Coleman-Derr 2018; Schimel 2018; Xu et al. 2018) with potential feedbacks on productivity, yet our understanding of these effects are limited. Drought has been shown to significantly alter the bacterial community and increase enzymatic activity with little effect on the fungal community (Ochoa-Hueso et al. 2018). Drought has further been shown to destabilize bacterial networks (de Vries et al. 2018) and disproportionately increase monoderm (single layer membrane) bacterial abundance (Xu et al. 2018). The soil microbiome (hereafter referred to as the "microbiome") serves a central role in ecosystem functioning through nutrient cycling (Wagg et al. 2014; Delgado-Baquerizo et al. 2016), decomposition (Glassman et al. 2018), and carbon (C) sequestration (Cotrufo et al. 2013; Kallenbach et al. 2016; Liang et al. 2017). Precipitation and soil moisture are the second most important indicators of microbiome structure and functioning next to pH (Fierer 2017); therefore, losses in function and structure of the microbiome will likely occur with intensifying drought (Naylor et al. 2017; de Vries et al. 2018; Naylor and Coleman-Derr 2018; Xu et al. 2018). This will cause further potential losses in grassland productivity, since plants rely heavily on the microbiome for essential nutrients (nitrogen (N), phosphorus (P), etc.) through nutrient cycling and decomposition (Jacoby et al. 2017). Further, grasslands store approximately one-third of all terrestrial C (White et al. 2000), which vastly mediates increases in atmospheric carbon dioxide due to human impacts. C sequestration will additionally be impacted by decreases in carbon mineralization (Hinojosa et al. 2019), soil microbial respiration (Hoover et al. 2016; Ren et al. 2018) (indicating a decrease in microbial activity) and decreases in stored soil organic matter (Ren et al. 2018) due to drought. Microbiome responses to drought will be a key indicator to belowground functioning; thus, making it critically

important to understand these responses and to mitigate potential losses in grassland function and structure.

The soil microbiome serves several key functions central to ecosystem functioning such as nutrient cycling, decomposition, carbon sequestration, and exchange of nutrients between plant and microbe. The soil microbiome is directly correlated with multiple ecosystem functions (Delgado-Baquerizo et al. 2020) and increasing microbial diversity has been shown to directly increase these ecosystem functions (Wagg et al. 2019). Thus, the soil microbiome serves a vital part in ecosystem functioning with certain microbial taxa even having been shown to increase plant growth (Lakshmanan et al. 2014), making understanding soil microbial response to drought highly critical to understanding belowground and plant response to drought. We currently still have a poor understanding of the soil microbiome and specifically how the soil microbiome responds to global change drivers, warranting further research. Shifts in the soil microbiome from global change drivers could have cascading effects on the ecosystem, thus making understanding potential impacts on the soil microbiome important.

Generally, we have a poor understanding of how the microbiome community responds to drought, but we have an even worse understanding of how the microbiome responds post-drought. Legacy effects describe impacts post-drought and are the negative or positive differences from the control that persist post-drought. Positive legacies in the microbiome would be increased diversity, abundance (biomass), functioning (increased enzyme activity). Negative legacies would be decreased diversity, abundance, functioning, or destabilized communities measured through networks. Microbiome legacy effects are largely unstudied, as is detailed in chapter 1 of this dissertation. A synthetic understanding of ecosystem response after drought

ends is further compounded by the various and inconsistent terms that researchers use to describe this period.

Thus, through this dissertation I first aimed to disentangle the words used to describe the post-drought period and redefine them for consistence use in the future (chapter 1). I then aimed to understand how the belowground community in grasslands responds after a long-term drought in both nutrient cycling (chapter 2) and microbial community composition (chapter 3). Finally, I aimed to generalize the response of microbial communities during and after drought across a climatic gradient (chapter 4).

CHAPTER 1: What happens after drought ends: synthesizing terms and definitions

1. Summary

Drought is intensifying globally with climate change, creating an urgency to understand ecosystem response to drought both during and after these events end to limit loss of ecosystem functioning. The literature is replete with studies of how ecosystems respond during drought, yet there are far fewer studies focused on ecosystem dynamics after drought ends. Furthermore, while the terms used to describe drought can be variable and inconsistent, so can those that describe ecosystem responses post-drought. With this review, we sought to evaluate and create clear definitions of the terms that ecologists use to describe post-drought responses. We found that legacy effects, resilience, and recovery were used most commonly with respect to post-drought ecosystem responses, but the definitions used to describe these terms were variable. Based on our review of the literature, we propose a framework for generalizing ecosystem responses after drought ends, which we refer to as 'the post-drought period'. We suggest that future papers need to clearly describe characteristics of the imposed drought, and we encourage authors to use the term post-drought period as a general term that encompasses responses after drought ends and use other terms as more specific descriptors of responses during the post-drought period.

2. Introduction

Climate models predict an intensification of the hydrological cycle (Dai 2011; IPCC 2014; Asadieh and Krakauer 2015). Increases in greenhouse gases are likely responsible for this intensification (IPCC 2014), and if nothing is done to mitigate the rise in global temperatures (below the 1.5°C benchmark), drought will become more frequent, widespread, severe and long-

lasting over time (Cook et al. 2015; Lehner 2017). This predicted intensification of drought has the potential to significantly impact future ecosystems, if past droughts are any indication of the future response (Cook et al. 2015). Indeed, extreme drought has been estimated to cause annual losses of about 1% of earth's terrestrial ecosystem function and reduce carbon uptake by 0.14 PgC/yr globally (Du et al. 2018). With climate-change driven intensification of drought, reductions in C uptake and more permanent losses in ecosystem function are expected to be magnified over time. As such, there is a pressing scientific need to understand how ecosystems respond to drought to better mitigate potential negative effects. Additionally, these effects may persist after drought has ended and affect ecosystem responses to future drought events (Schwalm et al. 2017). Thus, understanding the potential lasting effects of drought will be vital in developing Earth system models that can predict the true impact of drought both during and after these events.

Existing literature on ecosystem responses during drought is synthesized in both reviews (e.g., Niu et al. 2014; Felton and Smith 2017) and meta-analyses (e.g., He and Dijkstra 2014; Sun et al. 2020; Castagneri et al. 2021), which provide a cohesive narrative of the impacts of drought on a myriad of ecosystem processes as these events unfold. Although there is still uncertainty in drought responses, this body of work allows us to begin to generalize ecosystem responses during drought. For example, there is strong evidence from meta-analyses that the mean effect of drought on aboveground productivity is negative (Wu et al. 2011, Gazol et al. 2020; Sun et al. 2020). Yet, our knowledge of drought responses is incomplete without understanding whether responses that occur during drought persist after drought and for how long they persist. The current literature is largely inconsistent (Stuart-Haentjens et al. 2018) and limited in how ecosystems respond after drought, and whether and how long drought effects

persist after these events end. Models often assume that ecosystems recover completely after drought ends, when in reality, full recovery may take a few years (Anderegg et al. 2015) or may extend over decades (Weaver 1944). The directionality of ecosystem response post-drought is often mixed, with some research showing positive effects (e.g. increase in ecosystem functioning) post-drought (Griffin-Nolan et al. 2018; De Long et al. 2019), with others finding negative or neutral effects (Rousk et al. 2013; Hofer et al. 2016; Kreyling et al. 2017). Sala et al. (2012) found that legacies of dry years or low precipitation had negative implications for the next year's growth indicating that growth is inhibited after drought. This inconsistency in directionality of responses could be driven by a myriad of factors making it difficult to synthesize the literature in a cohesive way.

A synthetic understanding of ecosystem response after drought ends is further compounded by the various and inconsistent terms that researchers use to describe this period. As we describe in detail below, our review of the literature found these terms include legacy effects, lag effects, resilience, recovery, re-wetting, drought memory and compound drought/double-stressed. We contend that before we are able to synthesize the literature and move forward with research in this area, we need to unify these terms and definitions. This paper aims to summarize how researchers use and define these terms, discuss potential biases in this literature, and make recommendations on how we can best combine these terms in a unifying framework to allow for a generalized understanding of how ecosystems respond after drought events. To accomplish these objectives, we conducted a literature review focused on papers that examined above- and belowground terrestrial ecosystem responses after drought events have ended.

3. Literature review

We conducted a literature review (Web of Science) to assess how researchers define and use terms related to ecosystem responses after drought ends. Based on an initial review of the literature, we identified the following terms for our search: “drought AND legacy effect*”; “drought AND memory effect*”; “drought AND lag effect*”; “drought resilience”; “drought recovery”; and “compound drought OR compounded drought.” We did not filter by year or subject area as to not miss any possible papers. This February 2021 search yielded 1,415 results, of which we deemed 94 papers relevant (Appendix 1 Table 1). A large majority of the papers from this search were excluded because they did not impose drought, mentioned drought in passing, or were not conducted during the post-drought period. Furthermore, many of the papers were experiments that mentioned drought in their abstracts but did not actually study drought while others were not ecological papers and were therefore excluded. From each relevant paper, we extracted information regarding ecosystem type; whether aboveground or belowground measurements were taken; whether the experiments were greenhouse, lab, field or remote sensing experiments; the reduction in precipitation that occurred; the length of drought; the time after drought that the measurement(s) was/were taken; whether there were one or two droughts; what term they used; how they defined that term; whether the effect was positive, negative, or neutral; whether the mechanisms for the effect were abiotic or biotic; and the mechanisms cited for the effects observed (Appendix 1 Table 1). A few of the 94 papers did not contain all of this information, so some categories had fewer than 94 entries.

4. Terms, definitions and context of terms

4.1 Terms and definitions

The 94 papers included in our review most commonly used the term legacy effect(s) (41%), followed by recovery (27%), resilience (19%), lag effect (4%), compound(ed) drought (4%), memory (4%), and re-wetting (2%) (Figure 1.1). Legacy effects, recovery, resilience, lag effect, and re-wetting all describe the period after typically one drought and the response seen during this time-period. The term legacy effect was the most common term used likely because it is a simple way to say that effects were present after the end of drought. Recovery and resilience also describe this post-drought period, whereas compounded drought and drought memory generally describe the response during a second drought. Re-wetting can be considered different than the other terms, because it implies a larger than average level of precipitation, which the other terms do not imply.

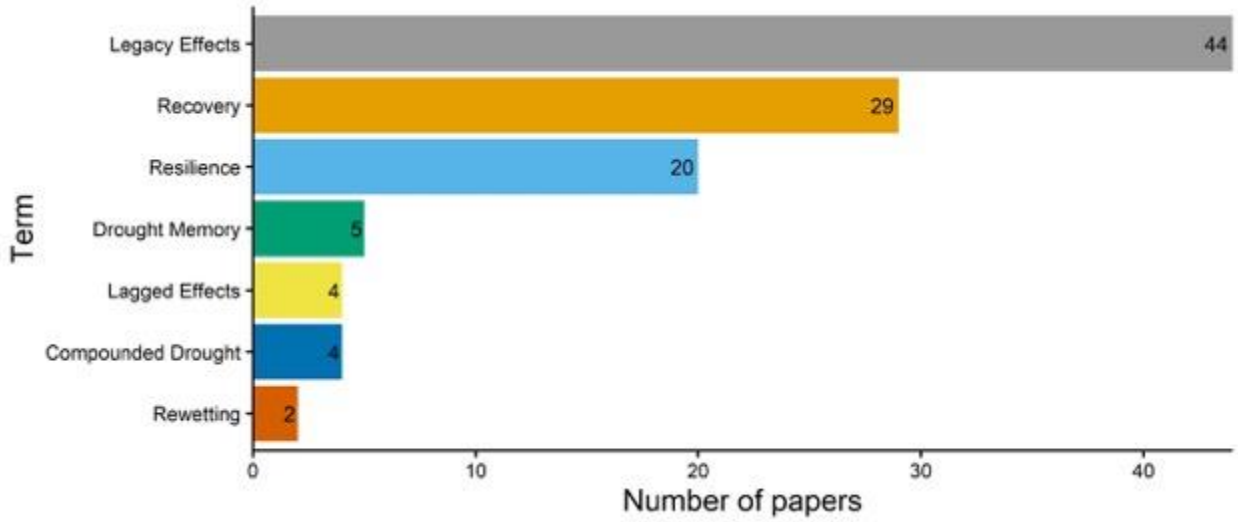

Figure 1.1. Summary of the terms used in the 94 papers reviewed and the number of papers that used each term.

After identifying the frequency in which terms were used, we extracted definitions authors used to describe the term or terms used in their papers. Based on the definitions provided by authors, we generated a list of definitions commonly used for each term (Table 1.1). Generally, the most

common definition used was ‘the effects of drought after drought has subsided’ (Table 1.1). The next most common definition used was ‘the ability to recover’. These terms were mostly associated with legacy effects and recovery/resilience, respectively. Terms were also defined with respect to the capacity to recover after drought, reduction in function, antecedent conditions, compound effects, or departure from typical growth. Only a small percentage (8%) of papers did not Table 1.1. Summary of the definitions of each of the terms assessed in this review. The different definitions for each term are provided with references for which papers the terms were used and defined. Our goal was to include the general definitions found across the papers and cite the most relevant papers.

Table 1.1. Summary of the definitions of each of the terms assessed in this review. The different definitions for each term are provided with references for which papers the terms were used and defined. Our goal was to include the general definitions found across the papers and cite the most relevant papers.

Term	Definitions	Reference
Legacy effect	• Effects of drought after drought has subsided • Indirect rather than direct effects of drought • Lasting physiological changes • How community responds after drought to re-wetting • Lag or incompleteness in recovery	Griffin-Nolan et al. 2018 Hicks et al. 2018 Kannenberg et al. 2019 de Nijs et al. 2019 Huang et al. 2018
Recovery	• Growth reaction following drought period • Post drought conditions / drought conditions • Well-watered conditions after drought • Time it takes to recover after drought	Gazol et al. 2017 Vitali et al. 2017 Panke-Buisse 2020 He et al. 2018
Resilience	• Capacity to recover to pre-disturbed conditions • Ability to recover from drought events • Post drought conditions/ pre-drought conditions • Post-drought recovery rate	Dang et a. 2019 Elsalahy et al. 2020 Vitali et al. 2017 Li et al. 2020
Lag effect	• Positive correlations the following year after drought	Zhao et al. 2018
Drought memory	• Memory that helps respond to future disturbance • Persistent effects of antecedent precipitation on productivity	Leufen et al. 2016 Liu et al. 2018
Re-wetting	• Wet period after drought	Van Sunder et al. 2019
Compounded Drought	• Effect of old perturbation to new perturbation • Effects of heatwave and drought at one time	Peltier et al. 2019 El-Madany et al. 2020

4.2 Context of terms

With our literature review, we extracted a suite of study attributes to determine if there were specific contexts in which terms were used (Figure 1.2). These study attributes included: ecosystem type, study type, measurement type, and types and direction of responses measured,

as well as the time after drought measurements were made (Figure 1.2a). In addition, we also examined key characteristics of the drought itself (Figure 1.2b). We found that several study attributes and drought characteristics stood out for differentiating the context in which terms are used.

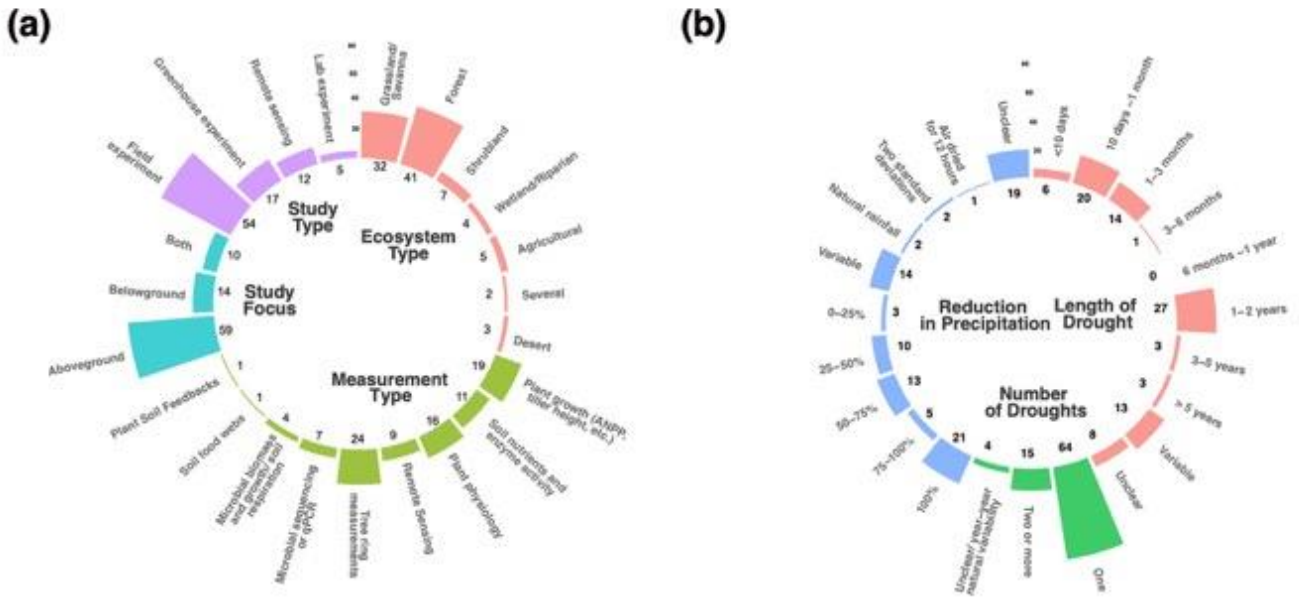

Figure 1.2. Attributes of the studies and characteristics of drought. The 94 studies included in our literature review varied in their study attributes and characteristics of drought. We were able to extract several study attributes including: ecosystem type; whether aboveground or belowground measurements were taken; whether the experiments were greenhouse, lab, field or remote sensing experiments; what measurements were taken. For studies that fit into more than one category (e.g. plant growth and nutrient analysis for measurement type), we counted the paper separately for each category they fell into. The drought characteristics examined were the reduction in precipitation that occurred; the length of drought; and whether there were one or two droughts. We found that studies were mostly from grassland and forest ecosystems, were mostly tree ring and plant growth measurements, consisted of mostly aboveground measurements, and were mostly field experiments (a). We also found that most studies imposed one drought and varied considerably in length of drought and reduction in precipitation imposed, although most studies imposed drought under a year and had mostly unclear (not mentioned in the manuscript) reductions in precipitation (b). a) Key descriptors of (a) study characteristics and (b) drought characteristics the 94 studies included in the literature review. Study characteristics included: ecosystem type (orange bars), study type (purple bars), study focus (aqua bars), and measurement type (green bars). Drought characteristics included: reduction in precipitation (blue bars), number of droughts (green bars) and length of drought (orange bars). Numbers at the base of each bar indicates the number of studies that fell into a category of each characteristic.

One such study attribute was the average time after drought that studies measured post-drought responses (Figure 1.3). When the terms recovery and resilience were used simultaneously, researchers measured the post-drought responses on average two years after the drought ended, which was longer than when other studies measured their responses after drought. Resilience and rewetting both individually on average measured responses 1.5 years after the drought ended. It is logical that papers that measured recovery and resilience would measure the effects post-drought at a longer time scale, since the papers claim to see recovery after some sort of time scale, which leads to the papers calling the system resilient. It is also possible that studies focused on particular vegetation types, such as forests, favor the terms resilience and rewetting which are often measured on a longer time scale. Investigators measured post-drought responses about 1 year after the event ended when using the terms recovery, legacy effects, and lagged effects. If a legacy effect or lagged effect is still occurring, this is likely closer in duration to when the drought ended. Lastly, drought memory studied the effects after drought on average after 0.5 years. Although the terms differed in on how long on average investigators measured the effects post-drought, the time frame in which effect post-drought responses/effects were measured averaged around 1 year after drought (range 0-20 years), which could be too short-term, since most papers observed lingering effects from the drought.

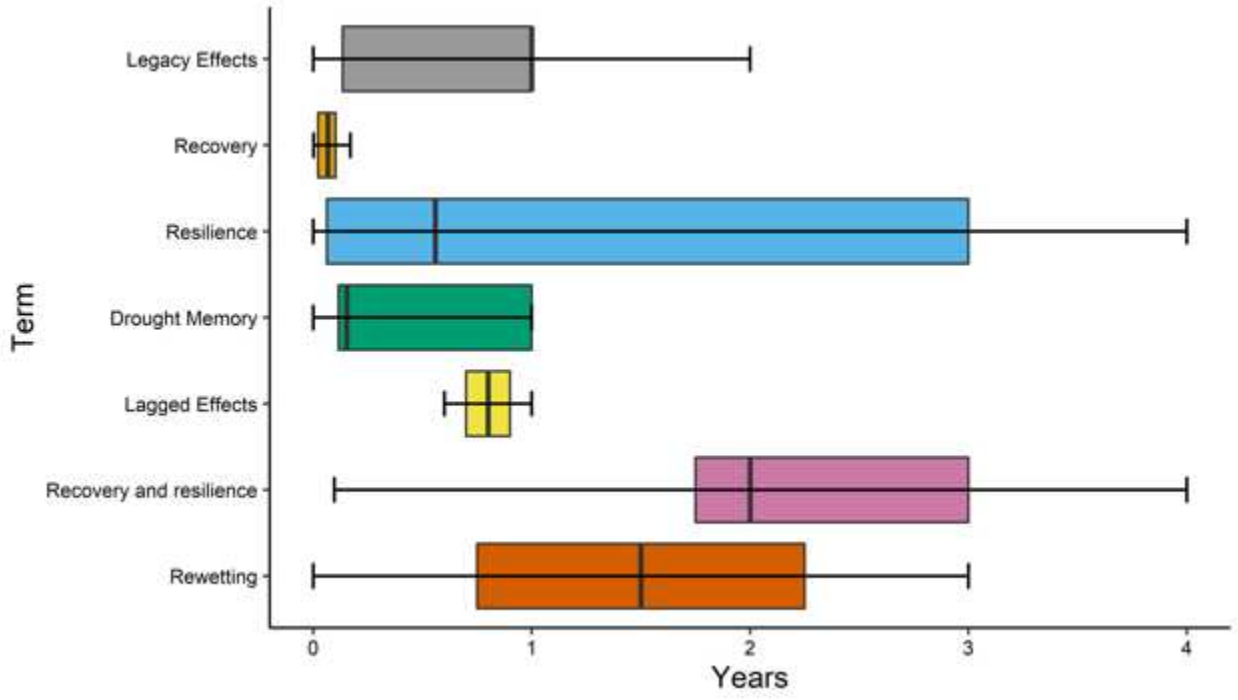

Figure 1.3. Box plots with median bars (outliers not shown) are used to show the average time after drought that each study measured responses with respect to the terms used. The category 'recovery and resilience' includes papers that used both the terms recovery and resilience, while the categories 'recovery' and 'resilience' include papers that only used the terms individually.

Another study attribute that provides important context for the post-drought terms usage was the direction (positive, negative, or neutral) of post-drought responses measured (Figure 1.4). We found that the 94 papers reviewed disproportionally reported negative effects of drought (Figure 1.4a). While it is possible that negative effects are more common than neutral or positive effects, it is not possible to distinguish this from a publication bias. This bias could skew future meta-analyses or syntheses toward more negative results than the true value of post-drought responses. Therefore, it is important to encourage the publishing of neutral (otherwise known as negative) results (Mlinaric et al. 2017). In addition to an overall bias in direction reported, we also found that direction of response differed by term used by a study (Figure 1.4b). Papers using the terms

legacy effects and lagged effects reported mostly negative responses after drought. In contrast, drought memory reported a variety of different responses (neutral and negative responses), even though one might expect only positive responses being reported. Recovery and recovery/resilience papers had more neutral and positive responses than negative responses. Finally, papers using resilience reported an equal number of positive and negative responses, while re-wetting papers reported only positive responses.

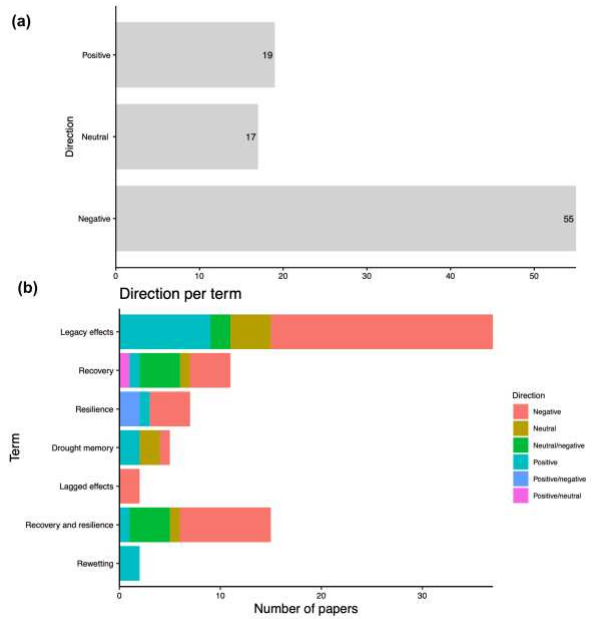

Figure 1.4. Direction of post-drought responses published. We identified papers as positive if the response after drought was positive (e.g. increased plant growth or increases in soil nutrients), negative if the response was negative (e.g. losses in plant growth or loss of soil nutrients), or neutral if there was no significant response found (e.g. plant growth was the same as the control or pre-disturbance). We counted the number of studies that by our definition had a positive, negative, or neutral response. When papers had both neutral and negative effects or neutral and positive effects, we counted each effect as a separate entry. We conducted a chi-square test for the number of papers that had positive, negative, or neutral responses post-drought to determine if the difference observed was by chance. Overall, 19 studies had positive effects, 55 had negative effects, and 17 had neutral (or no) effects. To assess if there was bias in the publications, we used R statistical software and the base R function "chitest" to obtain our p-value (R Core Team 2013). A significant p-value (<0.05) would indicate that our results were not due to chance and there is a bias involved. The test produced a significant p-value ($p = <0.001$), which supports that reported results were biased toward those that are negative. We checked if this bias applies to just legacy effects papers, which was the category with the largest number of papers. There were 9 studies with positive effects, 26 with negative effects, and 6 with neutral effects. We found a significant p-value in this test as well ($p = 0.002$), indicating that these differences were not due to chance. **(a)** The number of responses that were positive, negative, or neutral in the 94 papers reviewed. **(b)** The number of papers reporting a direction of response (positive, negative, neutral, or combined) for each term.

5. A synthesis of post-drought terms and definitions

Our review has illuminated the variety of terms used to describe ecosystem responses after drought ends and the variability in definitions of these terms and the contexts in which they are used. To provide a cohesive framework, we propose using the term 'post-drought period' to describe ecosystem responses that are observed after a drought ends (Figure 1.5) and using the terms highlighted in this paper to further describe the nature of the ecosystem responses observed in the post-drought period.

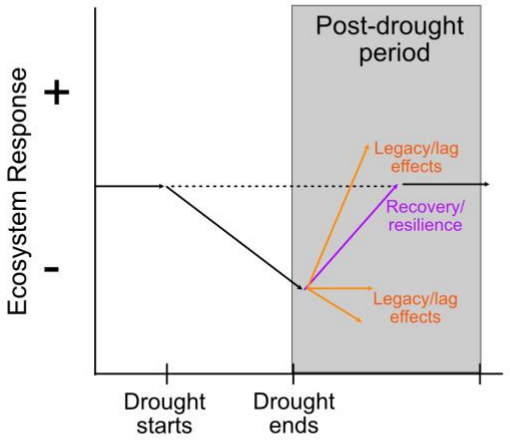

Figure 1.5. Framework for describing ecosystem responses after drought ends. We refer to this as the 'post-drought period'. Within this post-drought period, we propose the nature of the ecosystem response can be described by a set of terms. The start of the drought refers to either one drought or any subsequent droughts. We propose that the same terms should be used during the post-drought period regardless of how many drought periods have occurred. Legacy (which includes lag) effects describe either positive or negative effects observed during the post-drought period. Recovery and resilience are terms that describe neutral effects seen after a certain amount of time post-drought.

The first most commonly used term in the papers we reviewed was legacy effects. Legacy effects and lag effects have similar definitions and thus can be used interchangeably. However, the term legacy effect(s) is more commonly used over lag effects, and we suggest it is the more appropriate term to use for describing responses during the post-drought period. Further, we propose that legacy effects be used to describe responses observed in the post-drought period, whether they are positive or negative. These effects can last for an undefined period of time or could be indefinite. Sometimes the changes may be irreversible, indicating a state change, which would also be an example of a legacy effect. The term legacy effect(s) is most appropriate for describing the directionality of effects but would not be appropriate for describing a neutral effect. Legacy effects would also not be appropriate for describing the post-drought period generally, since it is a descriptor of what occurred, not a temporal description of the time-period after drought. As we observed across the papers used in this study, not all responses were positive or negative. Indeed, 19% of the papers had a neutral response, which would make legacy effects inappropriate for describing such responses, since our framework argues that legacy effects must have a directional response (Figure 1.5). Additionally, there actually may be more instances of post-drought neutral responses if potential publication biases are eliminated as discussed above. Lastly, legacy effects have also been described as the effect of past year's precipitation (Sala et al. 2012) or as historical conditions (Bunting et al. 2017), and we suggest that these be called antecedent conditions to avoid confusion with our definition of legacy effects.

The second and third most commonly used terms were recovery and resilience. Both terms are different than legacy effects because they describe a trend towards pre-drought conditions, but they are generally quantified in different ways (e.g., mathematical equations in ecophysiology;

Table 1.1). If there is a negative or positive response observed post-drought, but the response returns to a pre-drought levels, then by definition the system has recovered. Additionally, if a system recovers quickly or is not largely affected post-drought or during the drought event (i.e., neutral response), then we propose the system be called resilient to drought. Some systems may never recover after drought or experience a state change, which would make recovery inappropriate and misleading to use for describing the post-drought period. Given that systems may vary in how long it takes to return to pre-drought conditions, recovery is highly dependent on the time scales considered. Thus, recovery and resilience are appropriate terms when describing the directionality or speed of return to pre-drought conditions (otherwise known as a neutral response), but we contend these terms are not appropriate for generally describing the post-drought period.

The terms compounded drought and drought memory both describe the time point when a second drought occurs after the first drought ends. The two terms differ in their implication of the direction of the response. Drought memory implies a positive response to a second drought. The term memory implies that there is a "remembered" effect from the first drought that assists with the response to a second drought. Drought memory would in many cases be inappropriate for describing a response but would be best used to potentially justify or explain positive responses (legacy effects) if they are observed. Compounded drought depicts the sequential occurrence of drought events within a certain time period and is not related to characteristics of ecosystem responses. We suggest calling the period after a compounded drought the post-drought period and using the terms legacy effects, resilience, and resistance in the same way as after a single drought event to describe the nature of response. It is important to note that compounded drought has also been defined as another perturbation such as a heat wave occurring at the same time as

drought (Matusick et al. 2018; Zscheischler et al. 2018; El-Madany et al. 2020). Thus, we suggest using sequential drought to describe two or more drought events and use compounded drought to describe the co-occurrence of drought with heatwaves or other perturbations.

A key finding from our review is that the length of time of the post-drought period captured by the studies was often inconsistent or even undefined. We were able to assess this by looking across the terms used and the average amount of time that the paper measured responses post-drought (Figure 1.3). As discussed earlier, we found that the average time that these studies measured responses post-drought was relatively short. Although some studies measured drought effects up to 20 years post-drought, most studies measured the effects of drought on above- and belowground ecosystem responses for less than a year after the drought occurred. This is a short time frame and could also explain the bias we discussed towards negative results. Perhaps more papers would have observed a neutral effect had the responses been measured over a longer time scale or perhaps negative effects may have persisted longer than studies currently measure post-drought responses. This highlights the need for post-drought studies to measure responses for a longer time scale, particularly if they are interested in determining whether the system recovers or is resilient. Furthermore, many papers used the term recovery, yet they saw directional responses over the study period (Figure 1.3). Very few of the recovery and resilience papers saw full recovery and classified their effects as negative, since most still had negative responses. Using recovery or resilience for systems that have been unable to recover or have not yet recovered is misleading. These are instances when the term legacy effects would be more appropriate for describing the response in the post-drought period. Time-scales will be vital in future post-drought research with a strong preference for longer-term experiments along with defining the characteristics of drought clearly.

6. Mechanisms underlying post-drought responses

The papers reviewed attributed various mechanisms to explain positive, negative or neutral post-drought responses. Papers mostly cited biotic reasons (60%) as the only mechanism responsible for the effect in the study (Figure 1.6). Additionally, most papers cited a physiological reason as the mechanism for the response observed (Appendix 1 Table 2). Biotic, particularly physiological mechanisms, imply that the responses were plant-driven, which is highly possible, although belowground processes could also contribute to the aboveground responses observed. Some papers (18%) cited belowground reasons as the mechanisms such as changes in nutrient concentrations, elevated nitrogen levels, microbial community mediated, less active microbial community, or microbial turnover of plant carbon (Appendix 1 Table 2). Other papers most commonly cited water reserves or precipitation-based reasons as the mechanisms for the responses measured. There was variety in mechanisms used to explain responses observed in the papers, but it is unclear which mechanisms are most important and drive these effects. The biggest problem is that studies typically only cited one or a few mechanisms, which is unlikely to be the case in reality. This field of research will need to be driven forward by studying general mechanisms, focusing on mechanisms that link belowground and aboveground processes and responses. It is highly unlikely that the mechanisms (e.g. physiological or nutrient mediated) in the post-drought period driving the responses, whether it be recovery or a state-change, will be driven by only a single factor, as several factors have been shown to improve recovery post-drought (Xu et al. 2013; Jiao et al. 2021). This field of research would benefit from studies that holistically examine the mechanisms driving the responses seen during the post-drought period.

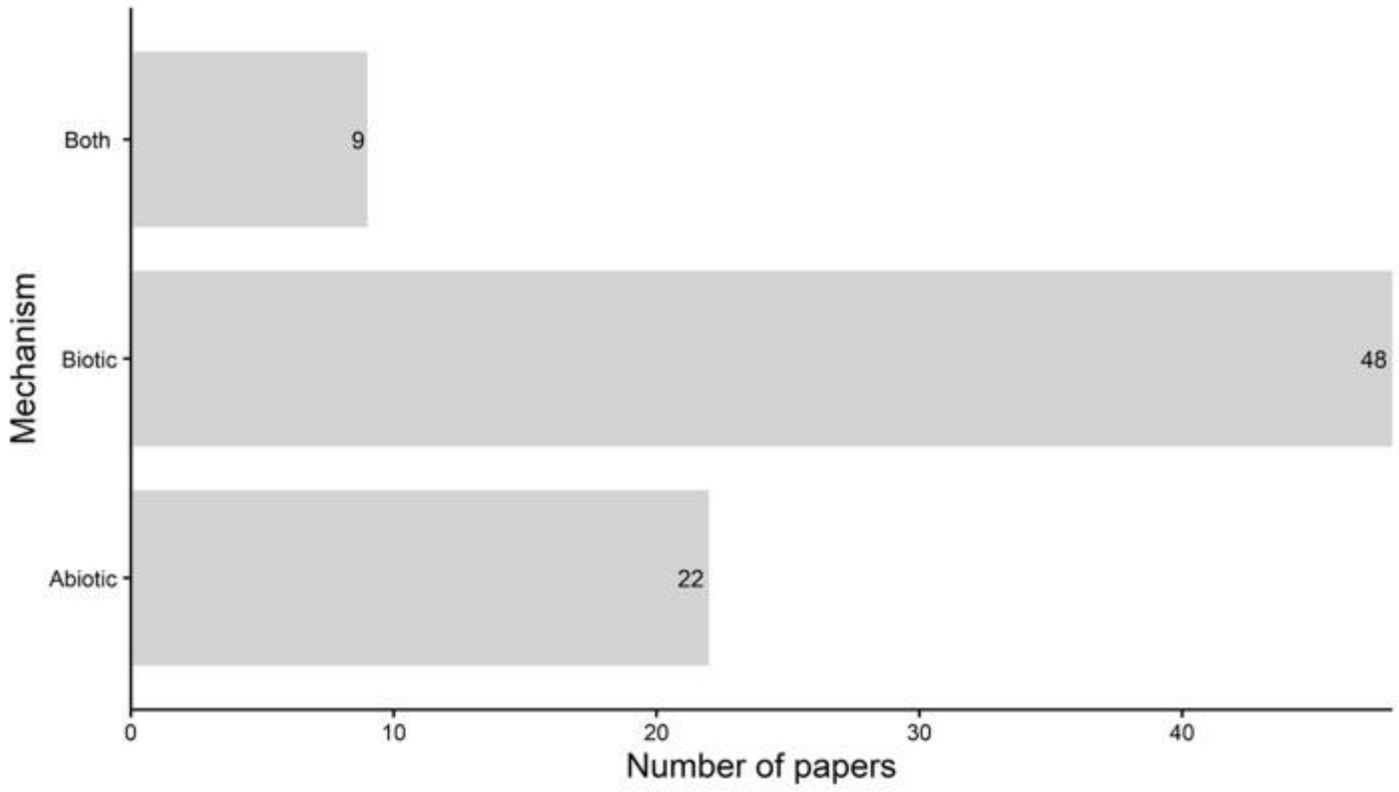

Figure 1.6. The number of papers that cited biotic, abiotic, or both abiotic and biotic mechanisms for the responses papers found post-drought. Mechanisms were grouped into categories (below) and then the numbers of papers that fit into these categories were counted. Mechanisms were determined by each paper's reasoning for the response post-drought typically highlighted in the discussion section. These mechanisms were then grouped and compiled as seen in Appendix 1 Table 2. We then split these into biotic and abiotic mechanisms as can be seen in Appendix 1 Table 2 and counted the number of papers that cited biotic only, abiotic only, or both abiotic and biotic mechanisms that led to the response seen post-drought. Papers mostly sited biotic responses and changes in physiology as the reasons for the response seen.

7. Knowledge gaps

Overall, our review suggests that we have limited understanding of the period after drought due to a dearth of studies and an undue emphasis on aboveground ecosystem responses, with potential publication biases making it difficult to parse out what happens after drought. Indeed, 71% of studies examined aboveground ecosystem responses alone, which leaves a large gap in the knowledge of belowground responses after drought (Figure 1.2a). There is a pressing need to understand belowground responses post-drought, since the belowground serves important functions such as nutrient cycling, decomposition, and carbon sequestration. Yet, our review suggests that belowground ecosystem responses are generally understudied, warranting further research.

Further, as a body of research in ecology matures, meta-analyses become an important way in which results from numerous studies can be synthesized to find generality (Gerstner et al. 2017), since ecological responses are often variable and occur at a large-scale. Meta-analyses of responses after drought will be important for providing general understanding in post-drought responses, which is critical in mitigating potential negative impacts of drought. At this point in time, we are approaching enough papers for a robust meta-analysis of aboveground responses in the post-drought period, although most papers focus on plant growth and tree ring measurements (Figure 1.2a). A meta-analysis becomes even more challenging for belowground responses. The only statistical analysis we were able to conduct in our review was a chi-square test to test for publication bias, because the number of papers were insufficient for any further analysis such as effect size of different ecosystem responses. This review has highlighted the absence of post-drought research, particularly that focuses on belowground responses. Furthermore, our review

revealed the variability in the ways in which drought is imposed and the resultant responses that were observed (Figure 1.2b). Adding to the post-drought research literature and using standardized approaches to imposing drought will allow for improved meta-analysis and synthesis in the future and increased understanding of post-drought ecosystem responses.

Research on the post-drought period will also be difficult to synthesize because researchers define drought inconsistently and the characteristics of drought are not clearly described (Figure 1.2b; e.g. timing, length, and magnitude of drought). Variability between how papers conduct drought research is inevitable, but it is crucial for papers to explicitly describe the characteristics of their drought even it was a natural drought. One such characteristic was the time that the response was measured after the drought ended. Eleven percent of studies had variable timing of their measurements post-drought, and in 5% of studies it was unclear at what time they measured effects post-drought. Papers were even more inconsistent in describing the magnitude of the drought. In 21% of the studies, we were unable to determine the magnitude of reduction in drought. The length of the drought period also was generally not explicit with 15% of studies having variable lengths of the drought period and 10% were unclear in their length of the drought period. It is clear from this review that a common definition of drought needs to be defined, as is discussed in Slette et al. (2019), and that papers need to clearly articulate the characteristics of their drought.

A second knowledge gap is the study of compounded droughts. Only 7.4% of papers used compound(ed) drought, double-stressed or drought memory to describe the effect of a second drought after a previous drought has occurred in the system. This could be important for future understanding of a drying climate because subsequent drought events may occur before the system has recovered from a previous drought (Schwalm et al. 2017).

A third knowledge gap is the need for further research examining mechanisms underlying post-drought ecosystem responses. We found that the mechanisms proposed were variable, but primarily focused on biotic mechanisms related to plant ecophysiology. How plants may be responding belowground and affecting soil processes and vice versa were lacking as potential mechanisms. This is important because plant-soil feedbacks and specifically the potential for decoupling, in which aboveground and belowground have different responses to drought and their interaction is changed, is likely an important in affecting post-drought responses (Bardgett et al. 2013). For example, if the soil microbial community changes after drought, but the plant community does not, functional decoupling could occur, because the interactions between the aboveground and belowground processes will change. It will not be enough to only study aboveground and belowground effects and mechanisms separately because studies on plants and soil must combined to measure potential decoupling and the feedbacks between them (van der Putten et al. 2016).

8. Conclusions

Our review highlights the need for consistency of terms used to describe the post-drought period and the knowledge gaps needed to advance research aimed at elucidating the effects of drought after these events end. Our review found that papers use a variety of terms to describe the period after drought, often do not fully describe drought characteristics, are short term in their study of post-drought responses and have potential biases that may impede future synthesis. Our review aimed to bring together the terms used to describe post-drought responses and proposed a common framework for these terms, which we refer to as the “post-drought period”. Within this post-drought period, we propose that terms often used to describe responses after drought ends be used as descriptors of the nature of post-drought responses rather than a

description of the period itself. We further propose that the term sequential drought be used to describe a drought event occurring after a previous drought event, but that the time after each drought event be called the post-drought period and use consistent terminology for describing the nature of post-drought responses. We hope that papers use our framework to increase consistency among studies. We also propose determining whether publication bias exists based on the preponderance of negative ecosystem responses reported post-drought; conducting more research on mechanisms underlying post-drought responses, plant-soil feedbacks, and the decoupling of above- and belowground processes; and to better describe the characteristics of the drought itself. These knowledge gaps must be remedied to provide a comprehensive and predictive understanding of ecosystem responses during the post-drought period, which almost certainly represents a longer time-period of impacts than those occurring during drought events.

CHAPTER 2: Limited legacy effects of extreme multi-year drought on carbon and nitrogen cycling in a mesic grassland

1. Summary

The intensification of drought throughout the US Great Plains has the potential to have large impacts on grassland functioning, as has been shown with dramatic losses of plant productivity annually. Yet, we have a poor understanding of how grassland functioning responds after drought ends. This study examined how belowground nutrient cycling responds after drought and whether legacy effects persist post-drought. We assessed the two-year recovery of nutrient cycling processes following a four-year experimental drought in a mesic grassland by comparing two different growing season drought treatments - chronic (each rainfall event reduced by 66%) and intense (all rain eliminated until 45% of annual rainfall was achieved) – to the control (ambient precipitation) treatment. At the beginning of the first growing season post-drought, we found that in situ soil CO_2 efflux and laboratory-based soil microbial respiration were reduced by 42% and 22%, respectively, in the intense drought treatment compared to the control, but both measures had recovered by mid-season (July) and remained similar to the control treatment in the second post-drought year. We also found that extractable soil ammonium and total inorganic N were elevated throughout the growing season in the first year after drought in the intense treatment. However, these differences in inorganic N pools did not persist during the growing season of the second year post-drought. The remaining measures of C and N cycling in both drought treatments showed no post-drought treatment effects. Thus, although we observed short-term legacy effects following the intense drought, C and N cycling returned to levels comparable to non-droughted grassland within a single growing season regardless of whether the drought was intense or chronic in nature. Overall, these results suggest that key

aspects of C and N cycling in mesic tallgrass prairie do not exhibit persistent legacies from four years of experimentally-induced drought.

2. Introduction

Climate models predict that semi-arid regions, such as the US Great Plains grasslands, are forecast to experience more and intense and widespread drought throughout the next century (IPCC, 2014; Guinard et al., 2015; Asadieh and Krakauer, 2015; Rahmani and Harrington, 2019). This predicted increased extremity of drought has the potential to catastrophically impact Central Plains grassland production if past droughts are any indication of future response (Cook et al., 2015; Lesk et al., 2016). For example, the widespread, extreme drought of 2012 affected 65% of the continental US and cost the US $30 billion in agricultural and rangeland losses (Rippey, 2015) and had large impacts on aboveground productivity (Knapp et al., 2015, Knapp et al., 2020). Further, extreme drought has been shown to deplete about 1% of vegetated land each year and consequently cause significant losses in C every year (Du et al., 2018). Thus, there is a pressing ecological, economic, and societal imperative to understand the impacts of intensifying drought on grassland and rangeland ecosystems, particularly those deemed most vulnerable to these events, such as grasslands of the Central Plains (Cook et al., 2015).

Numerous studies have shown that drought, by reducing soil moisture and water availability, can cause large reductions in aboveground production of grasslands (Hoover et al., 2014; Knapp et al., 2015; Arredondo et al. 2016; Hoover et al., 2016; Kreyling et al., 2017, Knapp et al., 2020) with an accompanying reordering of the dominant species (Fry et al., 2014; Hoover et al., 2014). Drought may also strongly affect the soil microbial community (Lucia et al., 2014; Naylor et al., 2017; Xu et al., 2018; Edwards et al., 2018; Naylor and Coleman-Derr, 2018; Schimel, 2018) with potential feedbacks on belowground processes (e.g. nutrient cycling,

decomposition). This will cause further potential losses in grassland productivity, since plants rely heavily on these belowground processes (Jacoby et al., 2017), for essential nutrients (nitrogen (N), phosphorus (P), etc.). Further, grasslands store approximately one-third of all terrestrial C (White et al., 2000) with the potential to mediate increases in atmospheric CO_2 due to human impacts. Carbon sequestration under drought may be additionally impacted by decreases in C mineralization (Hinjosa et al., 2019), soil microbial respiration (Hoover at al., 2016; Hinjosa et al., 2019), and decreases in stored soil organic matter (Ren et al., 2018). Belowground nutrient cycling and nutrient pool responses to drought will be key indicators of belowground functioning; thus, it will be critically important to understand these responses to mitigate potential losses in belowground grassland function.

The potential for legacy effects post-drought is a key way in which drought may inhibit recovery of biogeochemical cycling. Legacy effects are persistent positive or negative differences from pre-drought or control conditions after a drought ends (Cuddington, 2012). Positive legacies, as defined in our study, create conditions after drought that increase plant or microbial growth/functioning or increase nutrient availability, while negative legacies have the opposite effecty. If an ecosystem is unable to recover before a new drought occurs (e.g. exhibits negative legacies), then the ecosystem could be further damaged, making understanding legacy effects highly important (Schwalm et al., 2017). Thus, both positive and negative legacy effects could have important implications for the nature and pace of recovery of ecosystem functioning following drought.

However, current research is uncertain whether legacy effects occur after drought and, if so, how they impact belowground nutrient cycling post-drought. In particular, studies have found conflicting and mixed responses post-drought. Some studies report no legacy effects after

drought and high resilience to drought (Rousk et al., 2013; Hoover et al., 2014; Hofer et al., 2016; Bunting et al., 2017). Others report positive legacies, particularly in plant growth (Griffin-Nolan et al., 2018; De Long et al., 2019; Guo et al., 2020). Additional research reports recovery of all functions measured within two years, such as resource efficiency (Xu et al., 2017) and aboveground plant production (Griffin-Nolan et al., 2018, De Boeck et al., 2018). Other legacies include increases in soil enzyme activities (Alster et al., 2013) and N pools (Homyak et al., 2017). Negative legacy effects also can occur post-drought, including changes in microbial biomass (Hinojosa et al., 2019), shifts in plant-soil feedbacks leading to shifts in plant-community dynamics (Hassan et al. 2021), decreases in soil enzyme activities and microbial biomass (Legay et al., 2017; Zeglin et al. 2013), and decreases in C mineralization and available soil nutrients such as P, potassium, and soil organic matter (Evans and Wallenstein, 2012; Hawkes et al., 2017; Kreyling et al., 2017). Further, multi-site studies indicate that grasslands can be the least resilient ecosystem to drought (Peng et al., 2019) or the most resilient ecosystem to drought (Li et al., 2020). Thus, research is largely inconsistent with regard to post-drought responses, whether legacies persist, or whether recovery occurs. Moreover, we have shown that although a number of studies find post-drought legacies, few of these studies have examined belowground legacies, particularly nutrient cycling legacies post-drought (Vilonen et al., in prep).

To fill this major knowledge gap of whether belowground legacies exist post-drought, we took advantage of an existing project to evaluate post-drought impacts on soil C and N cycling in a mesic tallgrass prairie grassland. At the study site, a four-year, growing season drought was experimentally imposed in two ways: 1) chronically, i.e., by reducing each rainfall event throughout the growing season by 66%, or 2) intensely, i.e., by completing excluding rainfall

during the growing season until ~45% of mean annual precipitation (MAP) was removed. The drought decreased both aboveground net primary productivity (ANPP) and belowground net primary productivity (BNPP) throughout the four years of drought with stronger decreases in the intense treatment compared to the chronic treatment (Carroll et al., 2021). Changes in the plant species composition in the third and fourth years of drought were also observed (unpublished data). After four-years, the drought shelters were removed from both treatments, and we measured key indices (representing both pools and fluxes) of belowground C and N cycling over two post-drought growing seasons. The main objectives of this study were to determine whether legacy effects occurred and persisted after the drought, whether recovery occurred in some or all measures of C and N cycling, and whether measures of one nutrient cycle recovered more quickly than the other. We hypothesized that legacy effects would occur, but primarily in the first growing season, because carryover of soil water deficits typically are less than a year in duration (Liu et al., 2018), and more significant legacy effects would occur in the N cycle compared to the C cycle, since C pools at the study site are generally large and relatively stable (Wilcox et al., 2016), and the N cycle is generally more responsive to perturbations at this site.

3. Methods

3.1 Study Site and Climate Conditions

This study was conducted during the growing seasons (May – August) of 2018 and 2019 at the Konza Prairie Biological Station, a native, tallgrass prairie research site located in the Flint Hills of northeastern Kansas (39.09º N, 96.48º W). The climate consists of warm, wet summers and dry, cold winters. Mean annual precipitation is ~835 mm with ~75% of rainfall occurring during the growing season (April – September). Annual precipitation for the two years of the study was 811 mm in 2018 and 971 mm in 2019, with ~64% and 75% of the precipitation

occurring during the growing season in each year, respectively (Appendix 2 Figure 1). For this study, we utilized a large-scale, well-replicated drought experiment (the Extreme Drought in Grasslands Experiment, EDGE) that was established in 2013 in an annually burned and ungrazed native tallgrass prairie site. The site was located on a flat, level upland with relatively deep (~1 m or more), well-drained clay loam soils characterized as silty clay Mollisols.

3.2 Experimental Design

The EDGE experiment imposed drought in two ways from 2014-2017 using large rainfall exclusion shelters (n = 20 total), each 6 x 6 m in size and hydrologically isolated to a depth of ~1 m (see Griffin-Nolan et al. 2019 for more details). For the chronic drought treatment, 10 shelters were covered with strips of clear corrugated polycarbonate spaced so as to reduce each growing season rainfall event by 66% (April – September). For the intense drought treatment, the remaining 10 shelters were completely covered with panels of clear corrugated polycarbonate to exclude all rainfall events with no precipitation entering the intense treatment plots until a similar amount of total growing season rainfall was excluded as the chronic treatment (May – July), resulting in a shorter, but more intense reduction in rainfall. Both drought treatments resulted in a ~45% reduction in annual rainfall. Shelter roofs were put in place in May each year for both drought treatments. Roofs were removed each year in early Sept for the chronic treatment, while they were removed after a similar amount of rainfall was reduced in the intense treatment; this was typically reached after ~ 2 months of the panels being installed (typically May – July). The control treatment plots were unsheltered (n = 10), but still hydrologically isolated and received ambient rainfall throughout the growing season. The three treatments were arranged in blocks, each containing a replicate of each treatment, for a total of 10 blocks (n = 30 plots).

To assess post-drought legacy effects on C and N cycling, we removed the shelters after the four years of drought treatments and allowed ambient rainfall to fall onto all of the treatments in 2018 and 2019 (the first two years following drought). This allowed us to measure whether legacy effects were present and whether recovery occurred.

3.3 Soil Sampling

In 2018 and 2019, we collected soils monthly throughout the growing season (late May, early July, and mid-August) to measure soil C and N cycling. We homogenized four random soil core samples (15 cm depth x 5.7 cm diameter) collected from each "destructive plot" as detailed in Griffin-Nolan et al. (2019). The samples were immediately placed on ice and sieved to 2 mm within 24 hours. A subsample of these soils was kept fresh and unfrozen for laboratory-based microbial respiration measurements. The rest of the soil was transferred to a -20°C freezer until further analysis for all other non-in situ measurements. All analyses on frozen soils were performed within a year after collection.

3.4 Soil Moisture

We measured soil moisture in both the field and the lab to assess if soil moisture exhibited any legacies as a mechanism for the reponses observed. We used a hand-held TDR to measure in-situ soil moisture to a depth of 15 cm at each time of soil sampling. We additionally dried field-collected soil (the same soil used to measure nutrients) for 48 hours at 60°C to calculate moisture and soil wet soil/dry conversion factors for subsequent nutrient and enzyme analyses.

3.5 Soil Nutrient Fluxes and Pools

To characterize legacy effects of drought on C and N cycling, we measured in situ belowground respiration, lab-based soil microbial respiration, extractable inorganic N (ammonium and nitrate), extractable total dissolved organic C and N, in situ net N mineralization, and total soil organic C and N concentrations to measure main components of C and N cycling.

Belowground respiration was measured in situ using a Li-Cor 8100 infrared gas sampler (Lincoln, Nebraska). Two PVC collars were installed in each plot to a 6 cm depth and left in the field for the duration of the growing season. All biomass and living plants were removed from the collars at the beginning of the season and prior to every measurement. We then used a Li-Cor 8100 infrared gas sampler to measure CO_2 flux from the soil over a 60 second interval. Measurements were taken midday and during sunny and non-windy conditions to ensure uniform conditions for each measurement. Measurements were taken monthly in 2018 and weekly in 2019. More detailed methods can be found in Slette et al. (2021).

To measure soil microbial respiration in the lab, we placed 30 grams of sieved, fresh soil (the fresh unfrozen subsample; extracted from the field < 24 hours prior) from each plot in a sealable mason-jar (8 cm wide x 15 cm deep). We kept the soils at the same moisture from the field by sealing the soils in plastic bags and sealing the jars immediately after adding the soil. We measured microbial respiration once within 24 hours of extracting soil by opening the jars to allow re-equilibration with ambient CO_2 and then re-sealing the jars for 1-2 hours to measure accumulated headspace CO_2. Respiration was then quantified as detailed in Zeglin and Myrold (2013).

To measure extractable inorganic N, we extracted ammonium and nitrate from the previously frozen soil subset collected monthly. We shook 11 g of thawed field-moist soil with

1M KCl for 1 hour and then filtered the samples using Whatman filters (grade 42 – 2.5 μm filter). We then froze the extracts in a -20°C freezer until analysis. Extractable N was expressed on a per gram soil dry weight basis. To measure net N mineralization, a twelve-centimeter deep PVC tube (3.81 cm diameter) with the top two centimeters above ground was pounded into the ground next to the initial soil cores taken on the same date. The PVC tubes were capped, with holes in the aboveground portion of the tubes for gas exchange, and left in place for ~30 days. Cores were retrieved at the end of the incubation interval, then sieved within 24 hours, frozen in a -20°C freezer, and later extracted with 1 M KCl using the methods above. We used an Alpkem analyzer to measure extractable ammonium and nitrate on all KCl extracts (Saskatoon, SK). Net N mineralization was measured as the difference between extractable inorganic N in the initial and final cores. This was then divided by the days the cores were left in the field to calculate a daily rate.

To measure total dissolved organic C (DOC) and N (DON), we extracted 20 g field-moist subsamples of the previously frozen soil with 100 mL of 0.5M K_2SO_4. We shook the soils for four hours and filtered the samples using Whatman 42 filters, then froze the extracts in a -20°C freezer. We used a Schimadzu TOC-L analyzer (Kyoto, Japan) to measure DOC and DON.

To measure total C and N, we oven dried the soils at 60°C for several days until the soil was deplete of any moisture. The soils were then ground and analyzed for total C and N in a LECO TruSpec CN combustion analyzer (St. Joseph, MI) at the KSU Soil Testing Lab.

3.6 Extracellular Enzyme Activity

We measured the potential extracellular enzyme activities of several microbially-produced enzymes as an index of nutrient limitation. We measured C-cleaving enzymes: α-Glucosidase (AG), β-Glucosidase (BG), β-D-cellulosidase (CB), and β-Xylosidase (XYL); N-

cleaving enzymes: N-acetyl glucosaminidase (NAG) and leucyl aminopeptidase (LAP); and phosphorus-cleaving enzymes: phosphatase (PHOS). Substrates for each enzyme were attached to a highly fluorescent cleavage product. The substrates for AG, BG, CB, XYL, NAG, and PHOS were attached to 4-methylumbelliferyl (MUB), and the substrate for LAP was attached to 7-amino-4-methylcoumarin (MUC). We added a Tris buffer adjusted to a pH of 8 to our soils to create a soil slurry and shook our samples for 40 minutes. We then added our samples to a 96 well-plate and added substrates to our soil slurries with two replicates per sample. Additionally, we created MUB and MUC standard curves for each individual soil. To simulate standard soil conditions, the plates were incubated for 3 hours in the dark at 25°C. Fluorescence was measured using a multiplate reader (Tecan Infinite M200 plate reader, Switzerland) with a 365-nm excitation and 460-nm emission filters. A quench control was used. More detailed methods can be found in Bell et al. (2013) and Trivedi et al. (2016). We summed the C enzymes for total C enzyme activity and the N enzymes for total N enzyme activity (Bell et al., 2013; Dove et al., 2020).

3.7 Statistical Analyses

To compare treatments across each year's growing season, we calculated confidence intervals and standard error using mixed models that accounted for repeated measures over the growing season (monthly sampling). We conducted separate statistical analyses for 2018 and 2019 due to the different climatic conditions of the two years. Further discussion of why the two years were split can be found in results 4.1. Our mixed model contained both fixed and random effects. Time and treatment were both fixed variables with an interaction term to account for the repeated measures aspect of this experiment (lme4 package). As mentioned previously, our experiment had a blocked design. Blocks were treated as a random variable except for some

models where we had to treat block as a fixed variable. In our 2018 enzyme analysis, we ran into a problem of overfitting due to block variance being estimated as zero in the model. To correct this overfitting, we treated block as a fixed effect and used this model to draw conclusions. Additionally, we applied a natural log conversion to all enzyme activity data due to unequal variances detected from the residual vs. fitted plot of the original non-transformed models. For the belowground respiration models, we included soil moisture as a covariate, since soil moisture has strong effects on belowground respiration. Further, we calculated correlation coefficients for soil moisture and belowground respiration in both years. For all statistical analyses, we utilized R statistical software (R Core Team, 2013) and used several packages including lme4, lmerTest, pbkrtest, emmeans, and GGally. We also used R to create the graphics for this paper using ggplot2 and Hmisc to create 95% confidence intervals for each graphic.

4. Results

4.1 Soil moisture differences between the years

The differences in timing and amount of growing season precipitation that occurred each year post-drought led to drier soils in 2018 than 2019 (Figure 2.1). A majority of the rainfall fell after the growing season in 2018, which led to a dry growing season in 2018 (Appendix 2 Figure 1), followed by large rain events in September and October. We collected soil in the growing season, as we expected microbial activity to be low the rest of the year (Carson and Zeglin, 2018). In 2019, about 75% of the total annual rainfall fell during the growing season. However, the annual rainfall was 970.8 mm, which was well over the long-term average of 835 mm. This led to above-average soil moisture, especially since rain fell mostly in May, June, and July during the peak growing season. Therefore, we chose to assess the two post-drought years separately and compare legacy effects between a relatively dry year and a relatively wet year.

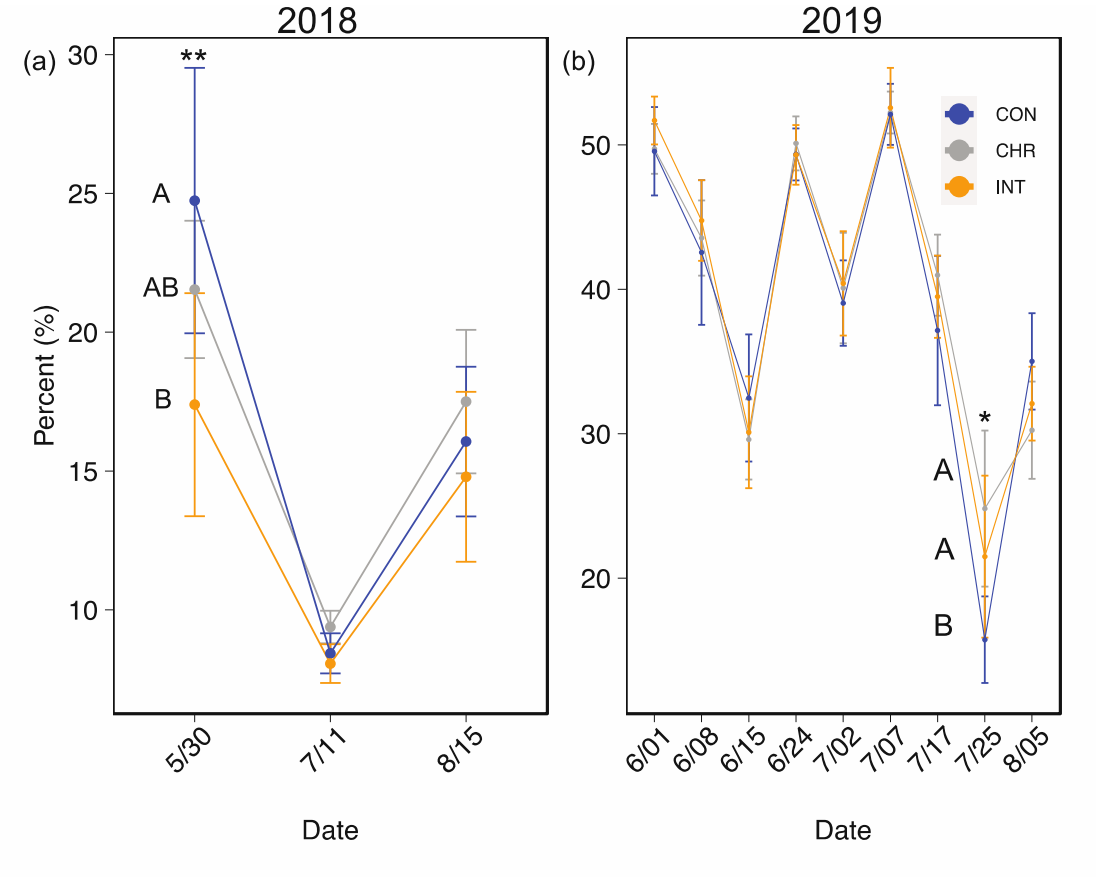

Figure 2.1. Soil moisture in 2018 (a) and 2019 (b). The control treatment (CON) is shown in blue, chronic (CHR) treatment in gray, and intense (INT) treatment in orange. The circles represent the average soil moisture for each treatment. The error bars represent the 95% confidence intervals. The asterisk represents significant differences among the treatments at $\alpha = 0.05$. Double asterisk represents significant differences at $\alpha = 0.01$.

4.2 Carbon Cycling

At the beginning of 2018 (May), in situ belowground respiration (root and microbial respiration) was significantly lower (~50%) in the former intense drought treatment compared to the control treatment (Figure 2.2a; Appendix 2 Table 2). Belowground respiration in the former chronic drought treatment was intermediate to the intense drought and control treatments, but not significantly different than the control treatment (Figure 2.2a). Lower belowground respiration in

the intense drought treatment was accompanied by lower soil water content (measured in situ) compared to the control treatment ($p=0.001$; Figure 1a; Appendix 2 Table 1). This decrease in soil moisture was likely the cause of the lower activity we observed in the intense treatment. We found that soil moisture and belowground respiration were significantly correlated in both 2018 and 2019 ($r = 0.695$, $p < 0.01$; $r = 0.322$, $p < 0.05$, respectively). By July and August 2018, there were no differences in belowground respiration among the treatments. Furthermore, we saw no statistical differences or changes in belowground respiration across treatments in the second growing season post-drought (Figure 2.2b; Appendix 2 Table 2). Belowground respiration in general was significantly higher (more than double) in 2019 than in 2018, mirroring higher soil moisture in 2019. Soil moisture in 2019 (Appendix 2 Table 1) was the same across all treatments except for on July 25th, when soil moisture was lower for the control treatment compared to the intense treatment. This was also at a point when the soil moisture was low for all treatments compared to the other time points (Figure 2.1b).

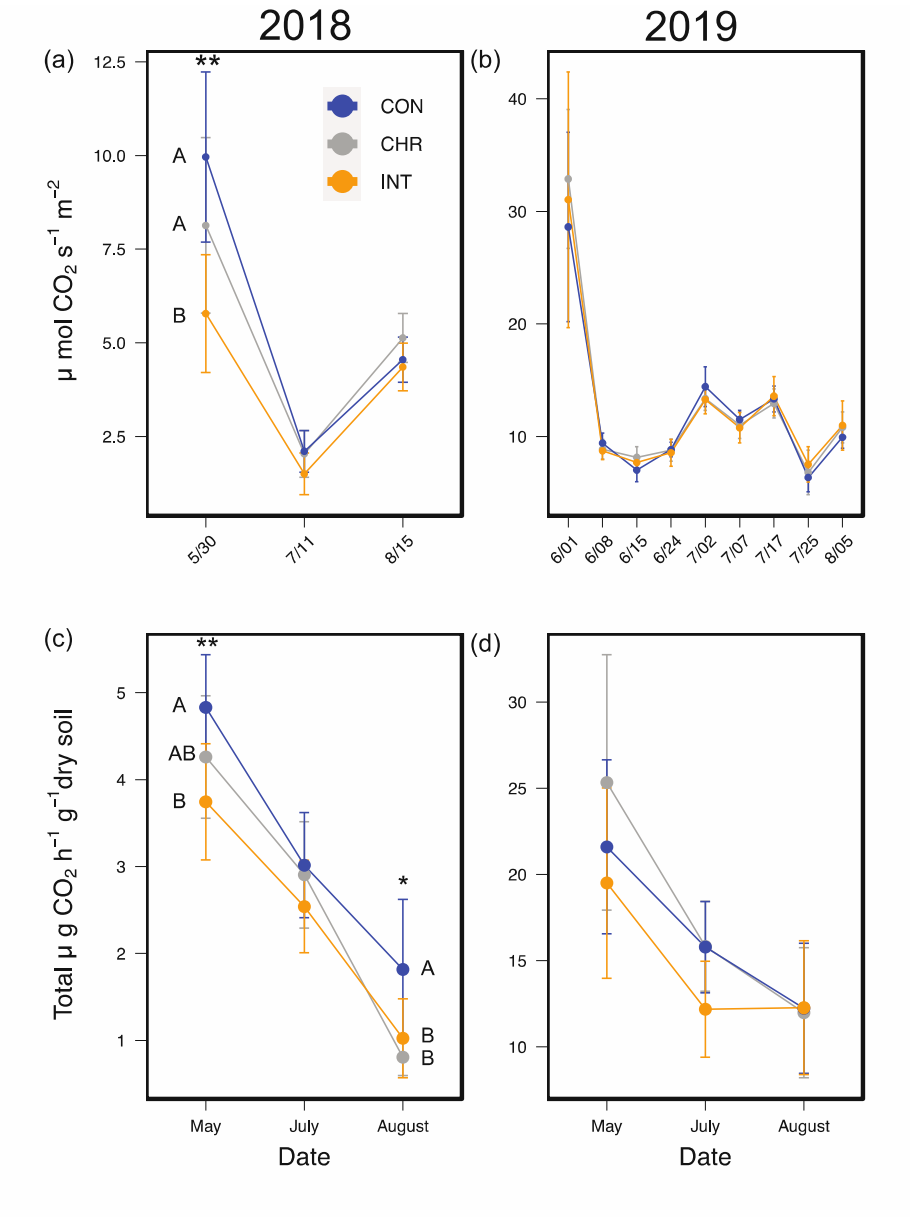

Figure 2.2. Belowground respiration measured *in situ* (a,b) and soil microbial respiration measured in the lab (c,d) in 2018 (left) and 2019 (right). The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average belowground respiration for each treatment. The bars represent the 95% confidence intervals. The asterisk represents significant differences among the treatments at $\alpha = 0.05$. Double asterisk represents significance at $\alpha = 0.01$. Note the difference in scales between 2018 and 2019.

We also measured soil microbial respiration in the laboratory (after removing roots) and found similar trends to the in situ belowground respiration data. Microbial respiration was

significantly lower in May of 2018 in the chronic drought compared to the control treatment ($p=0.0075$; Figure 2c; Appendix 2 Table 2). By July of 2018, we found no differences in microbial respiration among treatments, but in August 2018, we found that both the chronic ($p=0.014$) and intense ($p=0.066$) drought treatment had lower microbial respiration than the control treatment (Figure 2.2c). In 2019, there were no significant differences in microbial respiration among any of the treatments (Figure 2.2d; Appendix 2 Table 2).

There were no significant differences among treatments in total or individual C enzyme activities in 2018 (Appendix 2 Figure 2a; Appendix 2 Table 2) or 2019 (Appendix 2 Figure 2b; Appendix 2 Table 2; Appendix 2 Figure 3). Although, C enzyme activities were highest in the chronic drought treatment throughout 2018, once log-transformed to account for unequal variances the differences were non-significant. Additionally, we divided the ln C enzyme activity by the ln N enzyme activity to see if relative C and N limitations varied based on the ratios of the two activities. We found no significant differences either in 2018 or 2019 (Appendix 2 Figure 4, Appendix 2 Table 2).

We found no differences in DOC in either 2018 or 2019. There was large variability in the data in July of both years that could have obscured any differences among treatments (Appendix 2 Figure 5 a,b; Appendix 2 Table 2). However, total soil organic C was significantly greater in the chronic drought treatment in May ($p = 0.0070$) and July ($p = 0.0120$) of 2018 (Appendix 2 Figure 3; Appendix 2 Table 2). In 2019, total soil C was lower in the intense drought treatment ($p = 0.0333$) and only in July (Figure 3c; Appendix 2 Table 2).

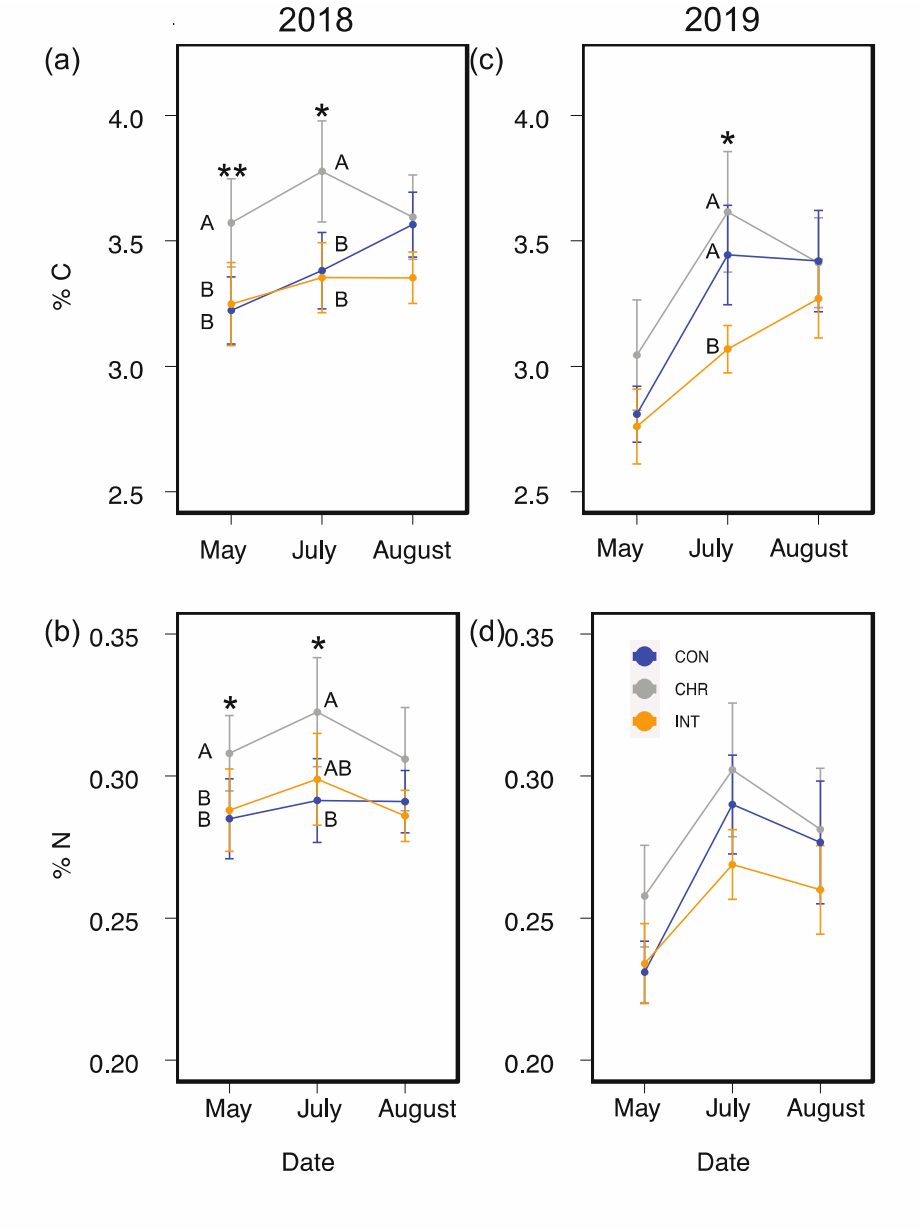

Figure 2.3. Percent soil organic C (a,c) and total soil N (b,d) in 2018 (left) and 2019 (right). The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average values for each treatment. The error bars represent the 95% confidence intervals. The asterisk represents significant differences among the treatments at $\alpha = 0.05$. Double asterisk represents significance at $\alpha = 0.01$.

4.3 Nitrogen Cycling

In 2018, levels of extractable ammonium generally were higher across the entire growing season in both the chronic and intense drought treatments compared to the control treatment, but these differences were not significant (Figure 2.4a; Appendix 2 Table 3). Further, levels of nitrate were higher across the entire growing season in the chronic and intense drought treatments, but only the intense treatment was statistically different than the control in July and August (Figure 4b: Appendix 2 Table 3). We also saw higher levels of total inorganic N in the chronic and intense drought treatments across the growing season of 2018, but only the intense treatment was statistically greater than the control in July and August (Figure 2.4c; Appendix 2 Table 3). These differences in total inorganic N were driven by the increases in nitrate. In 2019, these differences in inorganic N largely disappeared. In May of 2019, the control treatment had statistically higher levels of ammonium compared to the intense treatment and had greater non-statistically significant levels than the chronic treatment (Figure 2.4d; ANOVA table in Appendix 2 Table 3). There were no statistical differences among treatments in nitrate in 2019 (Figure 4e; Appendix 2 Table 3). We did see statistically higher concentrations of total inorganic N in May 2019 in the control treatment compared to the intense treatment. Inorganic N in the chronic drought treatment was lower than the control treatment, but the difference was not statistically significant (Figure 2.4f; Appendix 2 Table 3). Between years, ammonium and total inorganic N was much higher across all treatments in 2019 compared to 2018, but we found no differences among treatments in net N mineralization across 2018 or 2019. Notably, N mineralization values were mostly negative in 2019 (Appendix 2 Figure 6; Appendix 2 Table 3).

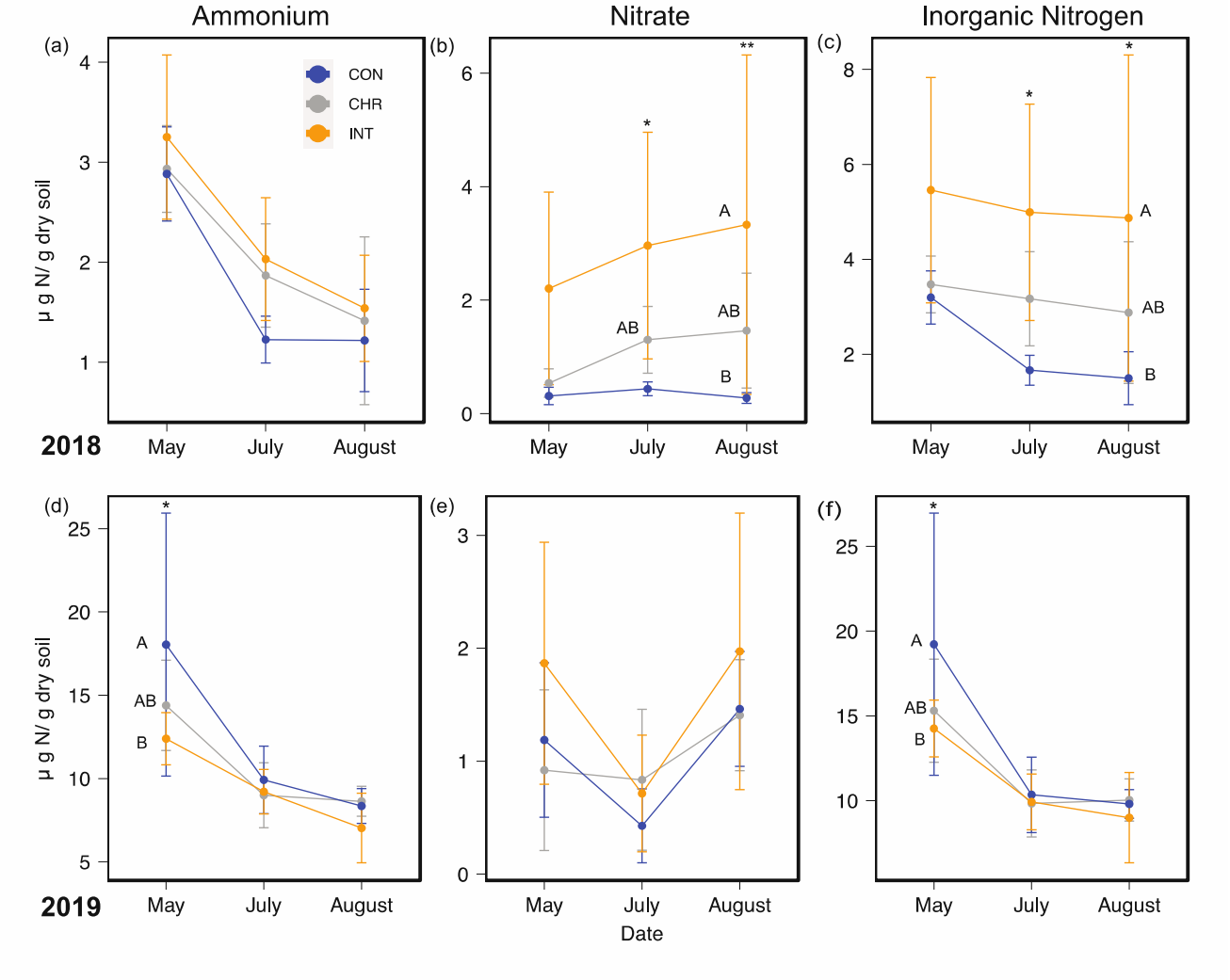

Figure 2.4. Extractable ammonium (a,d), nitrate (b,e), and total inorganic N (ammonium + nitrate; c,f) over the growing season in 2018 (top row) and 2019 (bottom row). The control treatment (CON) is shown in blue, chronic treatment (CHR) in gray, and intense treatment (INT) in orange. The circles represent the average value for each treatment. The error bars represent the 95% confidence intervals. The asterisk represents significant differences among the treatments at $\alpha = 0.05$. Double asterisk represents significance at $\alpha = 0.01$.

Total soil N in the chronic drought treatment was significantly higher in May ($p = 0.0216$) and July ($p = 0.0216$) of 2018 (Figure 3b; Appendix 2 Table 3). In 2019, there were no significant differences among treatments throughout the entire year (Figure 2.3d; Appendix 2 Table 3). We additionally found no significant differences in total and individual N enzyme

activities among treatments in either 2018 or 2019 (Appendix 2 Figure 7; Appendix 2 Table 3). Finally, there were no significant differences among treatments for DON in 2018 or 2019 (Appendix 2 Figure 5c,d; Appendix 2 Table 3).

5. Discussion

We aimed to understand if legacy effects in C and N cycling occurred and if they persisted post-drought in a mesic grassland following two prolonged drought treatments: chronic and intense drought. We predicted that legacies in C and N cycling will be short-lived and most prevelant in the first growing season due to short-term grassland drought memory (Liu et al, 2018). Given differences in rainfall in the two post-drought years, we further expected that any differences in the strength of legacies in the first year compared to the second year would be reinforced by lower levels of water availability inhibiting recovery in 2018. We also expected to see more significant legacy effects in the fluxes and pools of the N cycle compared to the C cycle, since C pools at the study site are generally large and relatively stable (Wilcox et al., 2016). Conversely, the N cycle in this grassland is generally more responsive to perturbations such as fire (Blair, 1997) or grazing (Johnson and Matchett, 2001), and N is often limiting at this site (Seastedt et al., 1991). Therefore, we expected to see available soil N build up over the drought period due to lack of uptake and leaching and decreased general belowground activity. By 2019, we expected these legacy effects to disappear due to short-term water memory (Liu et al. 2018) and short life cycles of belowground organisms. More specific predictions regarding C and N pools and processes are provided in the following paragraphs.

5.1 Legacies in C cycling

We expected that C cycling fluxes would be reduced in the drought treatments relative to control treatment, particularly in parts of the belowground C cycle that are more affected by

reductions in soil moisture, which include soil and microbial respiration, while C pools would remain unchanged. Specifically, we hypothesized based on previous research that soil and microbial respiration in previously droughted treatments would be lower (Hoover et al., 2016), C enzyme activity would be higher (Ochoa-Hueso et al., 2018), and C pools would remain relatively stable (Canarini et al., 2018;Wilcox et al., 2016). Our results mostly matched our hypotheses, but shifts in the C cycle were limited to soil/microbial respiration and only in the first year, with recovery happening much quicker than we expected. Belowground respiration (Figure 2.2a,c) was reduced relative to the control following four years of drought, but only in the first month of 2018 and only in the intense drought treatment, while microbial respiration was reduced in the first month of 2018 and August of 2018 in the intense treatment. These differences disappeared for the rest of the growing season and no differences in soil or microbial respiration were detected in 2019. There was a legacy of decreased soil moisture in May of 2018 in the intense drought treatment only (Figure 2.1a). This decrease in soil moisture was likely the cause of the lower activity we observed in the intense treatment due to the correlation we found in soil moisture and belowground respiration. Several possible reasons exist for this legacy of decreased soil moisture, such as changes in the water holding capacity (WHC) of the soil during the drought, which could be caused losses in organic matter or other changes in the soil that could lead to reduced WHC. Unfortunately, we did not measure WHC and therefore cannot confirm this as a mechanism. Although the decrease in belowground respiration was likely due to the legacy of lower soil moisture, other possible reasons for this decrease exist such as decreased microbial biomass or decreased root production, both of which were not measured in this study. Further, this decrease could have been due to changes in microbial community composition that led to differences in microbial C use efficiency.

We found no differences in dissolved organic C throughout our study (Appendix 2 Figure 5), which was consistent with our original hypothesis of stable C pools. Unexpectedly, we found higher percent total organic C in the former chronic drought treatment in 2018 relative to the control and intense drought treatment (Figure 2.3a). We expected organic C pools to remain unchanged due to the stability of C pools at our site (Wilcox et al., 2016) or decrease due to a decrease in plant contribution to the C cycle from reduced aboveground biomass (Hoover et al., 2014; Knapp et al., 2015; Hoover et al., 2016; Kreyling et al., 2017, Knapp et al., 2020). Other studies have reported decreases in soil organic C during drought in grasslands, including a meta-analysis of 148 publications (Deng et al. 2020), which is contradictory to what we found. On the other hand, drought has been shown to not limit root exudation, which could lead to an accumulation of C allowing for quick microbial recovery post-drought (Karlowsky et al. 2018). The increase in organic C we observed after drought in the chronic treatment could help explain quick recovery in key aspects of the C cycle. However, the increase in organic C disappeared by 2019, and thus appeared to be a short-term response post-drought, which could further indicate that microbial communities used the additional C leading to a lack of lasting legacy effects in the chronic treatment.

We also found no difference in C-releasing enzymes between the control and either of the drought treatments indicating no difference in the levels of relative C limitation among treatments (Appendix 2 Figures 2 and 4). Our results are contrary to other studies of drought in this grassland system. For example, Ochoa-Hueso et al. (2018) found increases in all enzyme activities throughout drought at this site, while another study showed a decrease in all enzyme activities after seasonal drought (Zeglin et al. 2013b). Our data indicate that for this measure of C cycling, the system recovered quickly post-drought and exhibited resilience to drought.

Interestingly, we only saw decreases in soil/microbial respiration and soil moisture in the intense drought treatment and observed no statistically significant differences between the chronic drought and control treatments. We expected to see changes in both drought treatments, since the chronic drought reduced similar amounts of rainfall, although we still expected to see more profound responses from the intense treatment due to complete exclusion of rainfall early in the growing season when plants are actively growing. Carroll et al. (2021) studied the four years of drought at our site and found decreases in aboveground net primary productivity (ANPP) and belowground net primary productivity (BNPP) throughout the four years of drought. Particularly, they found that the intense treatment led to greater decreases compared to the chronic treatment, although the decreases in ANPP and BNPP were still significant for the chronic treatment. This may partially explain why we only saw decreases in activity in the intense treatment, while the chronic treatment exhibited no legacy effects other than an increase in total organic C. The intense treatment also eliminated precipitation from entering the plots during the beginning of the growing season, which was likely much more detrimental than simply reducing each rainfall event and allowing some precipitation to enter the plots. Overall, although we saw some legacies in the C cycle, these legacies were relatively weak and did not persist. The C cycle was not strongly affected by four years of growing season drought and had few legacy effects.

5.2 Measures of N Cycling

For the N cycle, we predicted that we would find greater pools of inorganic N following drought due to decreased plant uptake, but also decreases in post-drought N cycling fluxes due to a decrease in microbial activity. Results were partially consistent with predictions. Although we observed no post-drought differences in the N cycle fluxes, we found higher levels of soil

inorganic N in the former intense drought treatment and higher total soil organic N in the former chronic drought treatment.

The increase in total inorganic N and ammonium in the intense treatment (Figure 2.4) in the first year after drought (2018) was likely due to a decrease in plant uptake or microbial immobilization, which led to an accumulation of inorganic N in the soil. It is unclear whether this difference was due to the accumulation of N during the drought due to reduced plant uptake or whether plants were still unable to recover to pre-drought growth levels and subsequently took up less N post-drought. Preliminary findings of ANPP post-drought indicate that ANPP fully recovers one-year after drought (unpublished data), thus it is likely that the former is true. Other studies also have found increases in inorganic N throughout drought events (Dijkstra et al. 2015; Canarini et al. 2016). In one study, uptake of nitrate by plants was shown to be sensitive to drought and less N was taken up during drought (Dijkstra et al. 2015).

In 2019, these differences disappeared for the most part. Ammonium was highest in the control treatment in 2019, and also significantly higher in 2019 than 2018. Nitrate was relatively lower in 2019 than 2018 (Figure 2.4). The large amount of precipitation in 2019 likely led to a large amount of losses from the system via leaching or denitrification and/or increased plant uptake and microbial immobilization. Ammonium generally is less mobile in clay soils, while nitrate is more readily leached (Cameron et al. 2013).

Importantly, these differences among treatments we observed in inorganic N were not present in measures of organic N (DON and TON) (Appendix 2 Figure 5 and Figure 3). Organic forms of N are much more stable (Kaye et al. 2002) than inorganic pools. Interestingly though, we did see higher percent total soil organic N in the chronic treatment (Figure 2.3). Generally, N tracks total C, which could indicate that the chronic treatment changed the balance of

belowground productivity and decomposition, allowing C and N to accumulate during the drought period. Deng et al. (2020) conducted a meta-analysis across 148 publications and found a positive effect size and increase in organic N throughout drought as well. This increase in organic N was likely due to decreased mineralization of the organic forms throughout drought. However, our assays showed that net N mineralization recovered quickly after drought, which is likely why we did not detect any differences in inorganic N in the chronic treatment. Preliminary data on aboveground productivity at this site has shown that the aboveground community recovers quickly after drought in the chronic treatment, which could further explain why inorganic N in the chronic treatment was comparable to the control treatment.

Further, we found no differences among treatments in any N-releasing enzymes (Appendix 2 Figures 7 and 8). An increase in N-releasing enzymes would indicate that soil microorganisms were experiencing higher N limitation (Schimel and Weintraub 2003). We saw no difference between the control and either drought treatment for the N-releasing enzymes indicating that there were no significant differences in relative N limitation amount in the treatments. This is supported by the increase in available N that we noted earlier. We also observed no changes in mineralization of N, indicating that the fluxes of the N cycle were unchanged by drought. Notably, mineralization was negative in 2019, which was likely due to the water-logged soils and potential denitrification or net N immobilization due to higher soil moisture and more favorable conditions for microbial growth and immobilization. In summary, N cycling was mostly resilient to drought, although there were increases in nitrate and total inorganic N throughout the first-year post-drought.

5.3 Lack of legacies of nutrient cycling post-drought

Our general findings indicate that only a few key measures of soil C and N cycing processes showed legacy effects of extreme, multi-year drought. The majority of C and N cycling measures showed no legacies (no statistical difference from the control plots) and of the few legacy effects observed, almost none persisted into 2019, which was comparably much wetter than 2018. Further, the intense treatment produced more legacies than the chronic treatment. This is likely due to the severity of the drought limiting plant and microbial activity. On the other hand, the chronic treatment seemed to recover immediately after drought or alternatively the chronic treatment did not significantly impact C and N cycling processes. But given the reductions in plant and root growth that occurred in this treatment throughout drought (Carroll et al. 2021) and the shift in plant species composition in the third and fourth years of drought (unpublished data), a lack of effect on belowground processes is probably unlikely. Other studies have found that legacy effects often are not as prevalent as expected. Wu et al. (2017) found that post-drought legacies only lasted one year in grasslands, while finding longer legacies in forest and shrubland systems. Rousk et al. (2013) worked across five shrubland ecosystems and found no legacy effects to drought resulting from short-term warming and drought. A study at Konza Prairie found that grassland production (ANPP) fully recovered one year post-drought, through increases in the dominant grasses compensating for the loss of biomass from less abundant species (Hoover et al. 2014). Another study found that after drought, recovery occurred quickly in a grassland system and normal levels of productivity were achieved (Hofer et al. 2016).

There are two possible hypotheses for why we observed some legacies in 2018 but not in 2019. One is that post-drought legacies were short-lived and the indices of nutrient cycling we examined had all recovered to control levels by 2019. Support for this hypothesis is shown by all

legacies disappearing in 2019. Such rapid recovery has been seen at other studies at this site which found full recovery in plant production in the first growing season after drought (Hoover et al. 2014, Griffin-Nolan et al. 2018). The second hypothesis is that when water is abundant and not limiting growth, drought legacies are obscured or overcome more rapidly. As support for the latter hypothesis, both precipitation and soil moisture in 2019 was above average, which likely made water a non-limiting resource. In contrast, precipitation in 2018 was below-average and there was a shift in seasonality, which led to lower soil moisture levels that could have limited both above- and belowground processes. This suggests that post-drought climate conditions that impact soil moisture availability could play an important role in the nature and pace of recovery and the potential for prolonged legacy effects. Future field studies would benefit from excluding all rainfall and manually adding back precipitation even to the control plots to control for natural variability in rainfall.

In summary, our study suggests that belowground processes in the mesic grassland studied is able to recover quickly after drought, similar to observations of rapid recovery of aboveground productivity (Hoover et al. 2014, Griffin-Nolan et al. 2018). It is plausible that this may be the case more generally for grasslands. Indeed, rapid recovery (or high resilience) after extreme drought appears to be a common feature of grasslands globally with aboveground productivity (Stuart-Haentjens et al. 2018). Our findings were mostly consistent with other studies, although we did not find the positive legacies that have been reported in some other (Griffin-Nolan et al., 2018; De Long et al., 2019; Guo et al., 2020). This may be due to the longer duration of our experimental drought.

However, additional research is needed to assess the role that drought magnitude and duration separately and together play in eliciting legacy effects on nutrient cycling post-drought.

Specifically, we suggest extending the duration of studies looking at legacies post-drought to better understand how long the legacies last and allow for more year to year climate variability. We also suggest studying across a larger set of sites to account for variability in MAP and mean annual temperature (MAT) to better find general patterns of drought legacy effects beyond one specific site. Further, within our study it was challenging to determine the mechanisms for the changes we saw. Thus, we suggest to measure a broader scope of soil characteristics and microbial measurements, such as WHC, soil pH, etc. and microbial biomass and community composition. Lastly, we suggest that studies use greenhouse or field studies to measure across a range of soil moistures or rainfall amounts post-drought to determine if soil moisture levels post-drought are a factor in determining whether legacies occur or do not occur after drought ends.

CHAPTER 3: Legacy effects of intensified drought on the soil microbiome in a mesic grassland

1. Summary

The soil microbiome remains largely unstudied, particularly with regard to response to global change drivers. One such driver, drought, is increasing in intensity and frequency and is expected to intensify with climate change. Further, legacy effects, or impacts after drought has subsided, could have lasting impacts on the soil microbiome with important consequences for ecosystem functioning. Thus, our study aimed to understand how the soil microbiome responds after intensified drought and whether legacy effects persist post-drought. We measured soil microbial community response in a mesic grassland for two years after a four-year experimental drought that imposed two drought treatments that either 1) chronically reduced each growing season rainfall event by 66% or 2) intensely reduced rainfall by completely eliminating growing season rainfall until a ~45% reduction in annual rainfall was achieved. The bacterial community had no legacies in the first season in response to either chronic or intense drought but showed a legacy of increased abundance of Verrucomicrobia and decreased richness in both treatments in the second growing season after the drought treatments ended. In the first and second growing seasons, we found small differences in beta diversity between the control and intense drought treatment for fungal communities but not for the chronic drought treatment. Further, we found that the two main phyla of fungi, Ascomycota and Basidiomycota, showed reduced relative abundance post-drought in the intense drought treatment. Overall, few legacies in soil microbial communities persisted after a four-year experimentally-induced drought. However, our results show that the nature of the drought – chronic vs. intense – can differentially impact fungal vs. bacterial short-term legacies. These results suggest that the soil microbiome is for the most part

drought resistant in this mesic grassland. This finding emphasizes the importance of long-term climate vs. current climate conditions in influencing the soil microbial communities.

2. Introduction

Drought is expected to intensify with forecast climate change. Increases in greenhouse gases and subsequent rises in temperature are likely responsible for this intensification (IPCC 2014), and if nothing is done to mitigate the rise in global temperatures (below the 1.5°C mark), drought will continue to intensify by becoming more frequent, widespread, severe, and long-lasting over time (Cook et al. 2015; Lehner 2017). The Central Plains grasslands of the US stand to be severely impacted by intensified drought, with significant ecological (Knapp et al., 2015; Knapp et al., 2020) and economic impacts (Rippey, 2015). Hence, there is a pressing need to understand the consequences of intensifying drought on these grassland ecosystems (Cook et al., 2015).

Intensified drought may also strongly affect the soil microbiome in grasslands (Naylor et al. 2017; de Vries et al. 2018; Naylor and Coleman-Derr 2018; Schimel 2018; Xu et al. 2018) with potential feedbacks on productivity and other ecosystem processes. The soil microbiome (hereafter referred to as the "microbiome") serves a central role in ecosystem functioning through nutrient cycling (Wagg et al. 2014; Delgado-Baquerizo et al. 2016), decomposition (Glassman et al. 2018), and carbon (C) sequestration (Cotrufo et al. 2013; Kallenbach et al. 2016; Liang et al. 2017). Precipitation and soil moisture are the second most important determinants of microbiome structure and functioning next to pH (Fierer 2017); therefore, alterations in function and structure of the microbiome will likely occur with intensifying drought (Naylor et al. 2017; de Vries et al. 2018; Naylor and Coleman-Derr 2018; Xu et al. 2018). This will cause further potential losses in grassland productivity, since plants rely heavily on the microbiome for essential nutrients (nitrogen (N), phosphorus (P), etc.) through nutrient cycling and decomposition (Jacoby et al. 2017). Further,

C sequestration will be impacted by decreases in carbon mineralization (Hinojosa et al. 2019), soil microbial respiration (Hoover et al. 2016; Ren et al. 2018) (indicating a decrease in microbial activity) and decreases in stored soil organic matter (Ren et al. 2018) due to drought. Given that microbiome responses to intensified drought will be a key indicator to belowground functioning, understanding microbiome responses is key to mitigating potential losses in grassland function and structure.

Generally, we have a limited understanding of how the microbiome community responds to drought, but we have an even more limited understanding of how the microbiome responds post-drought (Vilonen et al. in press). This is important given that the effects of drought are likely to persist long after drought ends and affect ecosystem functioning and responses to future drought events (Schwalm et al. 2017). Legacy effects describe impacts that persist post-drought. Positive legacies in the microbiome would be increased diversity, abundance (biomass), functioning (increased enzyme activity). Negative legacies would be decreased diversity, abundance, functioning, or destabilized communities measured through networks. While there are some lab and greenhouse studies (Evans and Wallenstein 2011; Meisner et al. 2018) (Preece et al. 2019) focused on microbial community legacy effects after drought ends, only a few studies have examined post-drought legacy effects on the microbiome in the field (Meisner et al. 2018; Preece et al. 2019). Thus, this research aims to understand whether legacy effects exist in the soil microbiome in intact native grassland.

Our study took advantage of an existing long-term project (the Extreme Drought in Grasslands Experiment, EDGE) to evaluate these legacy effects on the soil microbiome in a mesic grassland, since the soil microbiome at this site has been shown to be sensitive to drought (Ochoa-Hueso et al., 2018;). EDGE imposed a four-year growing-season drought in two ways: 1) chronically, i.e.,

by reducing each rainfall event throughout the growing season by 66%, or 2) intensely, i.e., by completing excluding rainfall during the growing season until ~45% of MAP was removed. After four years of drought, the shelters were removed from both treatments, and we measured the bacterial (16S) and fungal (ITS) community composition over two post-drought growing seasons. The two post-drought years had different climatic conditions as detailed in the methods and results section, allowing us to determine whether legacies were present in the soil microbiome and if so whether they persisted over time in a relatively dry year vs a relatively wet year after drought subsided. Our main objective was to determine if there were legacies post-drought in the structure of the microbial community in the first two years after extreme drought ended. And if legacy effects were present, our objectives were to determine whether: (1) bacteria or fungi exhibited stronger legacy effects, (2) the type of drought (chronic vs. intense) had an impact on severity of legacies, and (3) legacy effects were stronger in the first year post drought vs. the second year post drought. We expected (1) to find greater legacies in bacteria than fungi, since a study at our same site found stronger impacts on the bacterial community (Ochoa-Hueso et al. 2018); (2) the intense treatment to have greater legacies than the chronic, since other studies at our site have found greater legacies in nutrient cycling in the intense treatment (Vilonen et al. 2022, in press) and greater decreases in aboveground productivity in the intense treatment (Carroll et al. 2021); and (3) we expected to see stronger legacies in the first year post drought, since a study at the same site found stronger legacies on nutrient cycling in the first year vs the second year (Vilonen et al. 2022).

3. Methods

3.1 Study site/ climate conditions

This study was conducted in 2018 and 2019 at the Konza Prairie Biological Station. Konza is a native, tallgrass prairie in the Flint Hills of northeastern Kansas (39.09° N, 96.48° W) with

warm, wet summers and dry, cold winters. The climate is mesic with ~835 mm of mean annual precipitation (MAP) and ~75% of that rainfall occurring in the growing season (April – September). The climate differed between 2018 and 2019: MAP was 811 mm in 2018 and 971 mm in 2019, with ~64% and 75% of rainfall occurring during the growing season in each year, respectively. The site was annually burned, ungrazed, and located on a relatively flat, upland with ~1 m or more of well-drained clay loam soils, characterized as silty clay Mollisols.

3.2 Experimental Design

This study utilized a large-scale, well-replicated drought experiment (EDGE) established in 2013. EDGE imposed drought from 2014-2017 using large rainfall exclusion shelters (n=20 total), each 6 x 6 m in size and hydrologically isolated to a depth of ~ 1 m. For the chronic treatment, 10 shelters were covered with strips of clear corrugated polycarbonate spaced as to reduce each growing season rainfall event by 66% (April – September). For the intense treatment, 10 shelters completely excluded rainfall with clear corrugated polycarbonate panels until a similar amount of rainfall would be reduced as the chronic treatment reduces throughout the entire growing season (May – July). This resulted in an annual, average ~45% reduction in rainfall. Shelters were erected in May and typically taken down in July for the intense treatment and September for the chronic treatment. No shelters were erected for the control treatment (n = 10), but the plots were hydrologically isolated from one another and received ambient rainfall throughout the growing season. At the end of the 2017 growing season, the shelters were removed, allowing ambient precipitation to fall onto all of the plots in subsequent years. The plots each had four subplots with one subplot designated as a “destructive plot” as detailed in Griffin-Nolan et al. (2019), in which all soil samples were collected.

3.3 Soil sampling

In 2018 and 2019, we collected bulk soil mid growing season (July), since we expected soil microbial activity to be the most active at the middle of the growing season (Carson and Zeglin, 2018). We homogenized four random soil core samples (15 cm depth x 5.7 cm diameter) collected from each "destructive plot". Soils were immediately placed in a cooler, were sieved to 2mm within 24 hours, and frozen at -20° C. Ethanol was used to clean the soil-corer and sieve in between sample collection and sieving. DNA was extracted within a month of collection.

3.4 Soil moisture

We measured soil moisture in both the field and the lab. We used a hand-held TDR to measure in-situ soil moisture to a depth of 15 cm at the time of soil sampling. We additionally dried field-collected soil (the same soil used to measure nutrients) for 48 hours at 60°C to calculate moisture and soil wet soil/dry conversion factors.

3.5 DNA extraction

We extracted DNA using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, with the exception of increasing soil weight from 0.25g to 0.5 g and using nuclease free water. The DNA sample was tested for quality and quantity using a NanoDrop Lite Spechtrophotometer (Fischer Scientific, MA, USA). Extractions were repeated if 260/280 absorbance ratio was below 1.6. The DNA ranged from 40-85 ng/μL and all samples were diluted to 10 ng/μL and stored at -80° C.

3.6 qPCR

We quantified the numbers of copies of genes present of the extracted, diluted DNA using quantitative polymerase chain reaction (qPCR). We used 96-well plates using 2 μL of diluted DNA with forward and reverse primers and Bioline 2x SensiFAST SYBR No-ROX Mix. The

forward and reverse primers we used were Eub338- Eub 518 and ITS 1-5.8S, respectively, to amplify bacterial (16S) and fungal (ITS) genes. The plates with DNA, forward and reverse primers, and Bioline mix were then run on a CFX96 Touch Deep Well Real-Time PCR System (Bio-Rad, CA, USA). Resulting melt curves were visualized and any un-amplified samples were re-run. Number of copies of genes were calculated by dividing by initial DNA concentration and weight of soil added for the initial DNA extraction. The data was then ln-transformed for statistical analysis. We used R statistical program to run one-way ANOVAs in the car package and then ran post-hoc Tukey adjusted tests using the emmeans package.

3.7 Amplicon sequencing

Part of the DNA extracted from above was set aside for DNA sequencing to measure the diversity and community structure of bacteria and fungi. This DNA was amplified using a T100 PCR Thermal Cycler (Bio-Rad, CA, USA) using platinum and DNA marker bar codes specific to each sample to tag each sample to isolate samples after pooling and primer sets 515F/806R (Caporaso et al., 2012) and ITS1/ITS2R (Caporaso et al. 2012) to amplify a portion of the bacterial 16S rRNA gene and fungal ITS1 region. Bacteria (16S) and fungi (ITS) were then sequenced at the University of Colorado Anschutz Medical Campus Genomics Shared Resource. 16S (region v4; 515f-806r) and ITS (ITS1f-ITS2) paired-end 250-read sequencing was performed through amplicon sequencing using Illumina MiSeq Sequencing (Illumina Inc., CA, USA) to measure bacterial and fungal-associated sequences. Resulting data was returned as multiplexed FASTQ files for downstream analysis.

3.8 Bioinformatics

The sequencing results were received in the FASTQ format and processed using CUTADAPT to remove adapters from sequences. The USEARCH v.11 pipeline was used for

demultiplexing, denoising (UNOISE; Edgar 2016), quality filtering (UCHIME; Edgar 2011) and 97% operational taxonomic unit (OTU) generation (UPARSE; Edgar 2013). Specifically, we used cutadapt to remove adapters and primers (Martin 2011). Quality filtering was assessed using fastQC (Andrews 2010) and sequences were discarded if they had a low quality score (Q < 20), were short in length (<100 bp), or if they contained ambiguous nucleotides. We then merged paired-end reads. OTUs were clustered using DADA2 and DeNoised using uNoise 3 (Kang et al. 2021) and counted at the sample level. We assigned taxonomy to the OTUs using USEARCH and UCLUST against the SILVA (Quast et al. 2013) database for 16S sequences and UNITE (Nilsson et al. 2018) for ITS. We also removed sequences matching mitochondrial or chloroplast samples, using protocols per Edgar 2016. The OTU table was then exported as a txt file and used for further analysis in R statistical software.

3.9 Network analysis

To visualize combined network analyses with both bacteria and fungi, we created networks using the app CoNet (Faust and Reas 2016) using the program Cytoscape v 3.7.2 (Shannon et al. 2003). We created the visual aspect of the networks using the platform Gephi 0.9.2 (Bastian et al. 2009). OTUs with zero abundance and less than 60% occupancy were removed to limit noise in the visualizations. Interactions with a degree less than 10 were also removed.

3.10 Statistical analyses

Due to the differences in climatic conditions between 2018 and 2019, we analyzed each year separately. All statistical analyses were performed in R using the OTU table created. We used mctoolsr (https://github.com/leffj/mctoolsr/) to upload our meta data and OTU table. We first measured alpha and beta diversity to test for differences in diversity between the treatments. We rarefied data to 3,000 sequences per sample for both alpha and beta diversity using the

single_rarefy function in mctoolrs. We measured alpha diversity by calculating richness (number of OTUs) and Shannon's Diversity using the diversity function from the vegan package. We then created generalized linear models with drought treatment as a fixed factor to measure significance between treatments for each alpha diversity measurement. We ran one-way ANOVAs and post-hoc Tukey tests using the emmeans package to test for significance between treatments. We then ran Permanovas in the vegan package to test for significant differences between the treatments and used pairwise Permanovas to test for significance between treatments using the package pairwiseadonis. We then measured beta diversity using Canonical Analysis of Principal Coordinates (CAPs) using the CAPdiscrim function in the BiodiversityR package using treatment as a fixed factor.

To determine if there were differences from the OTU level to the phylum level in bacteria and fungi, we used fixed-effect negative binomial generalized linear models (GLM) from the MASS package in R. First, we transformed the data to relative abundance by dividing count per sample by the total per sample. We then normalized the data using TMM normalization and used the summarize_taxonomy function in mctoolsr to measure differences across the treatments for different taxonomic levels. We used the emmeans package to estimate abundance and standard error of each OTU. Emmeans calculates ln(counts), thus we back-transformed to counts. We were then able to run one-way ANOVAS across treatments depending on the taxonomic level and performed FDR adjustments. We extracted Tukey adjusted post-hoc comparisons across the treatments for phylum, order, class, and family and looked for differences within these groups using the emmeans package.

4. Results

4.1 Soil moisture and precipitation

The aforementioned differences in precipitation amounts during the two years of the study led to drier soils in July in 2018 than 2019 (Appendix 3 Figure 1). Specifically, almost no rain fell during the growing season prior to July in 2018, leading to relatively dry soil conditions. In contrast, the total annual rainfall in 2019 was well above the long-term average. As a result, soil moisture was 27% higher than in 2018 at the same time.

4.2 Alpha diversity

In 2018, there were no significant differences among treatments for bacteria or fungi in Shannon's diversity or richness; however, there were trends of lower Shannon's diversity and richness in both the chronic (6.4; 1167 species) and intense (6.4; 1168 species) drought treatments respectively compared to the control treatment (6.5; 1235 species) in the bacterial community (Figure 3.1a,b). There were no trends or differences for fungi in 2018. In 2019, both chronic (6.7; $p = 0.03$) and intense (6.6; $p = 0.01$) drought reduced Shannon's diversity for bacteria when compared to ambient conditions (6.9) (Figure 3.1c). Additionally, both the chronic (1706; $p = 0.02$) and intense (1696, $p = 0.01$) treatments had significantly lower richness for bacteria than the control (1801) treatment (Figure 3.1d). There were no significant treatment differences or trends for fungi for Shannon's diversity or richness in 2019 (Appendix 3 Figure 2).

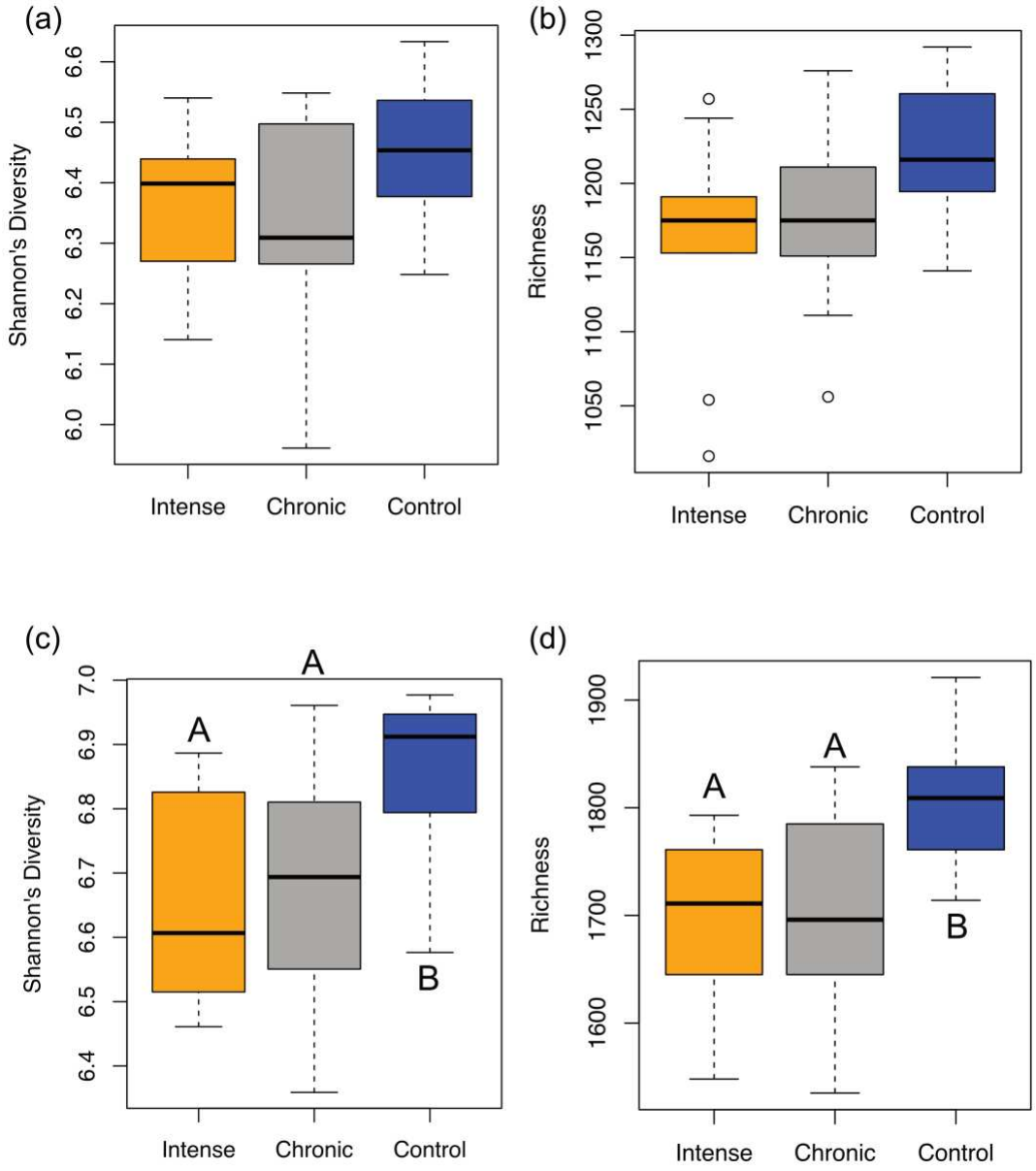

Figure 3.1. Bacterial Shannon's diversity (left) and richness (right) in 2018 (a,c) and 2019 (b,d),. Significant differences ($p < 0.05$) are indicated by different letters. Shown are boxplots with the black line indicating the median and whiskers indicating the 5 and 95 percentiles.

4.3 Beta diversity

In 2018, there were no significant differences in bacterial or fungal beta diversity (Figure 3.2a,b). In 2019, there were no significant differences in bacterial beta diversity (Figure 3.2c), but there was clear separation between the drought and control treatments for fungal communities in 2019 ($p = 0.03$, Figure 3.2d). Pairwise Permanovas showed that the intense treatment was significantly different from the control treatment ($p = 0.04$), but the chronic treatment was not significantly different from the control treatment ($p = 0.4$).

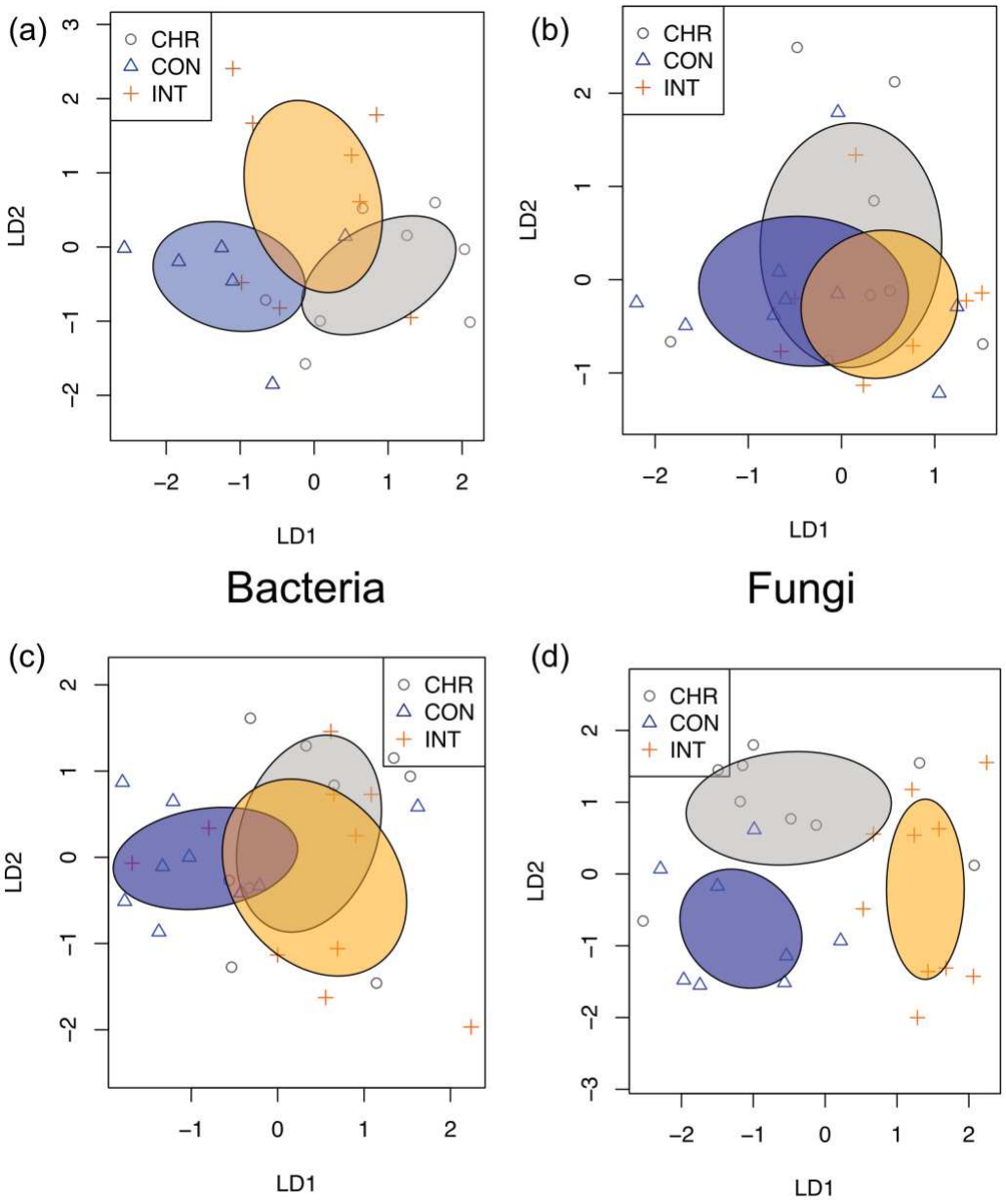

Figure 3.2. Beta diversity (constrained analysis of principle coordinates; CAP) of (left column) bacteria and (right column) fungi in (a,b) 2018 and (c,d) 2019. Significant differences were only found for beta diversity in 2019 for the fungal community.

4.4 Bacterial phylum, class, order, and family differences

In 2018, no differences in relative abundance among the bacterial phlya, class, order, or family were found. However, all treatments had high amounts of Verrucomicrobia (~21%) and Actinobacteria (~20%) in both 2018 and 2019 at the phylum level (Figure 3). In 2019, both the chronic ($p = 0.02$) and intense ($p = 0.02$) treatments had significantly higher relative abundances of Verrucomicrobia (Figure 3.3; Appendix 3 Figure 3a) as compared to the control treatment. Significant differences in relative abundances at from class to family were observed for only the phylum Verrucomicrobia. In 2019 at the class level, the same trend of higher relative abundances in both chronic ($p = 0.01$) and intense ($p = 0.009$) treatments relative to the control for the class Spartobacteria (Appendix 3 Figure 3b) was observed. While, the order Chthoniobacterales had higher relative abundances in both the chronic ($p = 0.01$) and intense ($p = 0.0009$) treatments (Appendix 3 Figure 3c). At the family level, the DA101_soil_group had significantly higher relative abundances in both the chronic ($p = 0.009$) and intense ($p = 0.002$) treatments relative to the control (Appendix 3 Figure 3d).

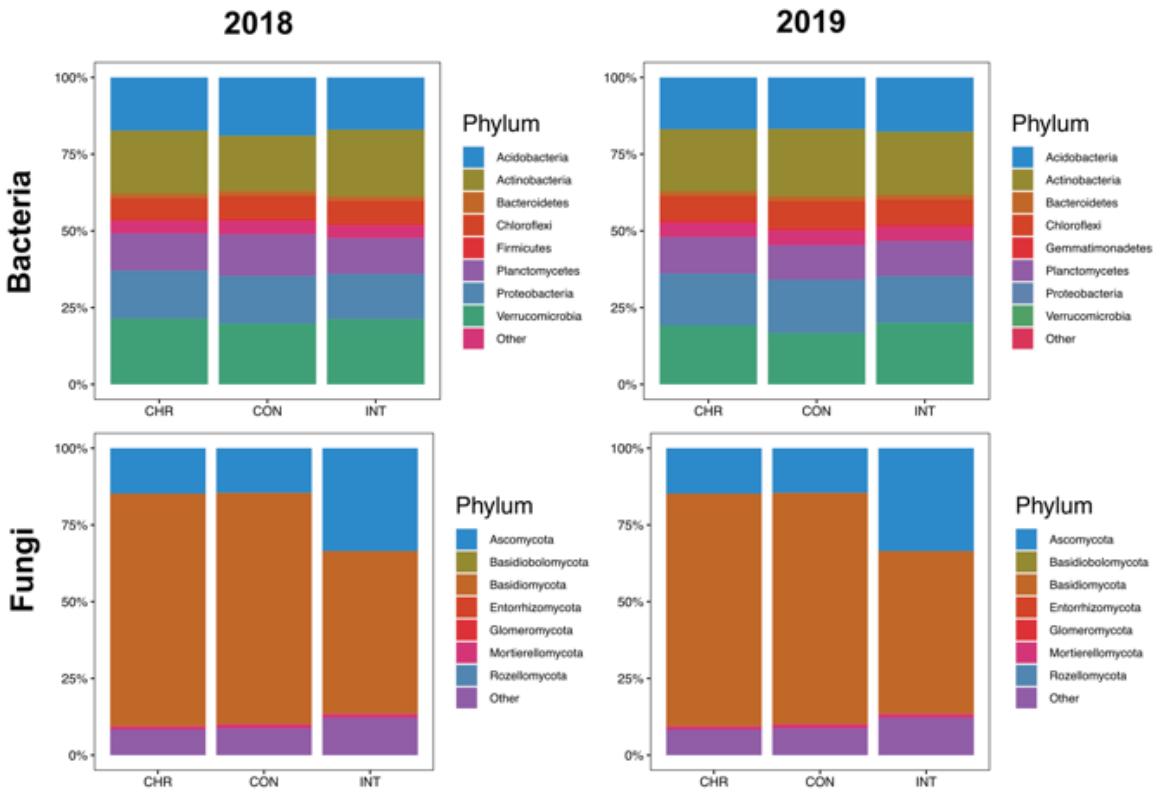

Figure 3.3. The relative abundances of phyla for bacteria (top row) and fungi (bottom row) in 2018 (left column) and 2019 (right column). The top eight most abundant phyla are shown with remaining phyla grouped in the "Other" category.

4.5 Fungal phylum, class, order, and family differences

In contrast to the bacterial phyla, our analysis revealed significant differences in multiple fungal phyla and several lower taxonomic levels with drought in both 2018 and 2019. In 2018, the relative abundances of the phylum Ascomycota were lower for the chronic treatment ($p = 0.03$; Figure 3.3; Appendix 3 Figure 4a) as compared to the control. In addition, abundance of the phylum Basidiomycota was lower in the intense vs. the control treatment ($p = 0.006$; Figure 3.3; Appendix 3 Figure 4b). In 2019, similar trends were observed for Ascomycota ($p = 0.04$; Figure 3.3; Appendix 3 Figure 5a) and Basidiomycota ($p = 0.01$; Figure 3.3; Appendix 3 Figure 5b). At the class level, the chronic treatment had a significantly lower abundance of phylum

Ascomycota class Eurotiomycetes (p = 0.01, Appendix 3 Figure 6a) in 2018. The intense treatment also had a significantly lower abundance of phylum Basidiomycota class Agaricomyetes (p = 0.007, Appendix 3 Figure 6b) in both 2018 and 2019 (p = 0.02; Appendix 3 Figure 9a). At the order level in 2018, abundance of phylum Ascomycota order Chaetothyriales was lower in the chronic treatment (p = 0.01, Appendix 3 Figure 7a), as was abundance of phylum Basidiomycota order unnamed in both the chronic (p = 0.008) and intense treatment (p = <0.001, Appendix 3 Figure 7b). Lastly in the order level, phylum Basidiomycota order Cantharellales (p = 0.04, Appendix 3 Figure 7c) were higher in the chronic drought treatment when compared to the control. For 2019, abundance of phylum Basidiomycota order Agaricales (p =0.01, Appendix 3 Figure 9b) was reduced. In 2018, the intense treatment reduced abundance of phylum Ascomycota family Herpotrichiellaceae (p = 0.04, Appendix 3 Figure 8a) in 2018, while abundance of phylum Ascomycota family unnamed was reduced in the chronic treatment (p = 0.02, Appendix 3 Figure 8b). We further found decreases in abundance in the chronic (p = 0.007) and intense (p = <0.001) of the phylum Basidiomycota family unnamed (Appendix 3 Figure 8c). In addition, abundance of phylum Basidiomycota family Clavariaceae was reduced in the intense treatment in both years (p = <0.001; Appendix 3 Figure 8d; Appendix 3 Figure 10a). Abundance phylum Basidiomycota family Tricholomataceae was also reduced in the chronic treatment (p = 0.04, Appendix 3 Figure 8e). Further, we found a decrease in the abundance of the phylum Basidiomycota family unknown in the intense treatment (p = 0.02, Appendix 3 Figure 8f). Finally, abundance of phylum Basidiomycota family Psathyrellaceae was reduced in the chronic treatment only in 2019 (p = 0.03, Appendix 3 Figure 10b).

4.6 Networks

In 2018, we found small differences between the coexistence networks for each treatment. For topological features, we quantified nodes and edges. In the control treatment there were 775 nodes and 6656 edges. In the chronic treatment there were 539 nodes and 4398 edges. In the intense treatment there were 547 nodes and 4326 edges. The contribution of bacteria in the co-existence networks in the intense treatment increased from 90% to 95% compared to the control but showed similar amounts of positive and negative interactions (Figure 3.4a). The chronic treatment had slightly more positive interactions (copresence) from 33% to 40% and had a slightly larger population of fungi (10% to 12%) than the control treatment (Figure 3.4a).

In 2019, we found more striking differences between network topology, microbial contributions, and interactions in the drought treatments as compared to the control. The control treatment had 439 nodes and 2747 edges; the chronic treatment had 780 nodes and 6242 edges; and the intense treatment had 1833 nodes and 34,475 edges. The contribution of bacteria in the co-existence networks increased from 66% to 84% in the chronic treatment and to 93% in the intense treatment compared to control. We also observed differences in the microbial interactions in the microbial co-existence networks in control as compared to drought treatments. The chronic treatment decreased in the amounts of positive (39% to 27%) and negative (40% to 24%) interactions (Figure 3.3b) compared to the control. The intense treatment had lower amounts of negative interactions (40% to 19%) compared to the control. Further, the intense treatment had several fungal taxa with positive interactions with bacteria (Figure 3.4b).

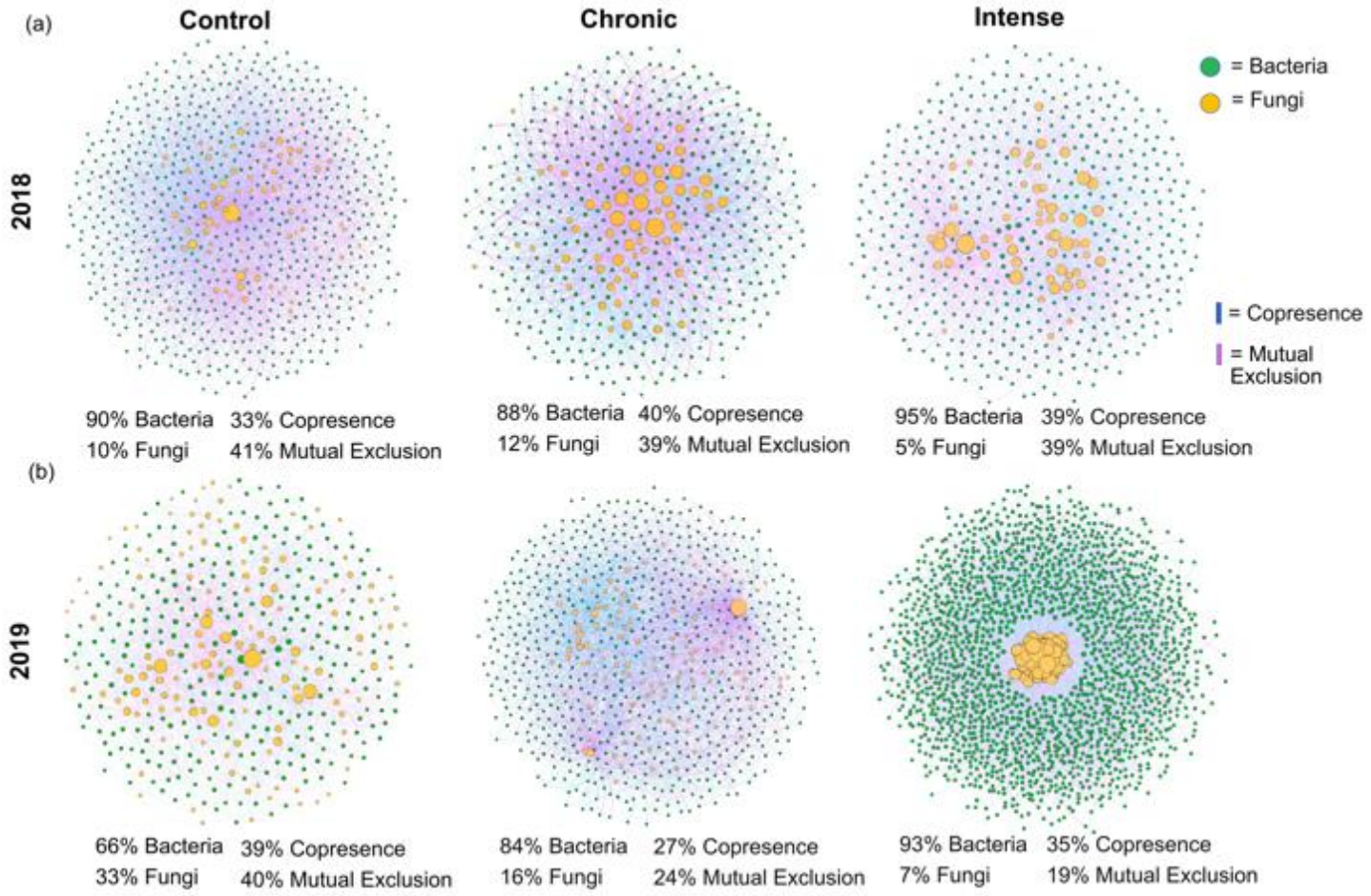

Figure 3.4. Combined bacterial and fungal networks for all three treatments in 2018 (top row) and 2019 (bottom row). Green dots represent bacteria and yellow dots represent fungi. Blue lines indicate co-presence (positive interactions) and pink lines indicate mutual exclusion (negative interactions). Unknown interactions are white and are not shown above.

4.7 qPCR

The bacterial data ranged from $1.0 \times 10^{10} – 3.5 \times 10^{10}$ copy numbers per gram soil and the fungal data ranged from $8 \times 10^{7} – 1.3 \times 10^{8}$. However, no differences ($p = 0.9$) between treatments for bacterial and fungal numbers were observed for 2018 or 2019 for either untransformed or transformed data (Appendix 3 Figure 11).

5. Discussion

Our study aimed to understand if legacy effects persisted in microbial community structure following extreme drought imposed in two ways – either chronically (66% reduction of each rainfall event) or as complete exclusion of rainfall for a period of time (intense). In our paper, we define legacy effects as positive or negative responses that persist post-drought such as changes in microbial community composition or function. A previous study at our site measured microbial response during the drought, but only in the chronic treatment (Ochoa-Hueso et al. 2018). The study was conducted in 2015 during the second year of drought and found significant differences in community structure (both alpha and beta diversity) of both bacteria and fungi with chronic drought. In particular, bacterial phyla Actinobacteria and Bacteroidetes increased in abundance, while Gemmatimondetes decreased in response to chronic drought. For fungi, no differences in any of the fungal phyla were found between the drought and control treatments (Ochoa-Hueso et al. 2018). Similar to the study conducted by Ochoa-Hueso et al. 2018 other studies have also reported stronger sensitivity of bacteria to drought compared to fungi (Naylor et al. 2017; de Vries et al. 2018). Thus, our study expected to see lingering impacts from the experimentally induced drought and thus see some of the impacts found in Ochoa-Hueso et al. 2018 of changes in beta diversity of both bacteria and fungi and shifts in bacterial phlya with

more pronounced legacies in the bacterial community compared to the fungal community. Further, we expected stronger legacies in the intense treatment based on the results from Carroll et al. 2021 and Vilonen et al. 2022 and that if legacies would persist mainly in the growing season following functional trends seen in Vilonen et al. 2022.

5.1 Bacterial legacies

Overall, there was little evidence for legacy effects in bacterial community structure. Consistent with Ochoa-Hueso et al. 2018, we did not see changes in alpha diversity (Figure 3.1a,c) or in bacterial gene copy number (qPCR) in the chronic or intense treatment, although we did observe decreased bacterial Shannon diversity and richness in the chronic treatment in 2019 (Figure 3.1b,d). This decrease may be due to the increase in relative abundance of Verrucomicrobia (see below).

Contrary to Ochoa-Hueso et al. 2018, we found that there were no significant changes in beta diversity between the drought vs. control treatments in either 2018 or 2019 (Figure 3.2 a,c), nor did we find the same bacterial phyla shifts. In 2018, we found no significant changes in any bacterial phyla. We expected to continue to observe an increase in Actinobacteria post-drought, as observed in 2015 during drought (Ochoa-Hueso et al. 2018), particularly since Actinobacteria generally increases in abundance during drought (Naylor et al. 2017, Naylor and Coleman-Derr 2018). This lack of response indicates that Actinobacteria and other bacterial phyla that shifted during drought recovered to control level abundances. Two possible explanations for this lack of response are that in 2015, the differences in Actinobacteria were brief and during the one time point measured or that 2015 was an above average precipitation year. Thus, either Actinobacteria quickly returned to control levels in 2018 or those shifts disappeared in the three years in between.

Our study showed the increased relative abundances of phylum Verrucomicrobia in both the chronic and intense treatments in 2019 (Figure 3.3). Typically, Verrucomicrobia has been found to decrease during drought (Sheik et al. 2011, Naylor and Coleman-Derr 2018), since Verrucomicrobia is gram-negative, and thus with only a single layer cell membrane is more susceptible to drought (Chodak et al. 2015). A possible explanation for the increase in Verrucomicrobia in 2019 in the drought treatments can be attributed to high water availability and subsequent high nutrient availability (increased carbon and nitrogen; Vilonen et al. 2022). Additionally, Verrucomicrobia has a high relative abundance in grasslands, compromising about 41% of reads in a typical grassland system (Bergmann et al. 2011). Verrucomicrobia is also typically oligotrophic (Bermann et al. 2011), although different classes of Verrucomicrobia have been shown to be copiotrophic and oligotrophic (Ho et al. 2017). Thus, this increase could have been due to high water availability allowing Verrucomicrobia to flourish.

The network analysis, in line with phyla abundance data, showed differences in nodes, edges and number of bacteria in the cooccurrence networks in 2019 only. Predominantly, there was a large increase of bacteria in our co-occurrence networks particularly in the intense treatment. The chronic and intense treatments not only had higher amounts of bacteria, but also significantly higher amounts of nodes and edges, with the intense treatment having higher amounts of all of these than the chronic treatment. High nutrient availability (Vilonen et al 2022) likely led to high recruitment of bacterial species with interactions not yet developing at the time point we measured (Moreno-Mateos et al. 2020), which could explain the higher number of unknown interactions in both the chronic and intense treatments (Figure 3.4).

Although there were small increases in species richness and increases in Verrucomicrobia in 2019, legacies on the bacterial community were either non-existent or small. One possible

explanation for the lack of legacies for bacterial communities is that our study site generally has a high abundance of Actinobacteria as compared to agricultural studies, which dominate drought microbiome studies. For example, Naylor et al. 2017 had <10% of reads compromising of Actinobacteria in their control treatments using crop plants. Actinobacteria has been shown to thrive under drought due to its spore forming ability (Naylor et al. 2017) and comprised about 20% of the reads in our study 2018 and 2019. Further, grasslands are typically drought resistant and have stable bacterial communities when compared to agricultural systems that are more sensitive to drought and have less stable C stocks (Delgado-Baquerizo et al. 2017a). Most of the studies on microbial response to drought have been conducted in agricultural systems (Naylor et al. 2017, Naylor and Coleman-Derr 2018). Another possible explanation for lack of legacies is that Delgado-Baquerizo et al. 2017b found that long-term climate had a much greater effect on microbial community composition vs. recent climate events. Any differences found during the drought disappeared quickly after the drought ended suggesting that these communities were able to recover quickly from drought likely due to stable C stocks (Delgado-Baquerizo et al. 2017a) or that longer-term climate conditions having stronger impacts on community composition (Delgado-Baquerizo et al. 2017b). To corroborate our results, lab studies conducting drying-rewetting cycles have found quick recovery after re-wetting post-drought or small legacies (de Nijs et al. 2019), including a drying-rewetting experiment done at our same site (Evans and Wallenstein 2011).

5.2 Fungal legacies

We also found several legacies of drought on fungal diversity. Ochoa-Hueso et al. 2018 found that fungal beta diversity was significantly different between the control and drought treatments, but no differences in alpha diversity or differences between fungal phyla were found.

Just as in the previous study during the drought, we found that the intense treatment had a significantly different beta diversity than the control in 2019 (Figure 3.2d), indicating that the communities were different for the two treatments. However, they did not find a difference in phyla, specifically Ascomycota and Basidiomycota. On the contrary, our results indicated that Ascomycota was reduced in the chronic treatment compared to the control treatment and that Basidiomycota was reduced in the intense treatment in both 2018 (Figure 3.3) and 2019 (Figure 3.3). Ascomycota is typically considered drought tolerant and oligotrophic meaning that Ascomycota is able to survive in lower nutrient environments (Li et al. 2020). Thus, the increase in water supply and nutrient availability post-drought could explain why Ascomycota was potentially outcompeted in the chronic treatment by copiotrophic phyla that have a competitive advantage in higher nutrient environments. The decrease we saw in Basidiomycota in the intense treatment could be a legacy from the drought. Since Ochoa-Hueso et al. 2018 only measured responses in the chronic treatment, we are unsure if the intense and chronic treatment saw the same responses during drought, particularly since plant, root (Carroll et al. 2021), and nutrient cycling (Vilonen et al. 2021) were more greatly diminished by drought in the intense treatment. Thus, it is possible that the intense treatment saw shifts in the fungal community not seen in the chronic treatment. Basidiomycota has been shown to be sensitive to drought and depleted during drought (Bastida et al. 2017; Sun et al. 2020; Buscardo et al. 2021). Thus, the most likely explanation for this decrease in Basidiomycota in the intense treatment post-drought is a negative legacy from the drought. Since previous studies did not measure the effects of drought on the intense treatment, further research is needed to determine whether intense drought more strongly affects the fungal community.

Generally, other studies have found that bacteria are highly sensitive to drought, while fungi are much more tolerant to drought (Naylor et al. 2017; de Vries et al. 2018). Our paper has found the exact opposite trend of greater sensitivity of fungi after drought. It is possible that fungi are resistant to drought, but not resilient after drought in a field setting. A possible explanation for the sensitivity of fungi after drought is that many fungi are saprophytic, and thus primarily decompose dead plant and animal material. The increased abundance of nutrients of both nitrogen and carbon could explain why fungi were negatively impacted after drought. Our network analysis further shows a significant decrease of fungi in the co-occurrence networks, indicating that bacteria were highly dominant and significant in the networks and fungi were less important likely due to the high availability of nutrients.

5.3 Lack of legacies in microbial communities

For both bacteria and fungi, we found fewer legacy effects than we expected. Further, we expected to see greater legacies for bacteria than fungi, since bacteria have been shown to be more sensitive to drought than fungi (Yuste et al. 2011; de Vries et al. 2018; Ochoa-Hueso et al. 2018). However, it seemed that the bacterial community recovered rapidly from drought with no significant differences in 2018 and small legacies in 2019. Although bacterial communities may be more sensitive to drought during the event, it seems that after the drought ends, these communities are able to recover rapidly. On the other hand, the fungal communities showed several potential negative legacies as observed by reduced abundance of key phyla. Thus, it seemed that although fungi were likely more resistant to drought, legacies persisted after drought ended. Still, the legacies found in the fungal community were relatively small compared to the legacies found in Ochoa- Hueso et al. 2018 and thus both communities exhibited few legacies to drought. Trait based microbiome models have found that legacy effects are likely to be non-

existent or short-lived in microbial communities (Wang and Allison 2021), thus our results are in line with current modeling.

Overall, we found that there were some legacies in both the bacterial and fungal communities, although in the bacterial community there were positive legacies, while the fungal community exhibited more negative legacies. It was difficult to parse out whether the intense treatment or chronic treatment had more significant legacies, since both had similar legacies. Lastly, for bacteria there were no legacies in 2018 and small legacies in 2019, while for fungal legacies were the similar both years, indicating that differences in water availability did not have a significant impact on the legacies observed. Our results have important implications for climate models that hope to include microbial data in large scale drought models. Previous research has raised the concern that systems may not fully recover from drought prior to a subsequent drought (Schwalm et al. 2017). This possibility could have unknown consequences for ecosystem functioning and structure. Our study encouragingly found that microbial communities seem to be highly resilient to drought, which matches several results of high resiliency to drought in grasslands (Hoover et al. 2014; Vilonen et al. 2022, in press). This high resiliency in grasslands could indicate that the effect of each drought does not carryover into future drought events, although more research is needed with more severe, longer droughts and the impacts of multiple subsequent droughts on belowground structure.

CHAPTER 4: Microbial resistance and resilience to short-term drought varies among grasslands spanning a broad precipitation gradient

1. Summary

Drought is increasing in severity and duration across the United States with disproportionately drier conditions expected in arid and semi-arid regions. Aboveground productivity stands to be heavily impacted by intensified drought, while belowground responses, such as nitrogen availability and soil microbial activity and composition, tend to be much more variable. Our study aimed to determine if belowground responses to drought were similar or different across a broad climatic gradient. To accomplish this goal, we collected intact soil mesocosms, each 9 cm diameter by 5 cm in depth, from four grassland sites spanning a broad climatic gradient in the Central US. The soil mesocosms were brought back to the greenhouse and half were subjected to a 30-day drought while the other half experienced control (well-watered) conditions. We assessed drought resilience (i.e., recovery to control conditions after drought) at one and two weeks after the experimental drought ended. Resilience of several key belowground responses were measured, including inorganic nitrogen, potential extracellular enzyme activity, and microbial community composition via sequencing. We found that the wettest and warmest grassland site (tallgrass prairie) was the most sensitive to the short-term drought with decreases in alpha diversity for fungi, differences in beta diversity for fungi, and decreases in all soil enzymes measured. Further, post-drought responses included increased enzyme activity across most enzymes, but these differences were quickly resolved two weeks after the short-term drought ended. Overall, we found that response to drought was highly site specific, which has important implications for predicting how belowground processes will be altered as drought intensifies in the future with changing climate.

2. Introduction

Climate change is predicted to intensify the hydrological cycle, with an increase in the frequency, magnitude, and duration of extreme drought as one outcome of this intensification (IPCC 2014). Drought can significantly impact ecosystem processes, such as plant productivity (Knapp et al. 2020), nutrient cycling (Hinjosa et al. 2018), and microbial processes (Schimel et al. 2018). Yet, we know much more about the impacts of drought on aboveground processes than belowground processes, and in cases where the two have been studied simultaneously there often is asymmetry in the responses of the two (Jaman et al. 2022). Of the belowground responses that can be studied, soil microbial structure and function responses to intensified drought are vital to understand since the soil microbiome plays a central role in ecosystem functioning through nutrient cycling (Wagg et al. 2014; Deldago-Baquerizo et al. 2016), plant and animal material decomposition (Glassman et al. 2018), and carbon (C) sequestration (Cotrufo et al. 2013; Kallenbach et al. 2016; Liang et al. 2017). Furthermore, the recovery of the soil microbiome from drought is a key part of the complex processes determining the fate of ecosystem functioning. However, our understanding of how soil microbial structure and function will respond to and recover from drought remains limited.

A critical part of understanding responses to and recovery from drought is framing belowground processes within a resistance-resilience framework. Resistance is defined here as the capacity to withstand a perturbation (high resistance = little change during an event), while resilience is the capacity to recover after a perturbation and recovery to pre-drought conditions (Pimm 1984; Shade et al. 2012). In this context, soil microbiome structure and function would be considered resistant if they change little with drought, and they would be considered resilient to drought if they rapidly return to pre-drought (or control) conditions. A number of studies have

measured resistance (e.g., Knapp et al. 2015; Kreyling et al. 2017) and resilience (e.g., Hoover et al. 2014; Hoover et al. 2016) of aboveground processes, such as plant productivity, but few studies have measured resistance and resilience of belowground processes to drought (Vilonen et al. 2022), particularly in non-agricultural systems. Some studies have measured microbial resistance to drought in agriculture systems (Naylor et al. 2017; Naylor and Coleman-Derr 2018; Xu et al. 2018) and natural systems (de Vries et al. 2018; Ochoa-Hueso et al. 2018; Schimel 2018), which suggests that the microbiome is often not resistant to drought. For example, decreases in diversity (Naylor et al. 2017; Naylor and Coleman-Derr 2018; Xu et al. 2018), enzyme activity (Ochoa-Hueso et al. 2018) and destabilization of bacterial networks (de Vries et al. 2018) have been documented. However, we know little about the resilience of the soil microbiome post-drought, although there is evidence for both recovery (Vilonen et al. 2022b) or lack thereof (Kaisermann et al. 2017; Meisner et al. 2018) in microbiome structure and function. Understanding whether or not soil microbiome structure and function is resistant or resilient to drought is crucial for predicting how belowground processes will respond in the future. Indeed, if the soil microbiome shows low resilience this could lead to cumulative impacts on belowground processes as droughts become more frequent with climate change (Schwalm et al. 2017).

Several studies have attempted to link measures of resistance and resilience to environmental factors such as mean annual precipitation (MAP) and mean annual temperature (MAT) to generalize responses across a spatial gradient (e.g., Knapp et al. 2015, Griffin-Nolan 2018, Stuart-Haentjens et al. 2018). Knapp et al. (2015) found that drier grassland sites were more sensitive to losses in aboveground productivity than wetter sites, while Griffin-Nolan et al. (2018) found for the same grassland sites that dry grasslands showed more positive legacies of greater aboveground growth after the drought ended than the wetter sites. Several other studies

have shown that responses to drought were more challenging to predict across a climatic gradient, although these studies found some similarities in responses across sites (Ochoa-Hueso et al. 2018; Carroll et al. 2021). Linking measures of resistance or resilience to environmental factors has important implications for future modeling, since this linkage would allow researchers to predict soil microbial structure and functional responses to drought in a changing climate and include these in ecosystem models predicting the impact of climate change. Indeed, Bardgett and Caruso (2020) suggested in their review of soil microbial response to climate extremes that the way forward in this field is to measure response to climate extremes across temporal and environmental gradients, since environmental factors and soil history play an important role in microbial response to drought (Griffiths and Philippot 2013).

Our study aimed to measure soil microbiome resistance and resilience to drought across a climatic gradient. The goal of our study was to determine whether responses were uniform or not across four grassland sites spanning a broad climatic gradient within the Central US to an experimentally imposed drought. For each of these sites, we measured the resistance and resilience of soil microbiome structure and function to an experimentally imposed short-term (30 day) drought. The goal of our experiment was to determine if resistance and resilience of soil microbiome structure and function to drought was similar or varied in grasslands spanning a climatic gradient. To isolate microbial impacts, we used a greenhouse experiment with intact mesocosms to eliminate plant effects. We measured several microbial community and functional measurements such as alpha diversity, beta diversity, phylum shifts, extracellular enzyme activity, qPCR, and inorganic nitrogen. We expected to find that generally across the microbial structural and functional measures that the drier sites would be the least sensitive, since dryland sites generally experience more frequent water limitation and because climate soil history

appears to be a large determinant of microbial responses to disturbance (Griffiths and Phillippot 2013). We predicted that alpha diversity, phylum shifts, and enzyme activities would be more resistant to drought for the drier sites than the more mesic sites. We further expected to see resilience at all the sites to drought with quick recovery of soil microbiome structure and function, since other studies have seen quick recovery of other measures of belowground processes in these grasslands (Vilonen et al. 2022b; Vilonen et al. 2022c).

3. Methods

3.1 Study Sites

Our study took advantage of the control plots at an existing well-replicated long-term drought project called Extreme Drought in the Grasslands Experiment (EDGE) and collected samples from four grassland sites: 1) shortgrass steppe in northern Colorado (SGS), 2) northern mixed grass prairie in Cheyenne, Wyoming (CHY), 3) southern mixed-grass prairie near Hays, Kansas (HYS), and 4) tallgrass prairie site at the Konza Prairie Long-term Ecological Research site located in northeastern Kansas (KNZ; Table 4.1). Our sites ranged from 375 mm to 892 mm in mean annual precipitation (MAP) and X and X in mean annual temperature (MAT). Soils ranged from sandy to clay-loam (Knapp et al. 2015). Further site characteristics can be found in Table 4.1.

Table 4.1. Site characteristics (adapted from Knapp et al. 2015 and Ochoa-Hueso et al. 2018).

Site	**Shorthand name**	**Grassland type**	**MAP (mm)**	**MAT (°C)**
Central Plains Experimental Range	SGS	Shortgrass steppe	375	9.5
High Plains Grasslands Research Center	CHY	Mixed grass prairie	400	7.9
Hays Agricultural Research Center	HYS	Mixed grass prairie	584	12.3
Konza Biological Research Station	KNZ	Tallgrass prairie	892	13

3.2 Intact soil mesocosm collection

To isolate the impacts of drought on the microbial community, we collected intact soil mesocosms from the control plots of the EDGE experiment at mid-growing season (July) to attain peak microbial activity (Carson and Zeglin 2018). Two soil mesocosms lacking plant material were collected from each control plot (n = 20 per site, n = 80 total). We used PVC piping (9 cm diameter) and cut each core to 7 cm. We collected intact cores from each plot by pounding the PVC tube 5 cm into the ground to collect the top 5 cm of soil, and then we removed the PVC tube using a shovel to prevent loss of soil from the bottom of each core. The PVC tube was then immediately stored in a plastic bag in a cooler. The tubes were then refrigerated at 4°C until they were placed in the greenhouse (~ 1 week). We attached fine screen mesh (50 micron nylon woven filter mesh, polyester food grade) to the bottom each PVC tube using nitrogen-free glue (silicone sealant). This mesh was added to prevent soil loss but allow water to leave the soil mesocosms.

3.3 Resistance-Resilience Greenhouse Experiment

The greenhouse experiment to assess resistance and resilience to drought consisted of two treatments: control and drought. The two cores collected from each control plot at each grassland site were designated as either the control or drought treatment (n =10 per treatment, n = 20 per site, n = 80 total). For the control treatment, we used ECH20 EC-5 soil moisture probes (Meter Group; Pullman, WA) to continuously monitor soil moisture throughout the experiment. We aimed to keep the average soil moisture that is typical of well-watered conditions at each site. We watered SGS to ~25% soil moisture, CHY to ~30%, HYS to ~35%, and KNZ to 45%. To maintain this moisture, we slowly added water to the cores until the ideal moisture was reached (+/- 5%) approximately every 2 days depending on the moisture measurements. For the drought treatment, we withheld water for 30 days. This full removal of water mimicked the intense treatment from the original EDGE experiment, in which all rainfall was excluded from each drought plot until a 66% reduction in growing season rainfall was achieved (see Carroll et al. 2021 for further details). We chose this drought treatment because previous experiments have shown more significant impacts to the intense treatment vs. the chronic treatment (where each rainfall event was reduced by 66%) in the field (Carroll et al. 2021; Vilonen et al. 2022). We also monitored soil moisture in the drought treatments over time, although soil moisture quickly approached 0% and remained that way throughout the drought treatment.

After 30 days of the drought and control treatments, we collected soil samples from all of the cores. We used a metal spoon to collect the soil, using ethanol to clean the spoon in between soil samples. We were careful to take the full 5 cm depth of the cores, since our experiment continued to assess recovery. The collected soil was stored in a cooler and returned immediately to the lab and stored in the fridge until they were sieved (within 48 hours of collection). Soils

were passed through a 2 mm sieve, and ethanol was used to clean sieves between samples. Half of the sieved soil was used for extracellular enzymes and inorganic nitrogen analyses, and thus were stored and frozen at -20°C immediately post-sieving. The remainder of the soil sample was stored at -80°C for DNA extraction immediately post-sieving.

To measure resilience to drought, we watered all cores every 2-3 days maintaining each site's average soil moisture after the 30-day drought period ended. After one and two weeks of watering, we collected samples from all the cores and processed the soils in the same manner as described above.

3.4 Enzyme activity and inorganic nitrogen

We measured several microbial functional measures to assess resistance and resilience to drought. To assess nutrient limitation due to drought, we measured the potential extracellular enzyme activities of several microbially-produced enzymes. We quantified C-cleaving enzymes: α-Glucosidase (AG), β-Glucosidase (BG), β-D-cellulosidase (CB), and β-Xylosidase (XYL); N-cleaving enzymes: N-acetyl glucosaminidase (NAG) and leucyl aminopeptidase (LAP); and phosphorus-cleaving enzymes: phosphatase (PHOS). Detailed methods can be found in Vilonen et al. 2022 and Trivedi et al. 2016. We summed the C enzymes for total C enzyme activity and the N enzymes for total N enzyme activity (Bell et al., 2013; Dove et al., 2020).

To assess potential nitrogen limitation, we extracted ammonium and nitrate from the -20°C frozen soil subset. We shook 11g of thawed greenhouse moist soil with 1M KCl for 1 hour. The samples were then filtered through Whatman filters (grade 42 – 2.5 μm filter). The extracts were then frozen at -20°C until further analysis. We used spectrophotometry to measure extractable

ammonium and nitrate on all KCL extracts using an Alpkem analyzer (Saskatoon, SK). Extractable N was expressed on a per gram soil dry weight basis.

3.5 DNA extraction

DNA was extracted from the -80°C soil sample subset following the manufacturer's protocols using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), except for using nuclease free water as the final step. We tested the DNA sample for quality and quantity using a NanoDrop Lite Spectrophotometer (Fischer Scientific, MA, USA). We repeated extractions if 260/280 absorbance ratio was below 1.6. DNA ranged in concentration from 16.7 to 520 ng/μL, thus we diluted all samples to 10 ng/μL and stored all extractions at -80°C.

3.6 qPCR

Using the diluted DNA above, we quantified the number of copies of genes present for bacterial and fungal specific genes using quantitative polymerase chain reaction (qPCR). We utilized forward and reverse primers specific to amplify bacteria (Eub338-Eub518) and fungi (ITS 1-5.8S). We then used 96-well plates and added 2 μL of diluted DNA with forward and reverse primers and Bioline 2x SensiFAST SYBR No-ROX Mix. The plates were then run on a CFX96 Touch Deep Well Real-Time PCR System (Bio-Rad, CA, USA). Melt curves were visualized and any un-amplified samples were re-run. We calculated the copy number of genes using standard curves of known concentrations of plasmid and divided by initial DNA concentration and the weight of soil used for the initial DNA extraction. Data was ln-transformed and tested for significance using one-way ANOVAs in the car package in R with post-hoc Tukey adjusted tests using the emmeans package.

3.7 Amplicon sequencing and bioinformatics

The diluted DNA extracted from above was used for DNA sequencing to assess diversity and community structure of bacterial and fungal communities. We amplified the diluted DNA using a T100 PCR Thermal Cycler (Bio-Rad, CA, USA) with platinum, DNA maker bar codes to tag each sample for pooling, and primer sets 515F/806R (Caporaso et al. 2012) and ITS1/ITS2R (Caporaso et al. 2012). These primer sets were used to amplify a conserved portion of the bacterial 16S rRNA gene and fungal ITS1 region, respectively. Our pooled DNA was then submitted to the University of Colorado Anschutz Medical Campus Genomics Shared Resource for Illumina MiSeq Sequencing (Illumina Inc., CA, USA) for amplicon sequencing using 16S (region v4; 515f-806r) and ITS (ITS1f-ITS2) paired-end 250-read sequencing. Resulting data was returned as multiplexed FASTQ files for downstream analysis.

After we received the data, we ran the data through a bioinformatics pipeline to create an operational taxonomic unit (OTU) table. We then used the USEARCH v.11 pipeline to demultiplex, denoise (UNOISE; Edgar 2016), quality filter (UCHIME; Edgar 2011) and generate 97% OTUs (UPARE; Edgar 2013). Initially, we used cutadapt to remove adapters and primers (Martin 2011) and used fastQC to quality filter the samples (Andrews 2010) and discarded sequences that had low quality scores ($Q < 20$), had short sequencing lengths (< 100 bp), or had ambiguous nucleotides. Then, we were able to merge paired-end reads and cluster and count OTUs at the sample level using DADA2 and DeNoise using uNoise 3 (Xiong et al. 2021). We also removed sequences that matched with mitochondrial or chloroplast samples (Edgar 2016). We assigned taxonomy to each OTU using USEARCH and UCLUST against the SILVA (Quast et al. 2013) database for bacterial (16S) sequences and UNITE (Nilsson et al. 2018) for fungal (ITS) sequences. Finally, we exported the OTU table and exported it as a txt file for statistical analyses in R statistical software.

3.8 Statistical analyses

We used the OTU table created above to run statistical analyses in R statistical software. We used the package mctoolrsr (Leff et al. 2017) to upload our OTU table and meta data file into R. We calculated basic diversity metrics such as alpha and beta diversity to test for differences in diversity between treatments. For alpha and beta diversity only, we rarefied our data to 4,000 sequences per sample using the single_rarefy function in mctoolsr. We used the diversity function in the vegan package to calculate richness and Shannon's diversity. We were able to evaluate statistical significance for by generating generalized linear models with OTU as the response variable and Treatment and Site and their interaction as fixed factors. We used one-way ANOVAs and post-hoc Tukey tests from the emmeans package to test for significance among treatments at each site. For beta diversity, we used PERMANOVAS in the vegan package to test for differences in beta diversity between the treatments. To visualize beta diversity, we utilized non-metric multi-dimensional scaling from the vegan package using the metaMDS function and Canonical Analysis of Principal Coordinates (CAP) using the CAPdiscrim function in the BiodiversityR package.

To evaluate differences in OTU to phylum level in bacteria and fungi, we used fixed-effect negative binomial generalized linear models (GLM) from the MASS package in R. We transformed the data to relative abundance and normalized the data using TMM normalization. We utilized the function summarize_taxonomy function in mctoolsr to summarize across taxonomic levels. We then used the emmeans package to estimate abundance and standard error of each OTU. We then ran one-way ANOVAs across treatments using FDR adjustments and extracted Tukey adjusted post-hoc comparisons. We summarized significant differences from the phylum, order, class, and family. To visualize this comparison, we used volcano plots to separate

sensitive and opportunistic taxa to drought. We took the log2 of the fold change (drought abundance/control abundance) and plotted it against the -log10 of the p-value (p-values of less than 0.001 were changed to 0.001 for better visualization).

For all resistance (measurements taken during the drought) and resilience (measurements taken after the first and second week of drought during the post-drought period) measures, effect-size was calculated using cohen's d and confidence intervals were calculated. Effect size was calculated to compare between the sites, and account for site differences by standardizing by the standard deviation.

4. Results

4.1 Resistance to drought

At the end of the 30-day experimental drought, we measured alpha diversity and qPCR to assess differences in the numbers of bacterial and fungal species present in the soil mesocosms. Based on 16S-sequencing, we found no evidence for differences in richness or Shannon's diversity between the drought and control treatments for the four sites (Figure 4.1). However, based on ITS sequencing, we found decreased richness and Shannon's diversity in the drought treatment at KNZ only (Figure 4.1). We found no significant differences or trends in qPCR for bacteria or fungi at all sites (Appendx 4 Figure 1). Further, we ran PERMANOVAS for bacteria and fungi to assess differences in community composition (Table 4.2) and community differences were visualized with NMDS plots (Appendix 4 Figure 2). We only found significant differences between the drought and control treatments for fungal communities at SGS ($p = 0.08$) and KNZ ($p = 0.06$). We plotted the fungal CAPs of these two sites (Figure 4.2) to show the difference between the treatments at the two sites. Lastly, we measured amount of nitrogen and enzymatic activity to assess nutrient limitation and microbial activity. We found no evidence for

the drought treatment affecting either ammonium or nitrate for the four sites. For enzymes AG, BG, XYL, NAG, and PHOS, we found decreases in their activity in the drought treatment compared to the control at the CHY and KNZ sites. For the enzyme CB, we found decreased activity at CHY, HYS, and KNZ in the drought treatment compared to the control, while we found increased LAP enzyme activity in the drought treatment at only CHY.

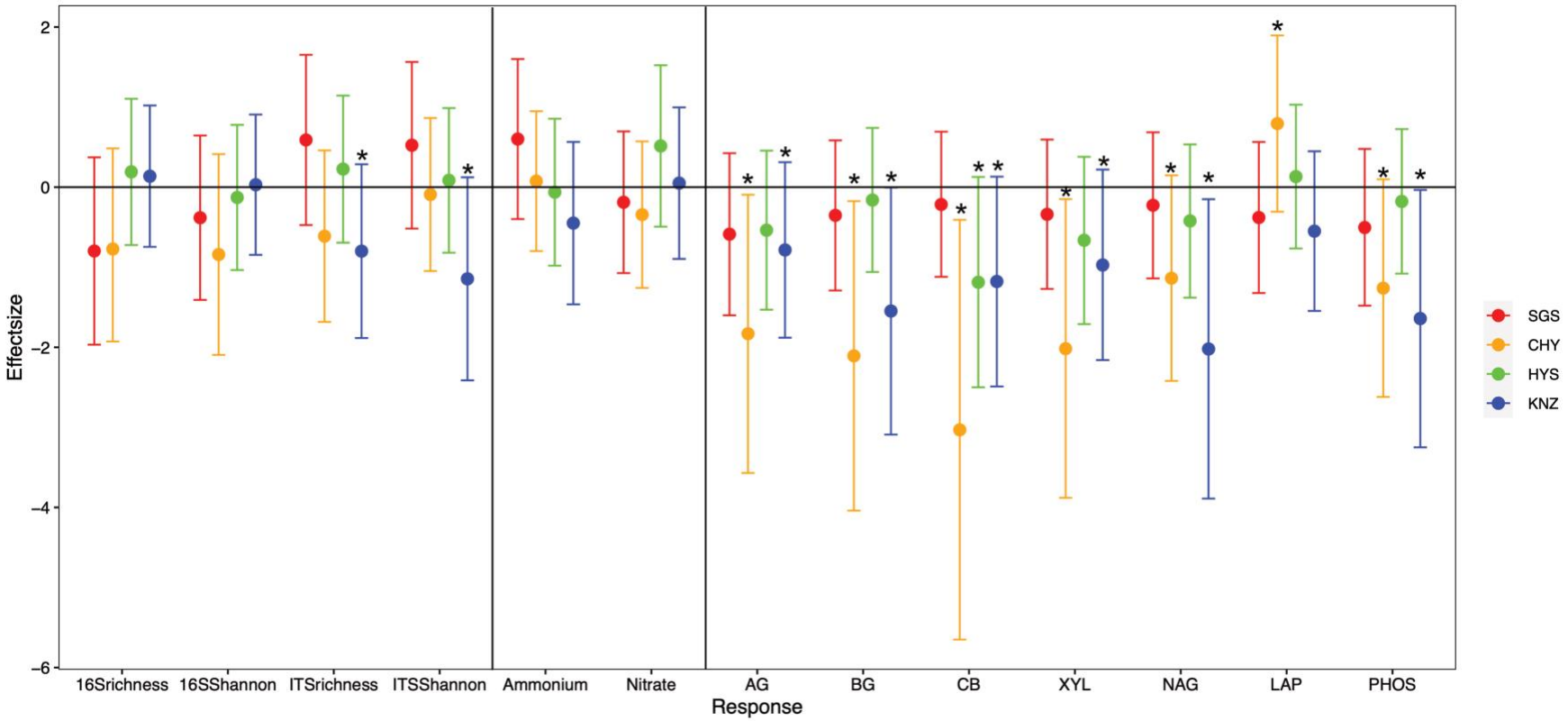

Figure 4.1. Effect size (cohen's d) of all measures taken during the drought. Effect size was measured as (Control-Drought)/ pooled standard deviation. Negative effect sizes indicate a decrease with drought. Positive effect sizes indicate an increase with drought. Star indicates significance level of p <0.05. 95% confidence intervals are shown. Sites are ordered from least to greatest MAP.

Table 4.2. p-values from PERMANOVA analysis.

SITE	WEEK	16S P-VALUE	ITS P-VALUE
SGS	0	0.69	0.084
CHY	0	0.609	0.179
HYS	0	0.453	0.378
KNZ	0	0.494	0.061
SGS	1	0.913	0.929
CHY	1	0.461	0.492
HYS	1	0.965	0.968
KNZ	1	0.695	0.774
SGS	2	0.708	0.815
CHY	2	0.339	0.894
HYS	2	0.716	0.924
KNZ	2	0.446	0.756

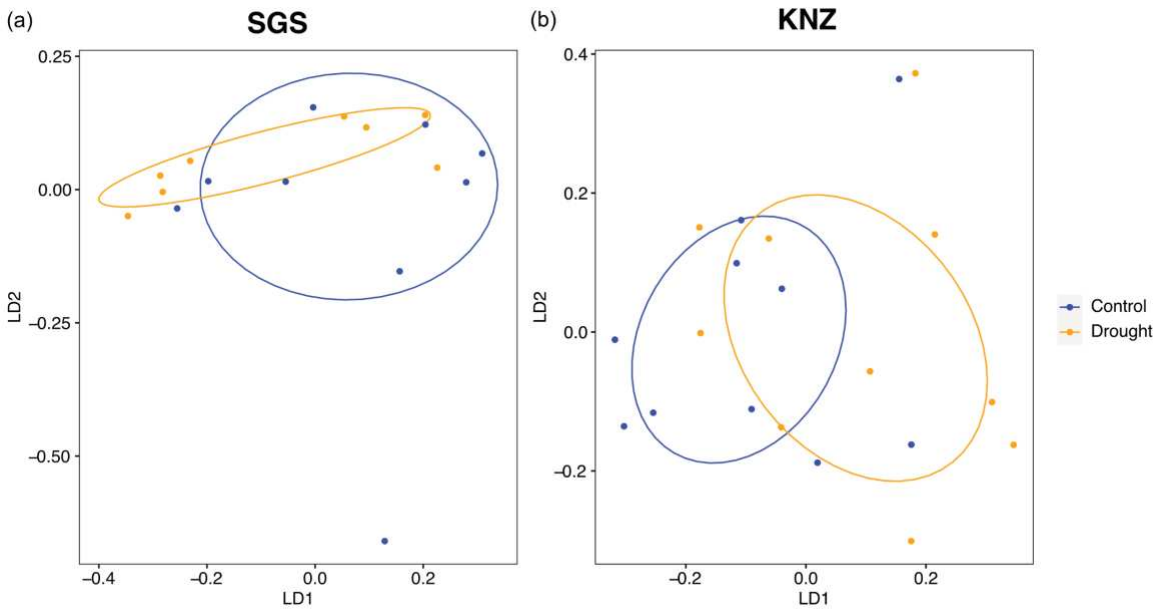

Figure 4.2. Canonical Analysis of Principal Coordinates (CAPs) of sites that had significant community difference (PERMANOVA, p-value =<0.1). Only fungal communities at SGS (a) and KNZ (b) were significantly affected by drought.

For phylum differences, we generated volcano plots to assess OTU response to drought for each phylum. Phyla that exhibited a >2-fold decrease with drought and were significantly different from the control ($p < 0.05$) are referred as sensitive, while those that experienced >2-fold increase with drought and were significantly different from the control ($p < 0.05$) are referred to as opportunistic. For bacterial phyla at SGS, we found that Firmicutes had more OTUs that were sensitive to drought and Actinobacteria, Proteobacteria, and Chloroflexi had more OTUs that were opportunistic with drought (Figure 4.3a). For bacterial phyla at CHY, we found that Acidobacteria had more OTUs sensitive to drought (Figure 3b). For bacterial phyla at HYS, we found that were generally more OTUs sensitive to drought and that Acidobacteria, Actinobacteria, Plantomycetes, Proteobacteria, and Verrucomicrobia all had more OTUs that were sensitive to drought (Figure 4.3c). For bacterial phyla at KNZ, we found that there were

more OTUs that were overall opportunistic with drought, with Acidobacteria, Firmicutes, Proteobacteria, and Verrucomicrobia each having more OTUs that were opportunistic to drought (Figure 3d). For fungal phyla at SGS, CHY, HYS, and KNZ, we found no differences in amount of OTUs that were sensitive or opportunistic across phyla or class (Figure 4.4; Appendix 4 Figure 3).

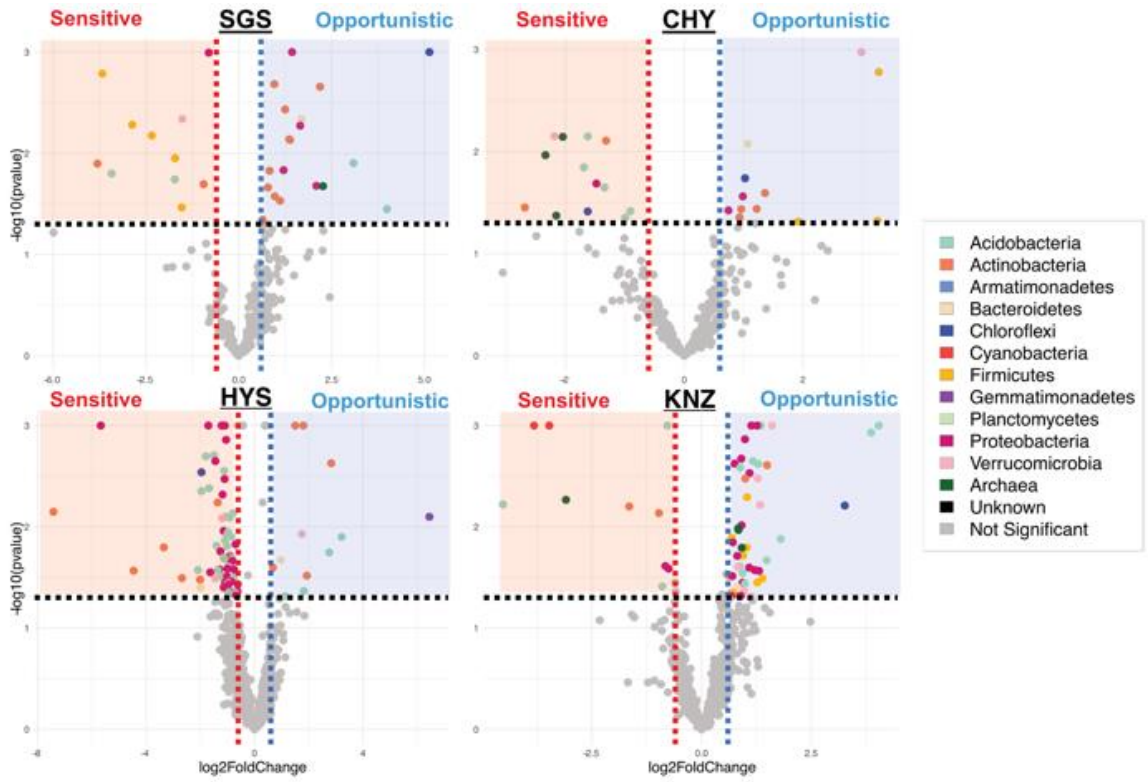

Figure 4.3. Plots of bacterial OTUs indicating which are sensitive or opportunistic under drought conditions. Each OTU is colored by phylum. All OTUs above the black dotted line indicate OTUs that had significant p-values ($p<0.05$). p-values of less than 0.001 were changed to 0.001 for better visualization. We divided the OTU estimated abundance in the drought treatment and divided by the estimated abundance in the control treatment as the fold change from control to drought. The log2 of this fold change is shown above. OTUs that decreased in abundance under drought are highlighted in red, and OTUs that increased in abundance under drought are highlighted in blue.

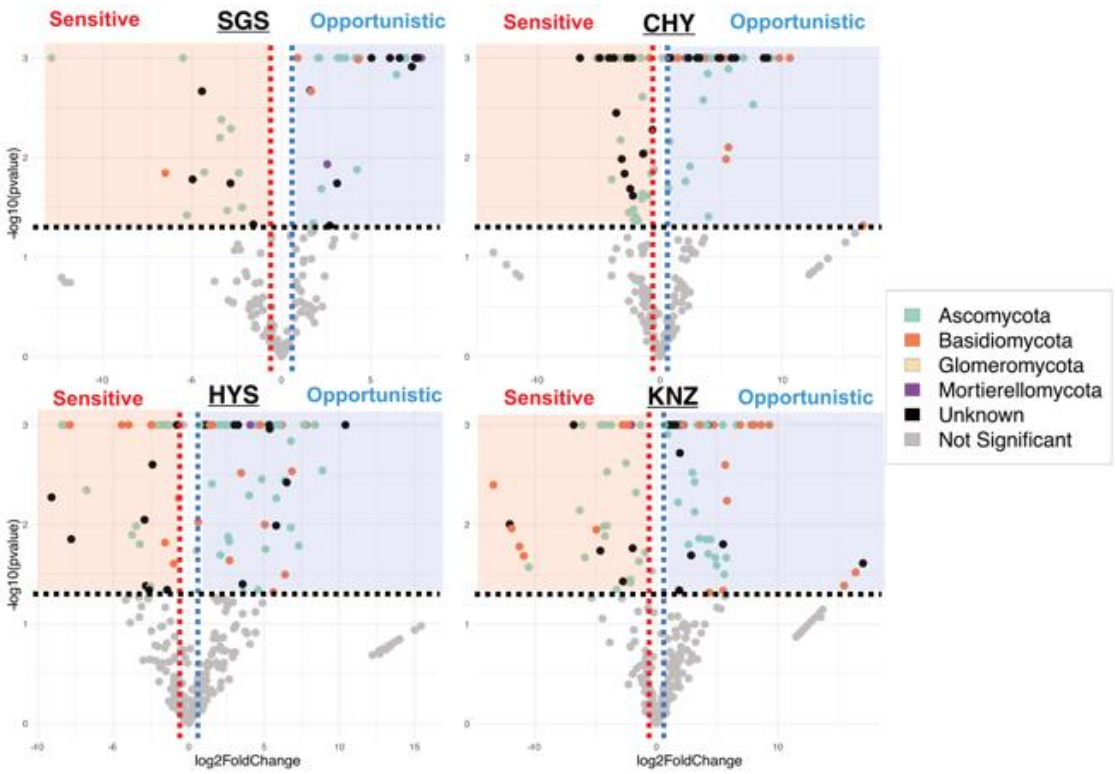

Figure 4.4. Plots of fungal OTUs indicating which are sensitive or opportunistic under drought conditions. Each OTU is colored by phylum. All OTUs above the black dotted line indicate OTUs that had significant p-values ($p<0.05$). p-values of less than 0.001 were changed to 0.001 for better visualization. We divided the OTU estimated abundance in the drought treatment and divided by the estimated abundance in the control treatment as the fold change from control to drought. The log2 of this fold change is shown above. OTUs that decreased in abundance under drought are highlighted in red, and OTUs that increased in abundance under drought are highlighted in blue.

4.2 Post-drought responses

After the 30-day experimental drought ended, we repeated the same measures as for resistance, measuring resilience using cohen's effect size divided by pooled standard deviation at one week and two weeks after the drought treatment ended. After one week, we found no significant differences in beta diversity (Table 4.2). Further, we found that there were evidence for differences in either 16S or ITS derived richness or Shannon's diversity for fungi or bacteria between the drought and control treatments for the four sites. Similarly, no differences in ammonium or nitrate amounts were observed. On the other hand, we found that enzyme

activities tended to exhibit the opposite response as during the drought and found significant differences for all of the enzymes. For AG and LAP, we found increases in enzyme activity in the drought treatment compared to the control across all sites. For XYL we found decreases in enzyme activity in the drought treatment compared to the control across all sites. For BG and CB, we found increases in enzyme activity in the drought treatment compared to the control for CHY and HYS. Lastly for PHOS, we found increases in enzyme activity in the drought treatment compared to the control for CHY, HYS, and KNZ (Figure 4.5).

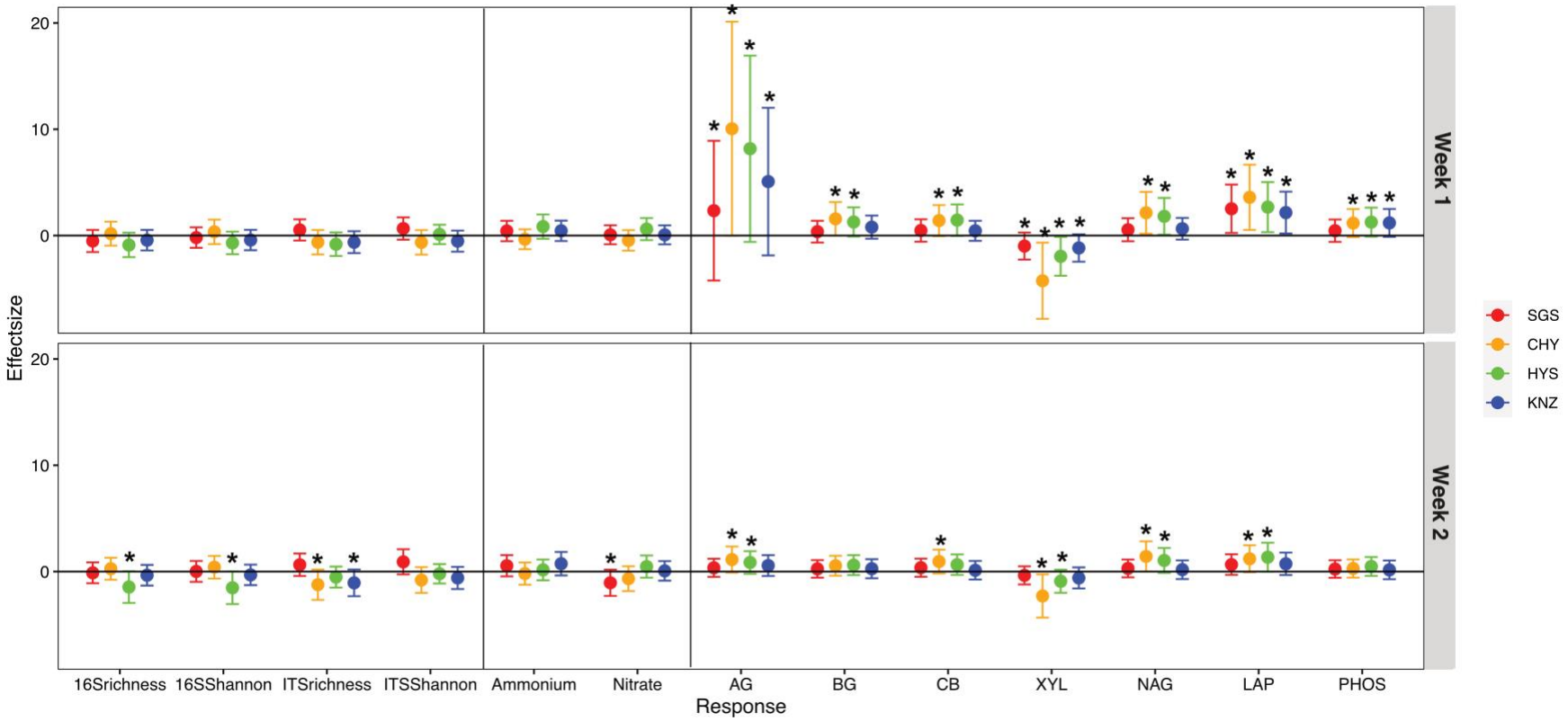

Figure 4.5. Effect size (cohen's d) of all measures taken one week and two weeks after drought ended. Effect size was measured as (Control-Drought)/ pooled standard deviation. Negative effect sizes indicate a decrease in the drought treatment compared to the control. Positive effect sizes indicate an increase in the drought treatment compared to the control. Star indicates significance level of $p < 0.05$. 95% confidence intervals are shown. Sites are ordered from least to greatest MAP.

After week 2, we found no statistical differences in beta diversity (Table 4.1). Further, we found no statistical differences for ITS Shannon's diversity; however, we found a decreases in 16S richness and 16S Shannon's diversity at the HYS site and ITS richness for CHY and KNZ. We also found small decreases in nitrate at the SGS site in the drought treatment compared to the control, but not for the other sites. For AG, NAG, and LAP, we found increases in their activity in the drought treatment compared to the control at CHY and HYS. For XYL, we found decreases in their activity at CHY and HYS in the drought treatment, while activity for CB was increased with drought only at CHY. Overall, the differences in enzymatic activity were smaller for the second week after the drought when compared to either the first week after drought or responses observed during the drought.

5. Discussion

Our experiment aimed to understand soil microbial functional and structural resistance to drought across four grassland sites in the Central US through microbial community sequencing, enzyme activity, and inorganic nitrogen. Our study achieved this by collecting intact soil mesocosms from each grassland site and conducting a drought experiment by completely eliminating watering for 30 days or maintaining soil moisture at average soil moisture conditions specific to each site. Our study also aimed understand if impacts from the drought persisted after drought or whether measures of soil microbial structure and function were resilient. Our study achieved this by watering the drought treatments for two weeks at control levels and measuring responses one and two weeks after drought ended. We predicted that we would find that dry sites would be the most resistant to drought (Griffiths and Phillippot 2013) and that all microbial communities would experience resilience to drought by showing quick recovery of all microbial

measures after the drought ended, in line with previous observations made at the most mesic grassland site (Vilonen et al. 2022a and Vilonen et al. 2022b).

5.1 High microbial resistance to drought

Overall, we expected to find that all four sites were not resistant to drought in their microbial functional responses, but they would experience similar structural responses primarily through phylum shifts. However, we expected to observe more pronounced differences in microbial structural and functional responses for the drier sites compared to the more mesic sites (Knapp et al. 2015). However, our initial predictions were not supported by our data. We found that the wettest site, KNZ, was the most sensitive to drought. The KNZ site had decreased fungal richness and Shannon's diversity. The KNZ site also exhibited decreases in enzyme activity for all enzymes measured (Figure 1), but these effects were also observed at the drier CHY site. However, the activity of LAP increased at only the drier CHY site, while the enzyme activity of CB decreased with drought for the wetter HYS site. Contrary to expectations, we found no significant differences for any measures except for fungal beta diversity at the SGS site, which is the driest of the four grassland sites (Figure 4.1). Lastly, the SGS and KNZ sites were the only sites where drought significantly affected fungal beta diversity (Figure 2).

A possible explanation for the decrease in all enzymes for both CHY and KNZ is that microbial activity was decreased by drought, although we did not measure microbial respiration or growth, so we are unable to confirm this hypothesis. Other studies have found that enzyme activity and microbial activity are both reduced by drought (Bogati and Walczak 2022) and other studies have found that enzyme activity correlates with microbial activity (Frankenberger and Dick 1983; Cui and Holden 2015). Thus, a likely explanation for this decrease in enzyme activity for all enzymes for CHY and KNZ was due to a decrease in microbial activity, since qPCR also

showed no differences in the amount of microbes present for any of the sites (Figure S1). Another possible explanation is that there were no nutrient limitations in the system during drought leading to decreased enzyme activity, although some studies have seen an increase in enzyme activity in response to drought (Ochao-Hueso et al. 2018) due to nutrient limitation, so this explanation is less likely than a general decrease in microbial activity as observed by Bogati and Walczak (2022). Lastly, the KNZ site might have been the least resistant to drought due to the microbial community experiencing natural drought and water limitation less frequently than the drier sites.

Overall, every site was much more resistant to short-term drought than expected. One possible explanation for this is that at these arid and semi-arid systems, the microbial community is highly resistant and tolerant to drought (Ochoa-Hueso et al. 2018; Sayer et al. 2021). Further grasslands in general typically have stable bacterial communities and C stocks (Delgado-Baquierizo et al. 2017a), which might protect them from negative impacts of drought. A previous study at the KNZ site showed that the microbial community had almost no change to drought (Vilonen et al. 2022) due to high levels of Actinobacteria and Verrucomicrobia, which could be a possible explanation for the high resistance to drought across our sites due to the high amounts of Actinobacteria and Verrucomicrobia. Another possible explanation for this high resistance to drought is that past climate or paleoclimate in natural systems is a greater determinant for microbial community composition compared to recent climate events (Delgado-Baquierizo et al. 2017b), and thus the microbial community was not impacted by the drought. Other studies have also shown that the microbial community is highly impacted by past legacies and soil histories and thus less impacted by current climate conditions (Evans et al. 2022).

5.2 Are there generalizable trends of the belowground to drought?

One of the main questions our study attempted to answer was if there were generalizable trends of the belowground to drought. Our main hypothesis of our paper was that we would find generalizable trends against MAP for alpha diversity and enzyme activity and perhaps some of the other measures. However, we did not find any data to support this. In fact, our data pointed to drought response being highly specific to each site. The first indication of drought response being site-specific is that only KNZ and CHY saw any significant shifts in enzyme activity during drought, and KNZ was the only site that saw decreases in alpha diversity and changes in beta diversity. The second indication that drought is highly site specific is that each site had very different phylum level responses for bacterial species. For example, at HYS Proteobacteria were mostly sensitive to drought, while at KNZ Proteobacteria were mostly opportunistic to drought. This dichotomy can further be seen when looking at Firmicutes at SGS and KNZ. At SGS, Firmicutes were mostly sensitive to drought, while at KNZ they were mostly opportunistic to drought. Lastly, this can be seen for Acidobacteria, where the phyla is sensitive to drought at CHY and HYS, but opportunistic at KNZ (Figure 4.3). There were no observable trends for fungi in phylum or class with the OTUs belonging to each phylum (Figure 4.4) and class (Appendix 4 Figure 4) being mostly evenly spread between opportunistic and sensitive.

Our findings that response to drought was highly site specific has important implications for future modeling. Many studies have been looking for generality across ecosystems to create better models to predict the impacts of climate change such as drought (Knapp et al. 2015; Ochoa-Hueso et al. 2018). However, our study found that finding generality in soil microbial functional and structural responses is highly challenging. Aboveground systems have seen trends in aboveground response (Knapp et al. 2015), but soil microbial responses to drought seems much more challenging to generalize (Ochoa-Hueso et al. 2018).

5.3 Resilience

Our second question aimed to understand if systems were all resilient to drought or whether some systems were more resilient to drought than others. We predicted that all systems would be highly resilient to drought, since previous research (Vilonen et al. 2022) found that the microbial community at the KNZ site was highly resilient to drought in a field experiment. High resiliency has also been shown in several other studies (Evans and Wallenstein 2011; Hueso et al. 2011).

In the first week post-drought, we saw several large increases of enzyme activity across all the enzymes for most of the sites. The differences were most pronounced in AG and LAP. AG releases glucose from starch (Stone et al. 2014), while LAP releases nitrogen substrates from peptides. The other carbon acquiring enzymes break down cellulose (BG and CB) and hemicellulose (XYL) (Stone et al. 2014), which are much harder to break down into glucose than starch as a usable product for the microbial community. Thus, we most likely saw this continued and high increase in the AG enzyme, because the active microbial community likely was nutrient deficient (de Nijs et al. 2019). Thus, the microbial community likely excreted an enzyme that could break down an easily digestible substrate into glucose for microbial growth. In the second week post-drought, many of the changes we found in the first week post-drought were either non-significant or had smaller effect sizes than the first week after drought (Figure 4.5). Thus, it seems that over the short two-week period of time we began to see full recovery of all measures

Overall, although we saw several increases in enzyme activities after the first week of drought and small significant differences in the second week, all four sites showed high resilience to drought. It has been shown that the microbial communities in dryland sites tend to recover quickly after a disturbance ends (Steven et al. 2021). Further, most of these sites also

showed high resistance to drought, so any of the effects we saw was likely due to re-wetting causing positive shifts in functioning as has been seen in other studies (de Nijs et al. 2019).

Although we expected to find generality across sites, our study was unable to do so, suggesting that response to drought is highly site specific. Importantly, the microbial community at most of the sites was highly resistant to short-term drought as well. This indicates that the microbial community in these four grasslands that encompass the Central Plains region of the US has the potential to be highly resistant to drought conditions in the future. Future studies would benefit from encompassing a larger geographic range and attempting to find generality across more sites and ecosystems. Further, future research would benefit from attempting longer time scales of drought, since we imposed a short drought period.

CONCLUSIONS

Throughout this dissertation, I sought to define the terms used after drought ends (chapter 1), evaluate legacy effects on the belowground in nutrient cycling (chapter 2) and microbial structure (chapter 3), and determine whether belowground impacts of drought are generalizable across a climatic gradient in grasslands. My dissertation defined the terms as legacy effects (positive or negative impacts after drought ends), recovery (return to pre-perturbation functioning or structure), and resilience (ability to return to pre-perturbation functioning or structure). Further, my dissertation found that there were some legacies on carbon and nitrogen cycling, but that these legacies were short-lived. Additionally, legacies on microbial community structure were also short-lived and small compared to effects found during the drought. Lastly, my dissertation found that microbial responses to drought were highly site specific, but that the site from the previous two chapters was the least resistant to drought. In sum, my dissertation found that microbial communities are encouragingly resistant and resilient to drought. These results are highly promising, since drought is projected to intensify over the next decade and we have shown that microbial communities have the ability to be highly resistance and resilient to these conditions.

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APPENDIX 1 CHAPTER 1

Table S1. *List of all the papers used in this study. In-text citations were omitted for general statistics and descriptions of these papers used below.*

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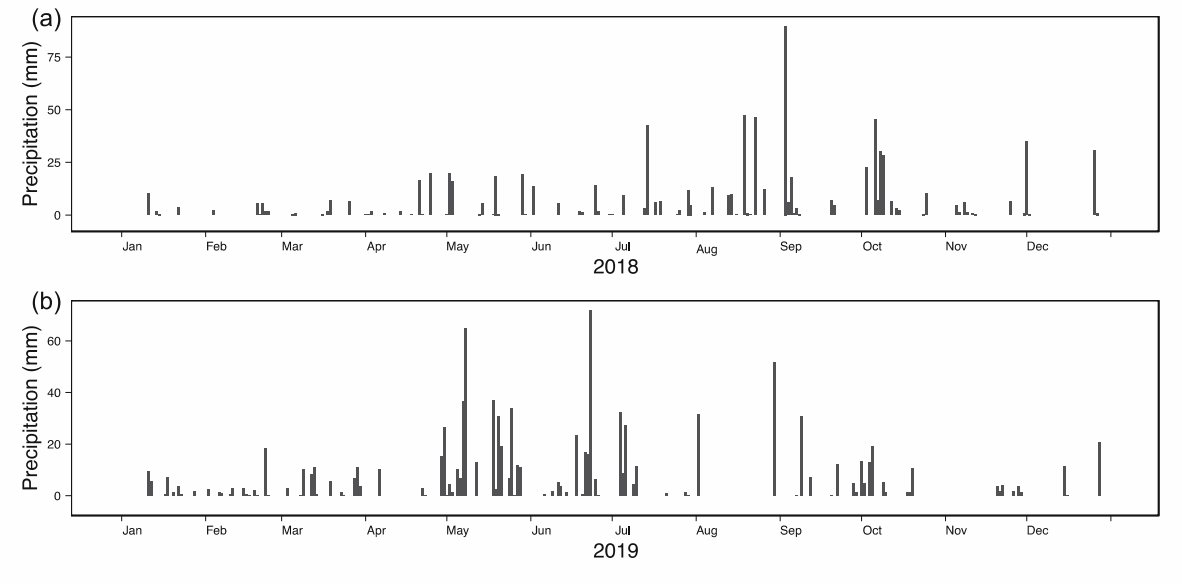
Table S2. Summary of the mechanisms that papers cited as the reason for the response observed.

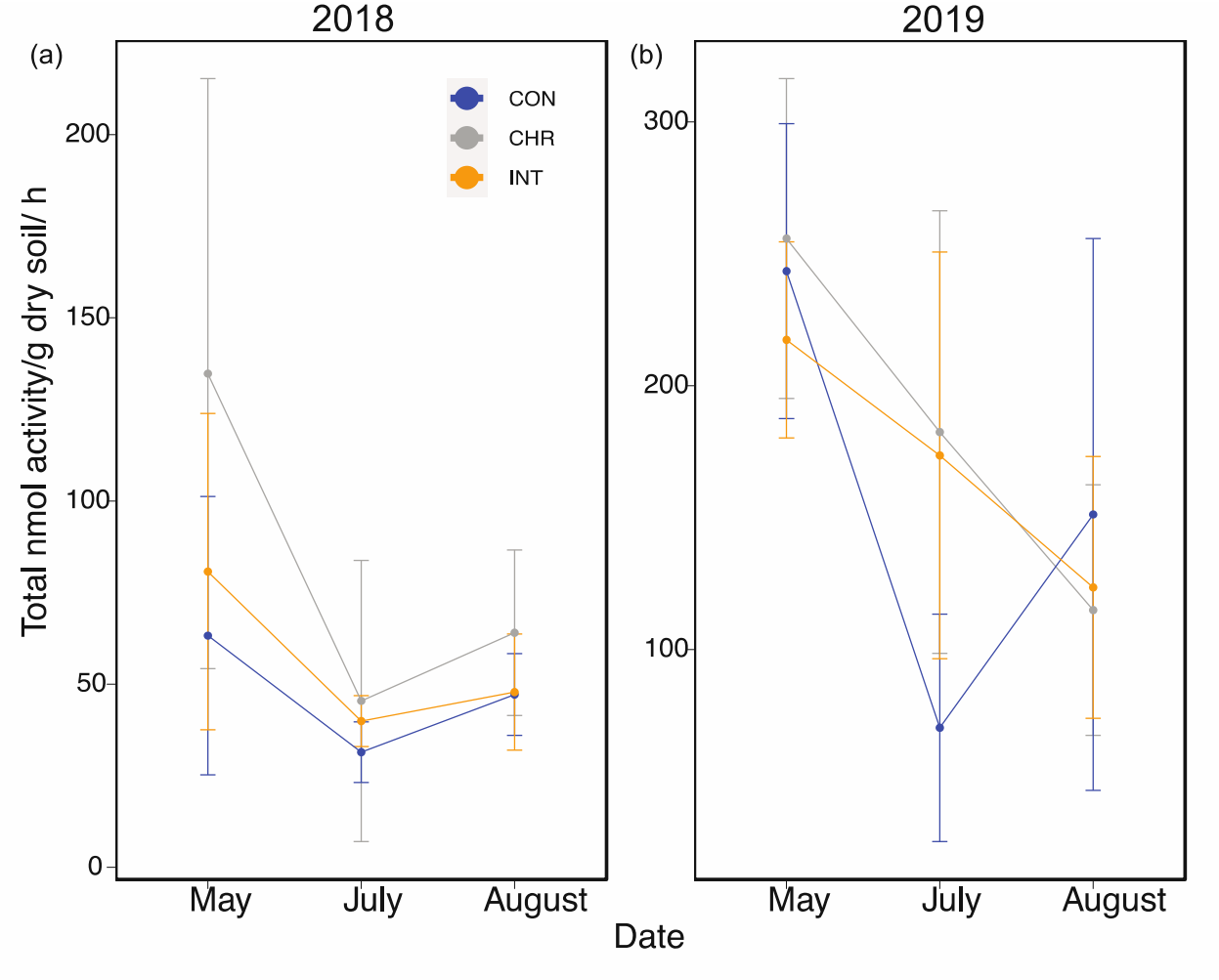
Mechanisms	Number of papers
Biotic	
Changes in physiology	13
Foliage losses	5
Loss of trees/ evapotranspiration	1
Species trait shifts	1
Photoprotection	1
Reductions in photosynthesis	1
Root to shoot ratios	1
Plant community change	2
Seedling establishment	1
Microbial community mediated	4
Higher levels of microbial biomass	1
Less active microbial community	1
Microbial turnover of plant-derived C	1
Age/height of plant	4
Effect of neighboring trees	2
Root exudation	2
Root regeneration/growth	1
Stomatal and biochemical traits	1
Plant enzyme activity change	4
Prioritization of leaf flushing	1
Biomass allocations and carbohydrate metabolisms	1
Carbon reserves	5
Storage of carbohydrates	1
Loss of carbohydrates	2
Abiotic	

Water reserves left over from year before	5
Change in nutrient concentrations	5
Elevated nitrogen levels	3
Increased light availability	2
Precipitation related (water amount)	5
Unclear	5

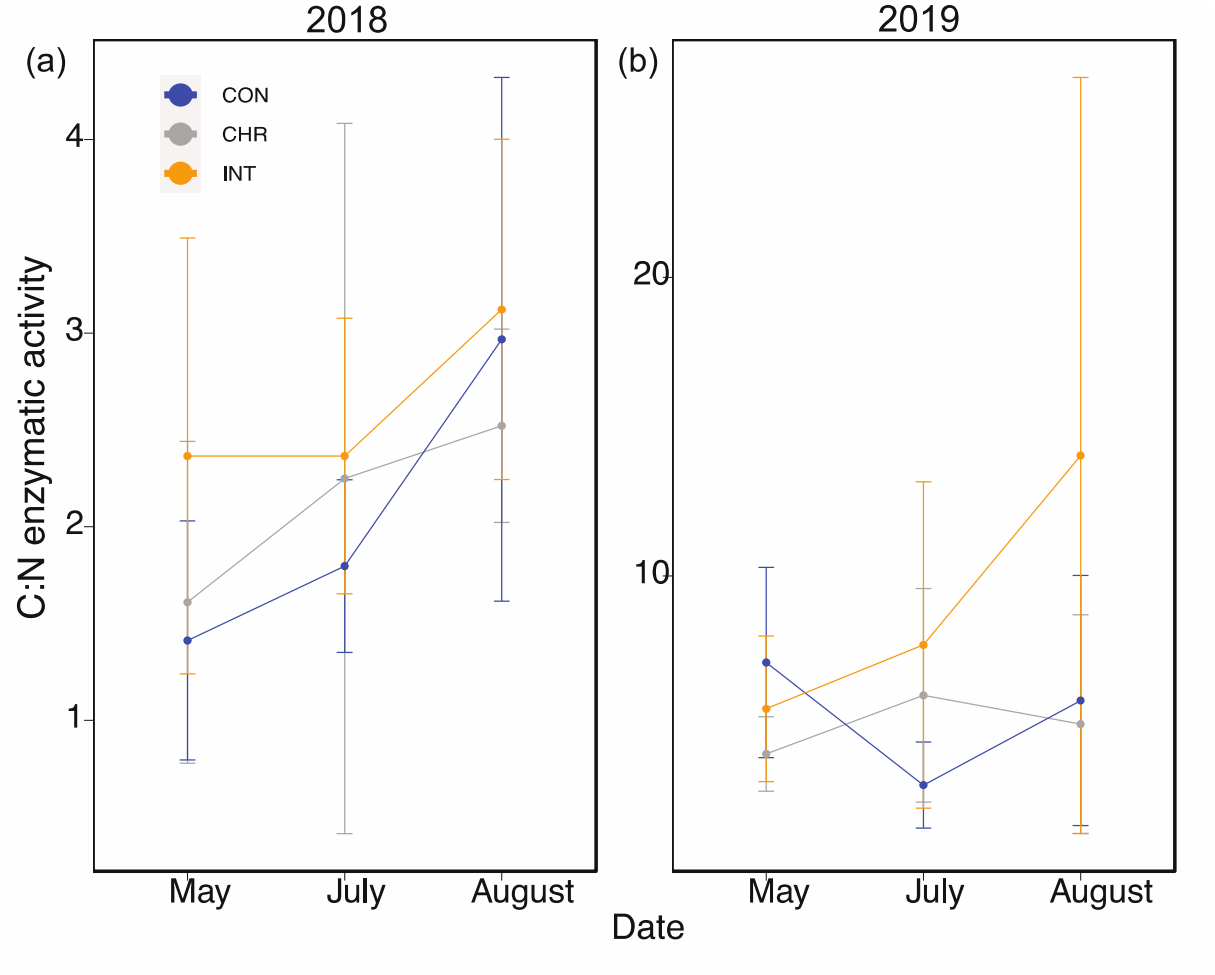
APPENDIX 2 CHAPTER 2



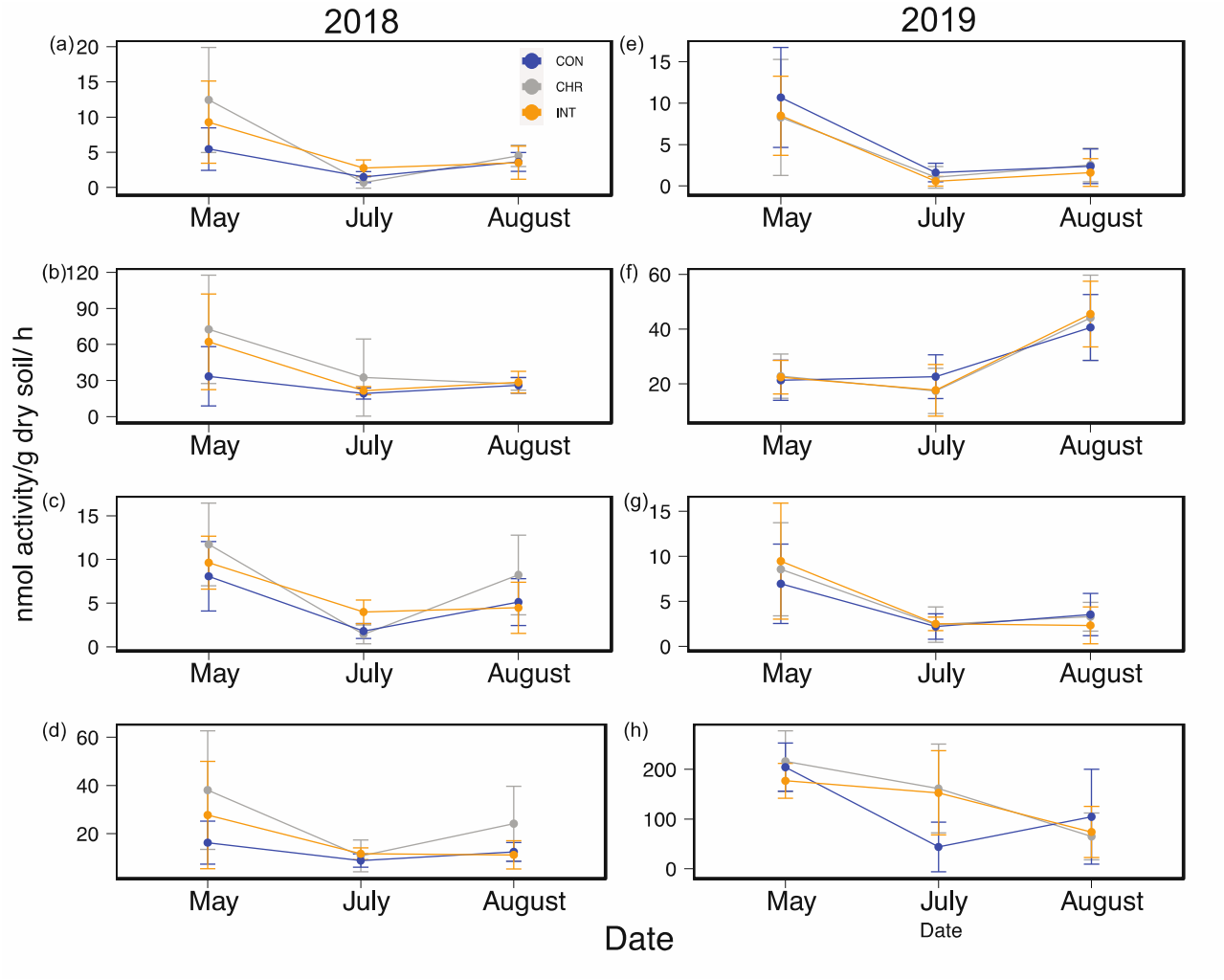
Appendix 2 Figure 1. Precipitation (mm) in (a) 2018 and (b) 2019mm. Each bar represents a single daily precipitation event.



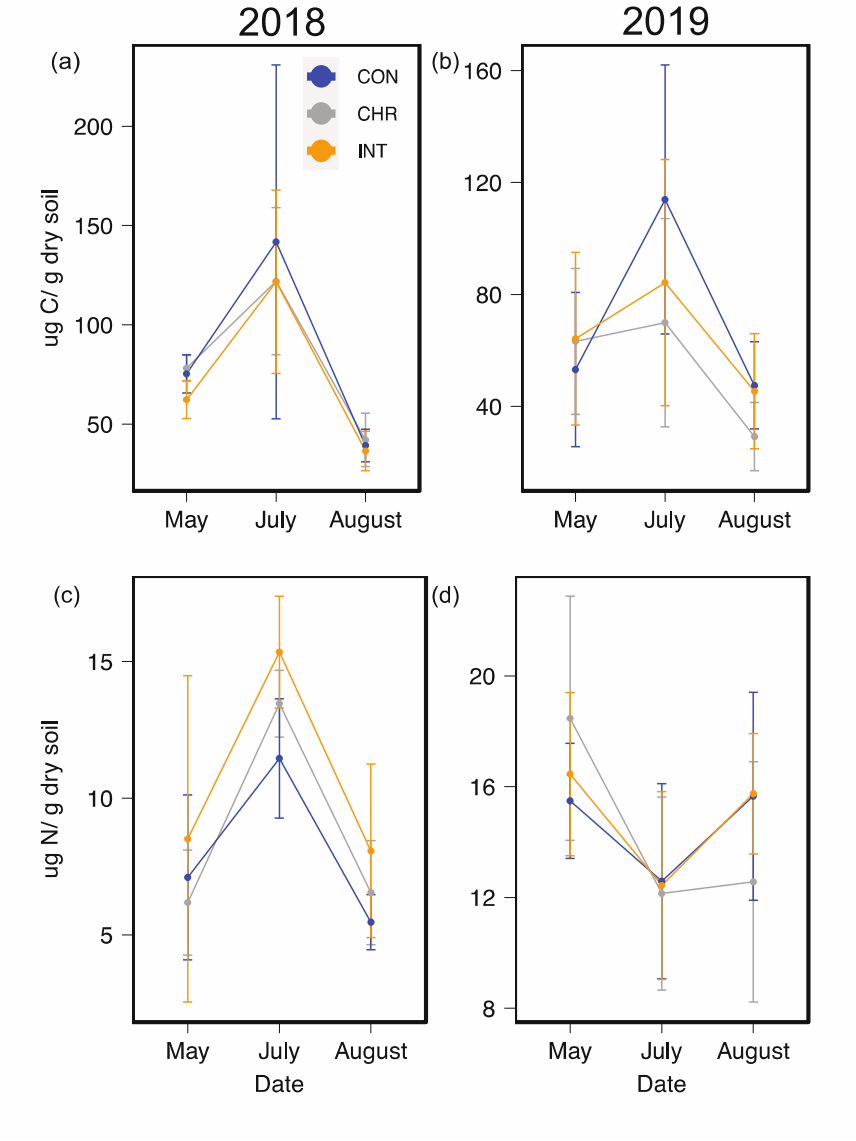
Appendix 2 Figure 2. Cumulative C enzymes (AG + BG + CB + XYL) in 2018 (a) and 2019 (b). Graphs show the raw enzyme data. Statistics were run with ln transformed data to account for unequal variance. The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average enzyme activity for each treatment. The error bars represent the 95% confidence intervals. No statistical differences among treatments were detected.



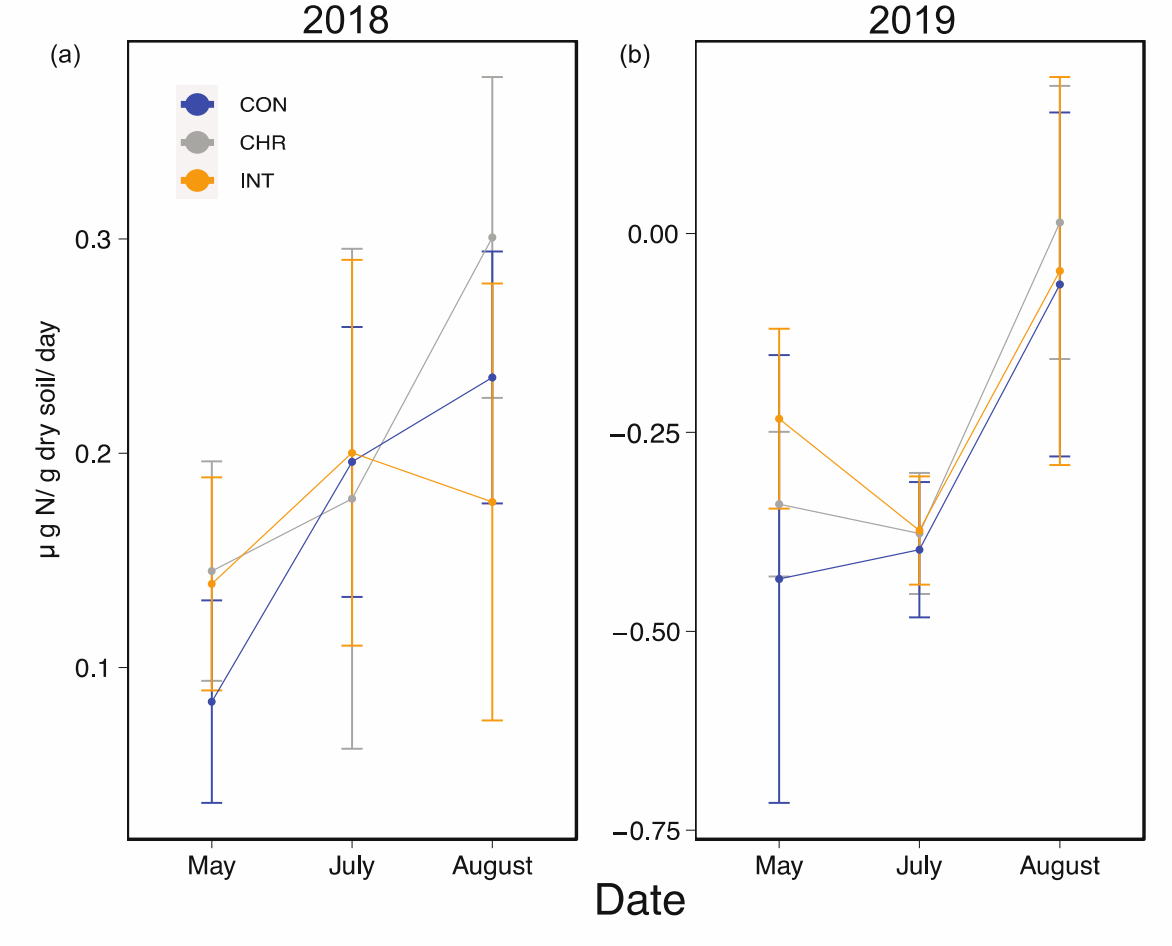
Appendix 2 Figure 3. Cumulative C enzymes (AG + BG + CB + XYL) divided by cumulative N enzymes (LAG + NAP) in 2018 (a) and 2019 (b). Graphs show the raw enzyme data. Statistics were run with ln transformed data to account for unequal variance. The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average enzyme activity for each treatment. The error bars represent the 95% confidence intervals. No statistical differences among treatments were detected.



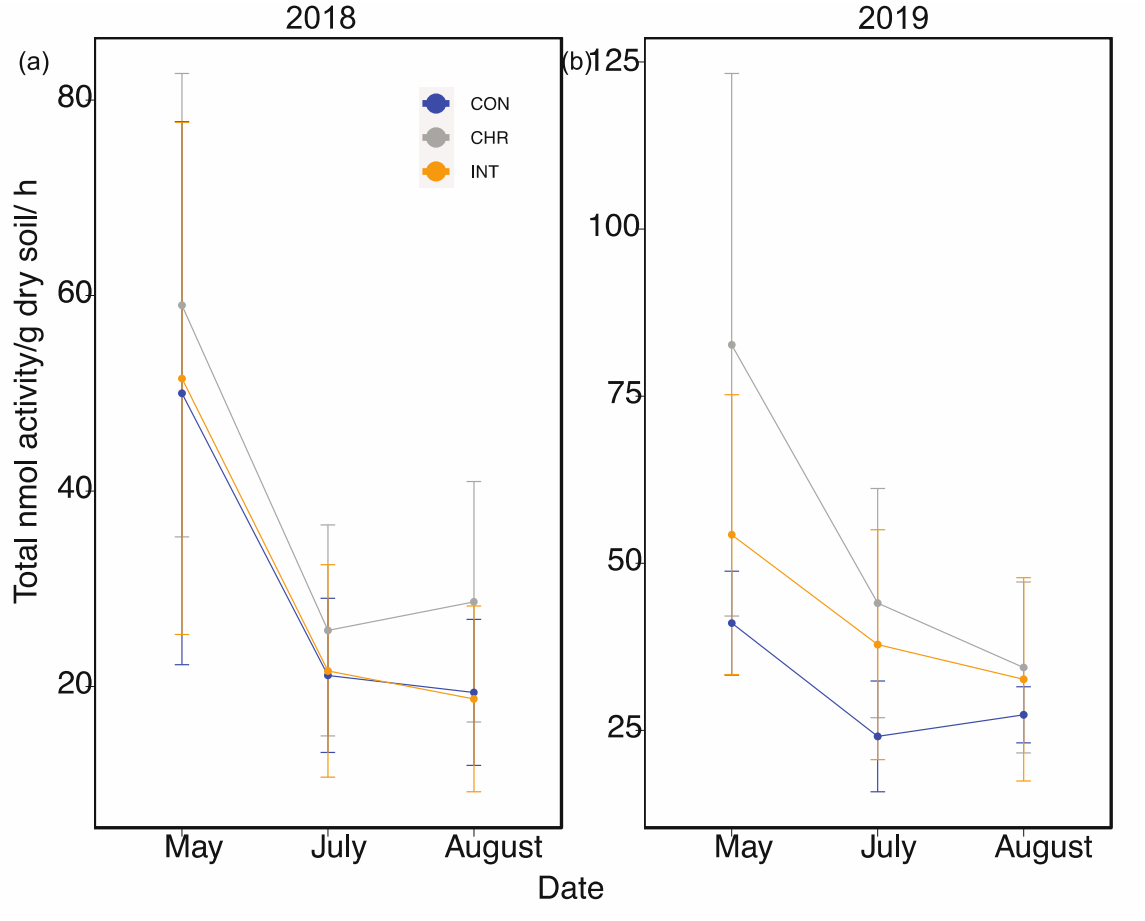
Appendix 2 Figure 4. All individual C enzyme activities in 2018 and 2019. 2018 AB (a), BG (b), CB (c), and XYL (d) are displayed on the left side. 2019 AB (e), BG (f), CB (g), and XYL (g) are displayed on the right side. Graphs show the raw enzyme data. Statistics were run with ln transformed data to account for unequal variance. The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average enzyme activity for each treatment. The error bars represent the 95% confidence intervals. No statistical differences among treatments were detected.



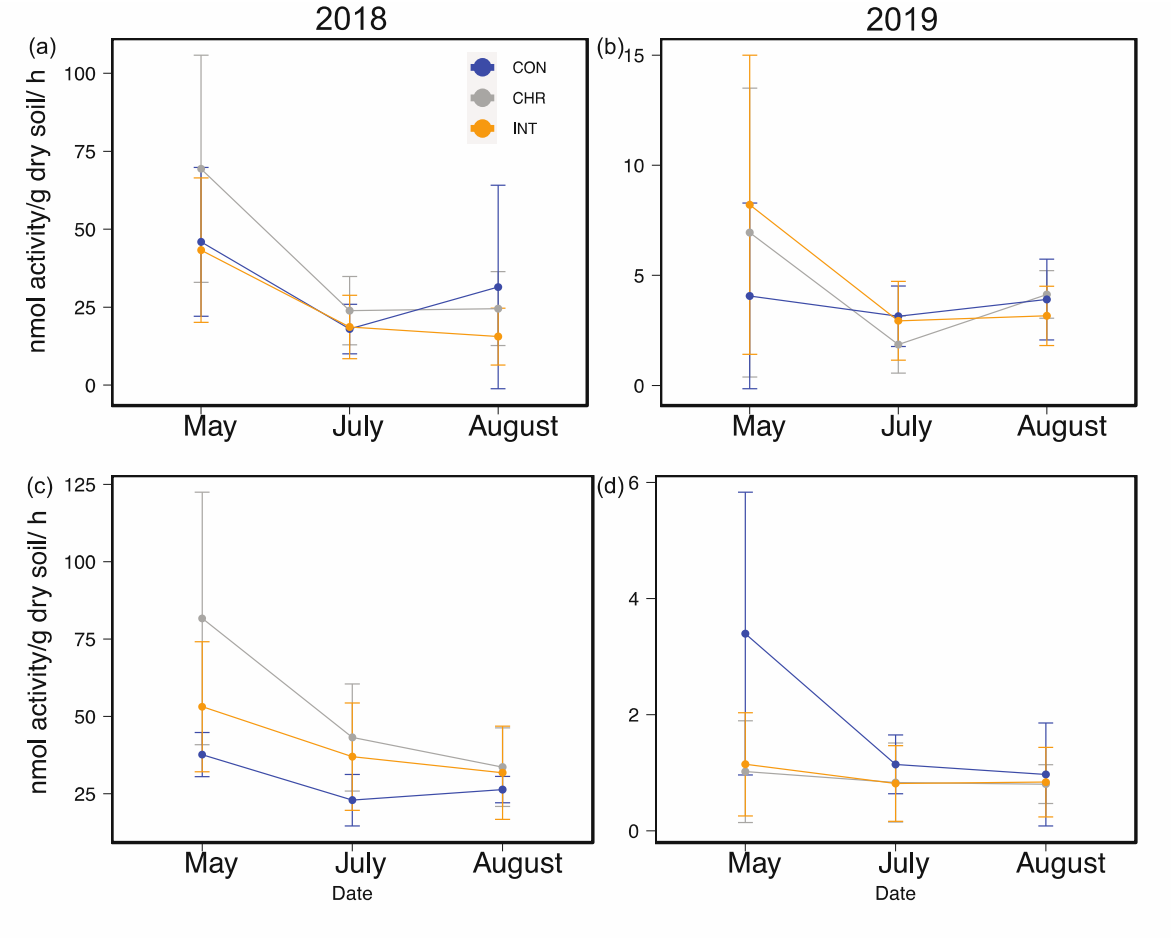
Appendix 2 Figure 5. Dissolved organic C and dissolved organic N in 2018 and 2019. 2018 dissolved organic C (a) and 2019 dissolved organic C (b) are displayed on the upper panel. 2018 dissolved organic N (c) and 2019 dissolved organic N (d) are displayed on the lower panel. The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average organic C or N for each treatment. The error bars represent the 95% confidence intervals. No statistical differences were detected.



Appendix 2 Figure 6. Net N mineralization in 2018 (a) and 2019 (b). The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average N mineralization for each treatment. The error bars represent the 95% confidence intervals. No statistical differences among treatments were detected.



Appendix 2 Figure 7. Cumulative N enzymes (LAP + NAP) in 2018 (a) and 2019 (b). Graphs show the raw enzyme data. Statistics were run with ln transformed data to account for unequal variance. The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average enzyme activity for each treatment. The error bars represent the 95% confidence intervals. No statistical differences were detected.



Appendix 2 Figure 8. All individual N enzyme activities in 2018 and 2019. 2018 NAG (a) and LAP are displayed on the upper panel. 2019 NAG (a) and LAP are displayed on the lower panel. Graphs show the raw enzyme data. Statistics were run with ln transformed data to account for unequal variance. The control treatment is shown in blue, chronic treatment in gray, and intense treatment in orange. The circles represent the average enzyme activity for each treatment. The error bars represent the 95% confidence intervals. No statistical differences were detected.

Appendix 2 Table 1. ANOVA table of soil moisture.

Measure	Variable	Sum Sq	Mean Sq	Num DF	F value	Pr (>F)
2018 Soil Moisture	Treatment	164.88	82.44	2	4.25	0.018*
	Date	2409.88	1204.94	2	62.16	<<0.001***
	Treatment: Date	152.78	38.20	4	1.97	0.11
2019 Soil Moisture	Treatment	55.80	27.90	2	1.12	0.33
	Date	26403.2	3300.4	8	132.67	<<0.001***
	Treatment: Date	671.7	42.0	16	1.69	0.0499*

Appendix 2 Table 2. ANOVA table of C cycling measures.

Measure	Variable	Sum Sq	Mean Sq	Num DF	F value	Pr (>F)
2018 Belowground Respiration	Treatment	30.44	15.22	2	3.97	0.023*
	Date	158.72	79.36	2	20.70	<<0.001***
	Soil Moisture	6.39	6.39	1	1.67	0.20
	Treatment: Date	34.77	8.69	4	2.27	0.071
2019 Belowground Respiration	Treatment	12.60	6.30	2	0.26	0.77
	Date	10633.7	1329.21	8	54.80	<<0.001***
	Soil Moisture	19.9	19.92	1	0.82	0.37
	Treatment: Date	114.0	7.13	16	0.29	0.99
2018 Initial Microbial Respiration	Treatment	9.83	4.92	2	8.084	<0.001***
	Date	140.81	70.41	2	115.80	<<0.001***
	Treatment: Date	2.96	0.74	4	1.22	0.31
2019 Initial Microbial Respiration	Treatment	100.91	50.46	2	1.63	0.20
	Date	1589.89	794.94	2	25.74	<<0.001***
	Treatment: Date	100.67	25.17	4	0.81	0.52
2018 C Enzymes	Treatment	1.23	0.65	2	1.81	0.17
	Date	8.03	4.01	2	11.16	<<0.001***
	Block	2.30	0.26	9	0.71	0.70
	Treatment: Date	1.43	0.36	4	0.997	0.42
2019 C Enzymes	Treatment	1.97	0.99	2	1.65	0.20
	Date	16.04	8.08	2	13.42	<<0.001***
	Treatment: Date	2.97	0.74	4	1.24	0.30
2018 C:N Enzymes	Treatment	0.18	0.088	2	1.86	0.16
	Date	0.88	0.44	2	9.3	0.00026***
	Treatment: Date	0.018	0.0045	4	0.095	0.98
2019 C:N Enzymes	Treatment	1.94	0.97	2	2.74	0.072
	Date	0.97	0.48	2	1.37	0.26
	Treatment: Date	2.21	0.55	4	1.56	0.19
2018 DOC	Treatment	1913	956	2	0.59	0.55
	Date	98701	49351	2	30.68	<<0.001***
	Treatment: Date	1704	426	4	0.26	0.90

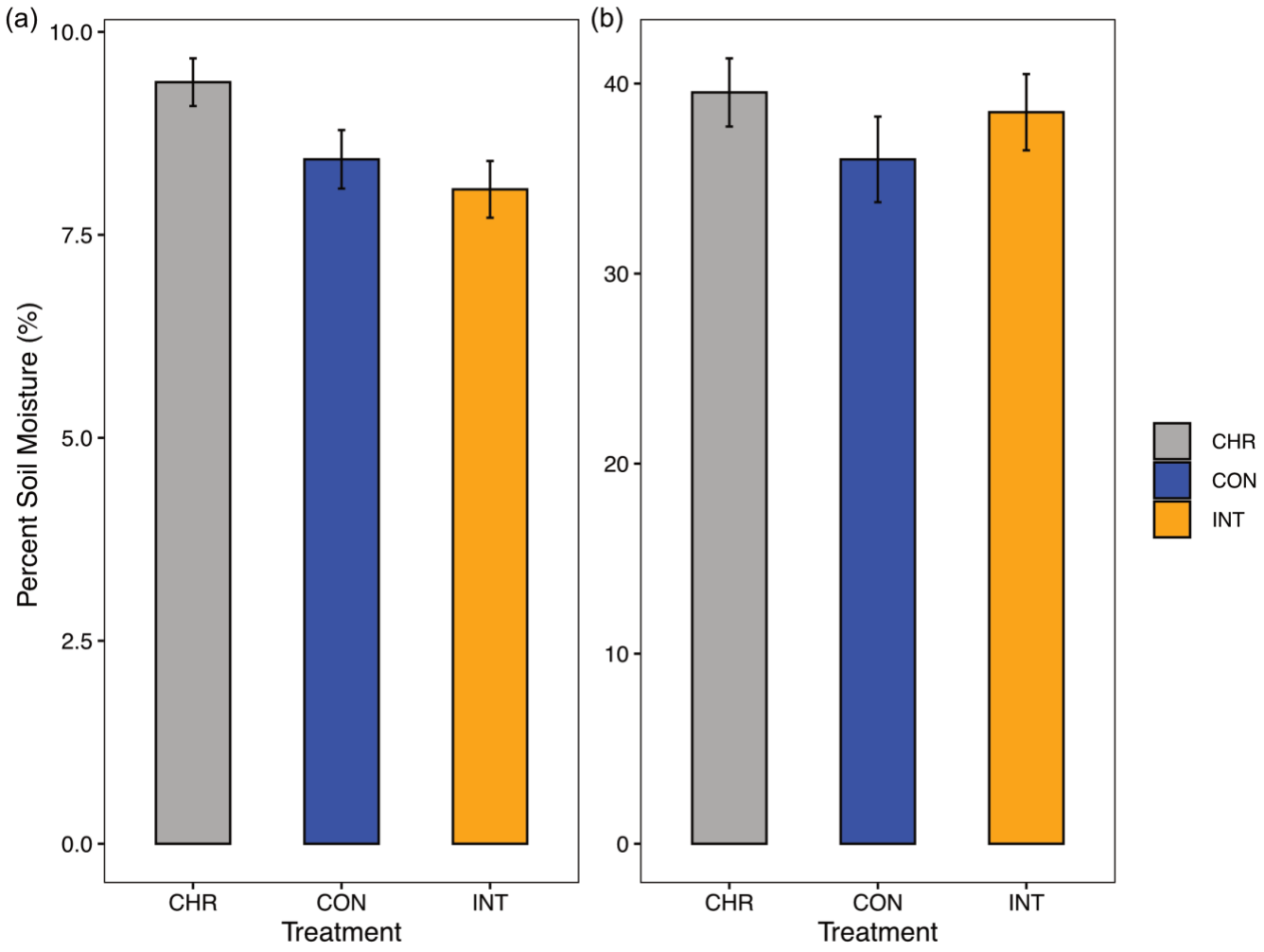
2019 DOC	Treatment	4600	2299.8	2	0.93	0.40
	Date	35405	17702.7	2	7.19	0.0014**
	Treatment: Date	8208	2052	4	0.83	0.51
2018 TOC	Treatment	2.27	1.14	2	18.40	<0.001***
	Date	0.72	0.36	2	5.80	0.0047**
	Treatment: Date	0.18	0.044	4	0.72	0.58
2019 TOC	Treatment	2.27	1.14	2	18.40	<0.001***
	Date	0.72	0.36	2	5.80	0.0047**
	Treatment: Date	0.18	0.044	4	0.72	0.58

Appendix 2 Table 3. ANOVA table of N cycling measures.

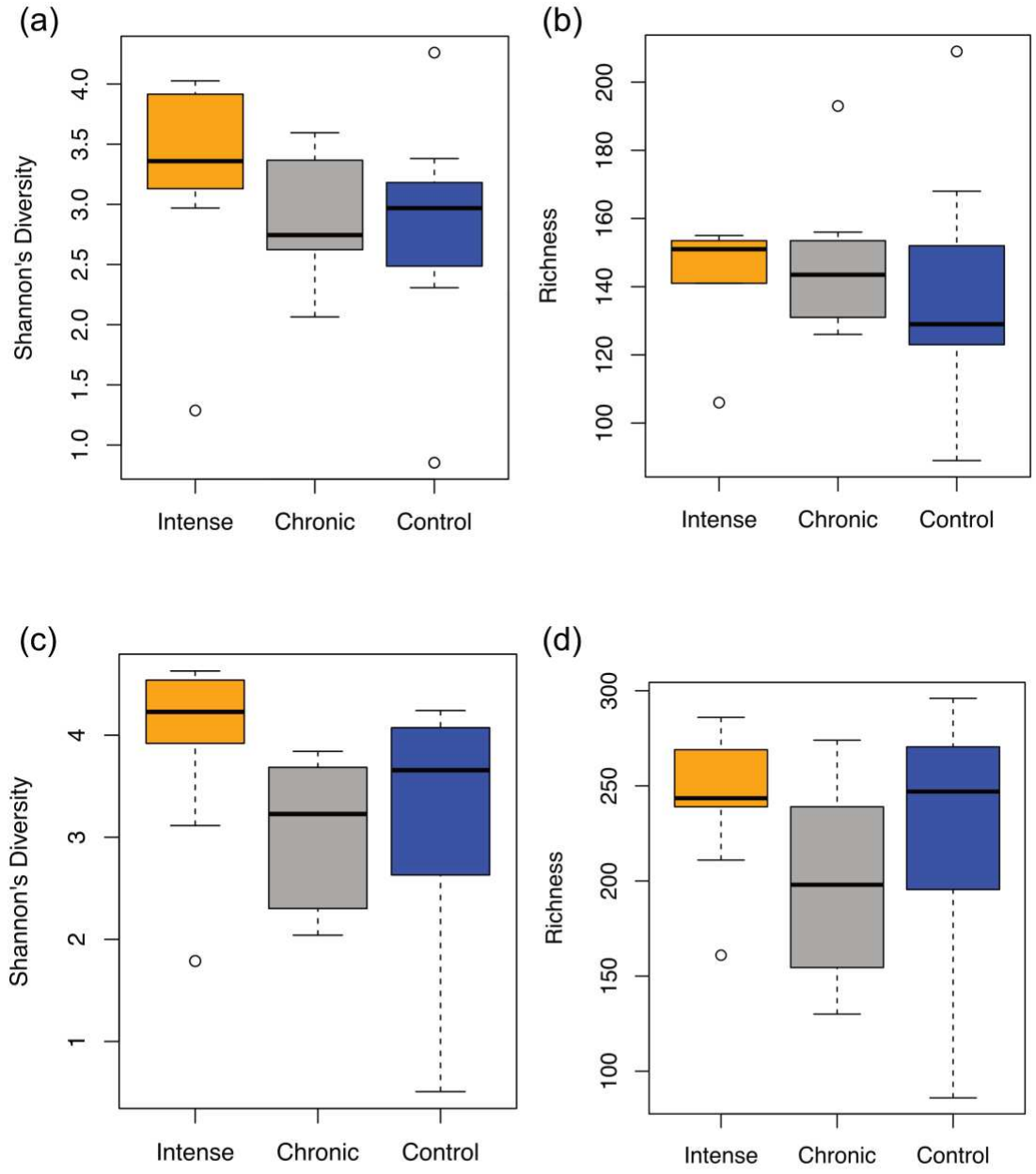
Measure	Variable	Sum Sq	Mean Sq	Num DF	F value	Pr (>F)
2018 Ammonium	Treatment	3.77	1.88	2	2.25	0.11
	Date	44.77	22.28	2	26.75	<<0.001***
	Treatment: Date	1.15	0.29	4	0.34	0.85
2018 Nitrate	Treatment	98.07	49.03	2	12.49	<0.001***
	Date	7.47	3.74	2	0.95	0.39
	Treatment: Date	3.87	0.97	4	0.25	0.91
2018 Inorganic N	Treatment	138.10	69.05	2	10.40	<0.001***
	Date	15.64	7.82	2	1.18	0.31
	Treatment: Date	5.53	1.38	4	0.21	0.93
2019 Ammonium	Treatment	84.51	42.25	2	2.07	0.13
	Date	756.73	378.36	2	18.51	<<0.001***
	Treatment: Date	77.03	19.26	4	0.94	0.45
2019 Nitrate	Treatment	4.23	2.12	2	1.83	0.17
	Date	12.98	6.49	2	5.62	0.0056
	Treatment: Date	2.97	0.74	4	0.64	0.63
2019 Inorganic N	Treatment	56.17	28.08	2	1.29	0.28
	Date	776.86	388.43	2	17.82	<<0.001***
	Treatment: Date	70.02	17.50	4	0.80	0.53
2018 N min	Treatment	0.05	0.012	2	0.89	0.41
	Date	0.19	0.097	2	7.042	0.0016**
	Treatment: Date	0.072	0.018	4	1.30	0.28
2019 N min	Treatment	0.096	0.048	2	0.77	0.47
	Date	1.94	0.97	2	15.70	<<0.001***
	Treatment: Date	0.12	0.030	4	0.48	0.75
2018 TON	Treatment	0.0080	0.0040	2	11.27	<0.001***
	Date	0.0019	0.00093	2	2.63	0.080
	Treatment: Date	0.00042	0.0001	4	0.30	0.88

2019 TON	Treatment	0.012	0.0061	2	7.78	<0.001***
	Date	0.033	0.016	2	20.97	<0.001***
	Treatment: Date	0.0016	0.00041	4	0.53	0.72
2018 N enzymes	Treatment	1.22	0.61	2	1.59	0.21
	Date	13.30	6.65	2	17.34	<<0.001***
	Treatment: Date	0.20	0.049	4	0.13	0.97
2019 N enzymes	Treatment	2.61	1.30	2	3.86	0.026*
	Date	7.65	3.83	2	11.33	<<0.001***
	Treatment: Date	1.02	0.25	4	0.75	0.56
2018 DON	Treatment	100.7	50.35	2	2.58	0.083
	Date	682.98	341.49	2	17.53	<<0.001***
	Treatment: Date	22.76	5.69	4	0.29	0.88
2019 DON	Treatment	3.34	1.67	2	0.062	0.94
	Date	292.54	146.27	2	5.40	0.0065**
	Treatment: Date	107.91	26.98	4	0.997	0.42

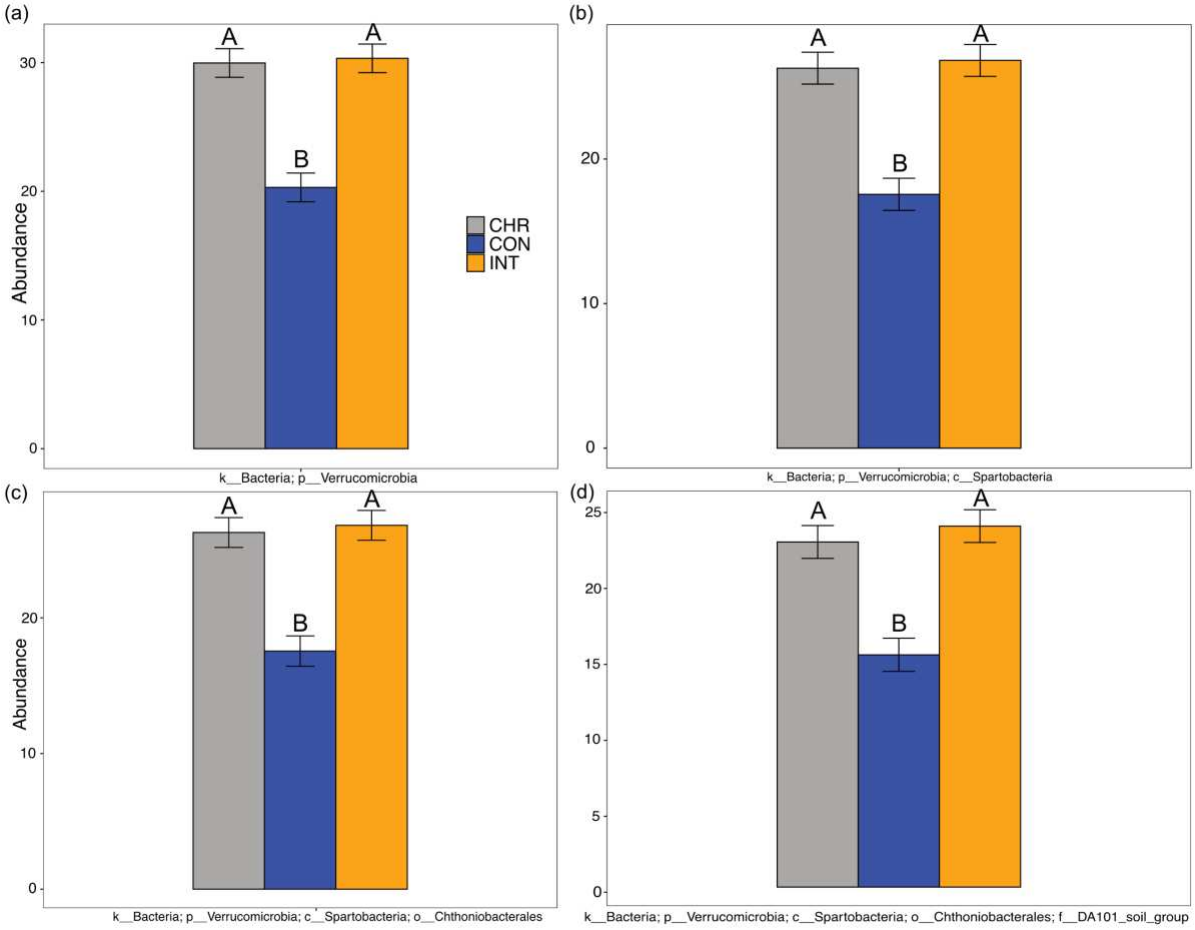
APPENDIX 3 CHAPTER 3



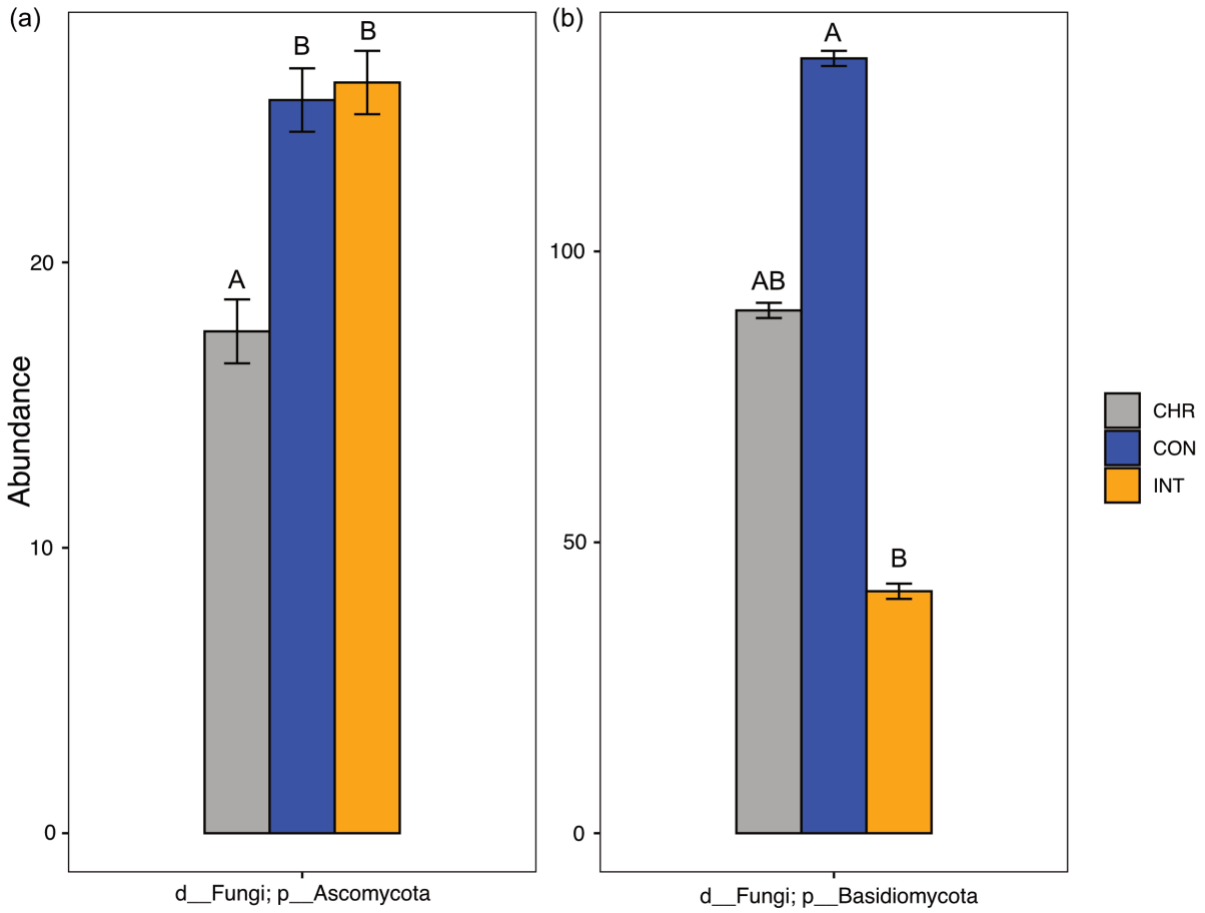
Appendix 3 Figure 1. Volumetric soil moisture (%) in 2018 (a) and 2019 (b) measured in July when the soil cores were collected for our study.



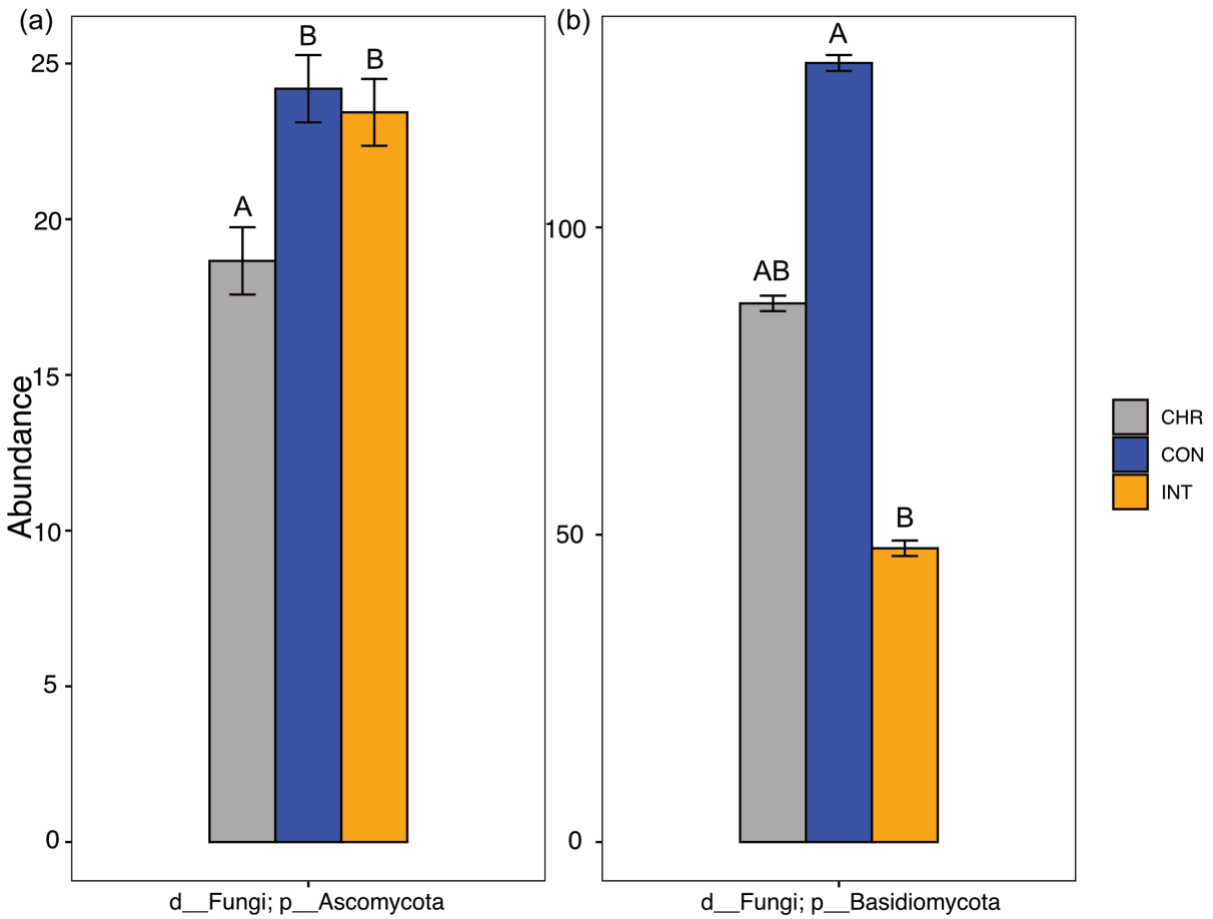
Appendix 3 Figure 2. Fungal shannon's diversity in 2018 (a) and 2019 (b). Fungal richness in 2018 is visualized in c and 2019 in d. Significance is indicated by letters and none are shown if there were no significant differences. The line within the box indicated the median and the box represents 50% of the data. Outliers are shown outside the box plot.



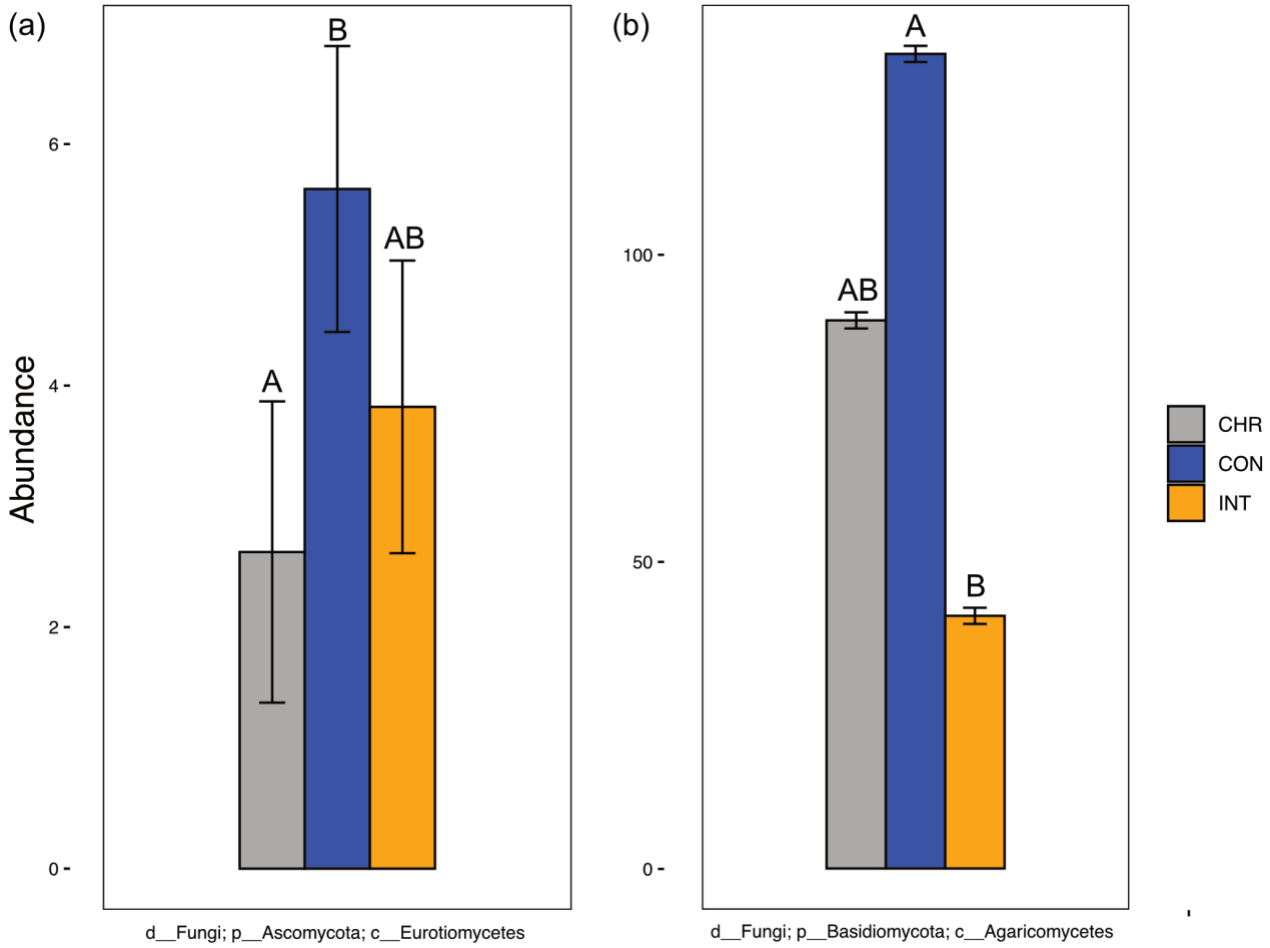
Appendix 3 Figure 3. Significantly different bacterial phyla (a), class (b), order (c), and family (d) found in 2019 with abundances shown above. Standard error bars and letters are used to represent significance.



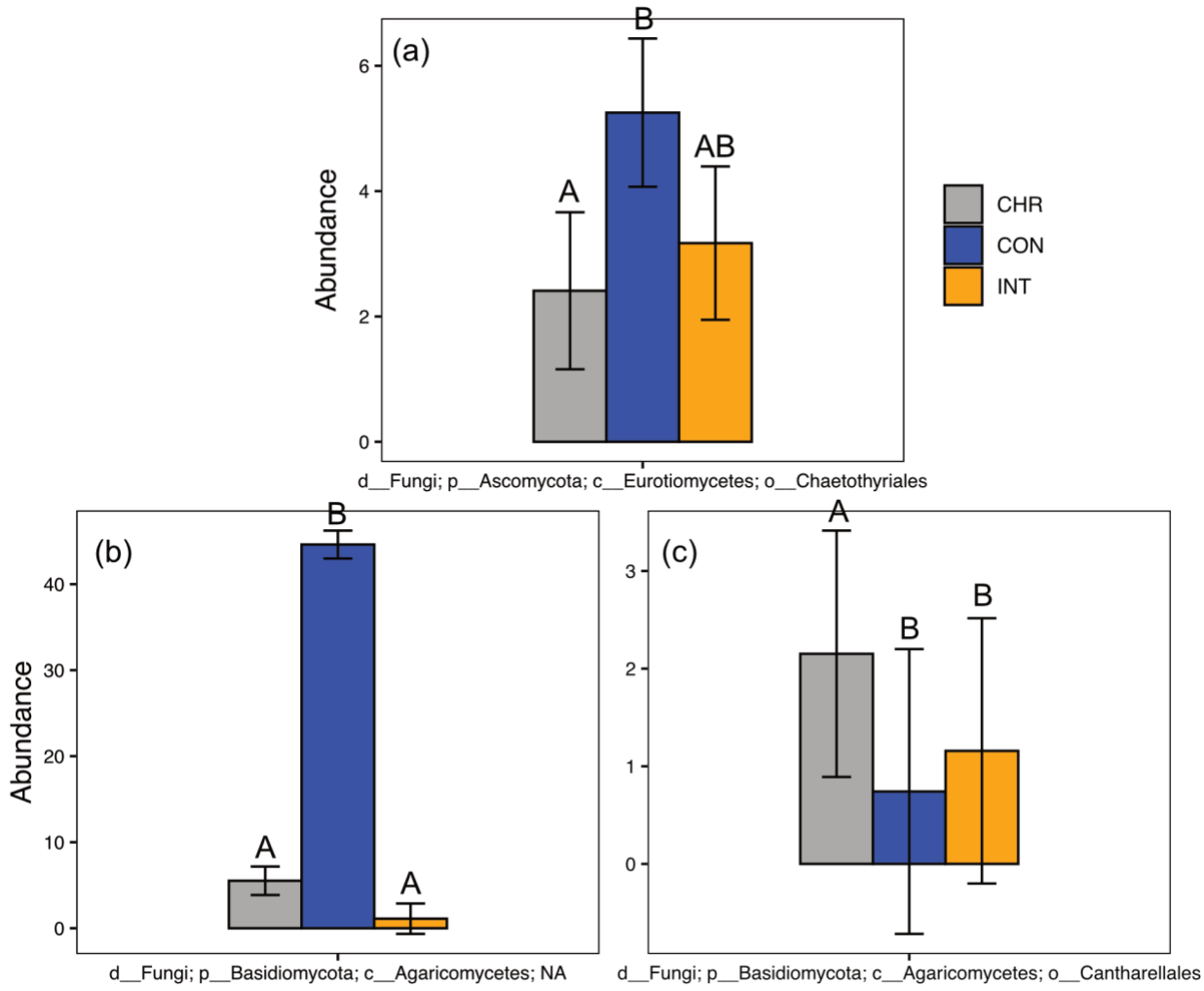
Appendix 3 Figure 4. The fungal phyla with significant differences in 2018. Ascomycota is on the left (a) and Basidiomycota is on the right (b). Standard error bars are shown and letters represent significance.



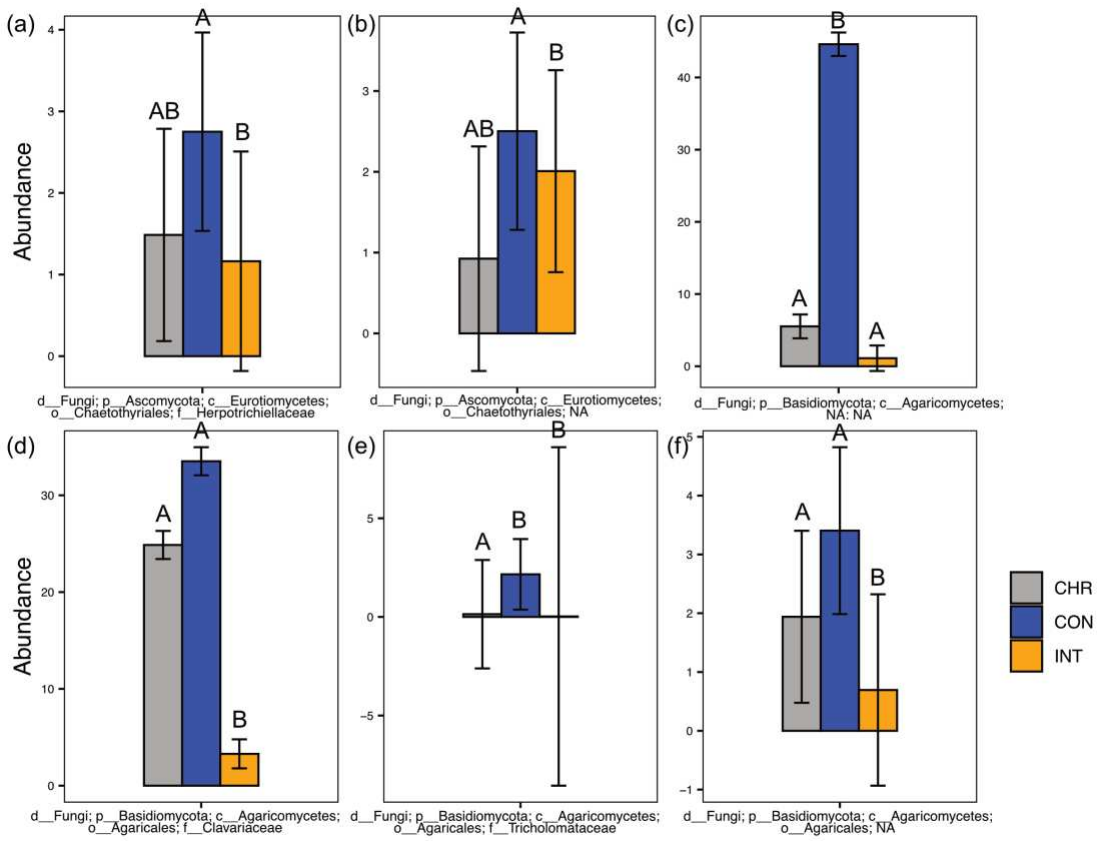
Appendix 3 Figure 5. Fungal phylum that showed statistical differences in 2019. Ascomycota is shown on the left (a) and Basidiomycota is shown on the right (b). Standard error bars are shown and letters represent significance.



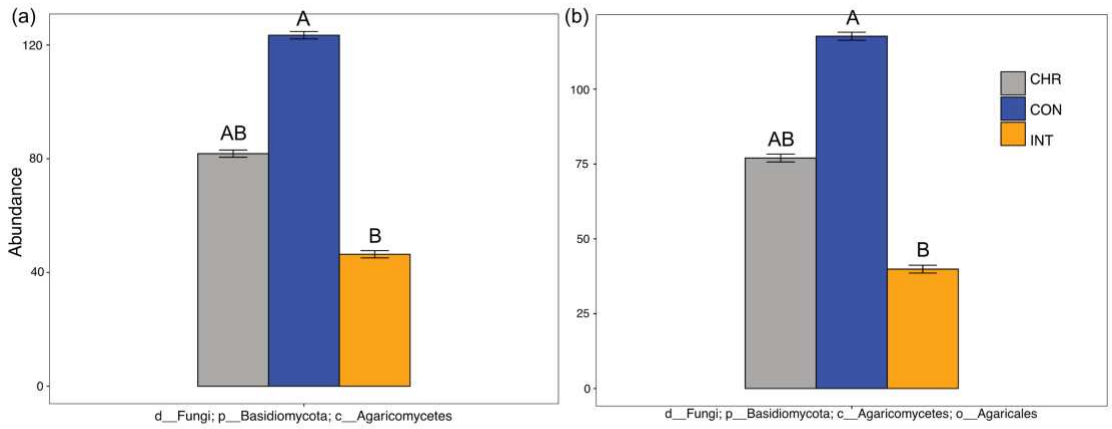
Appendix 3 Figure 6. The two significant classes for fungi in 2018. Eurotiomycetes is shown on the left (a) and Agaricomycetes is shown on the right (b). Standard error bars are shown above and letters represent significance.



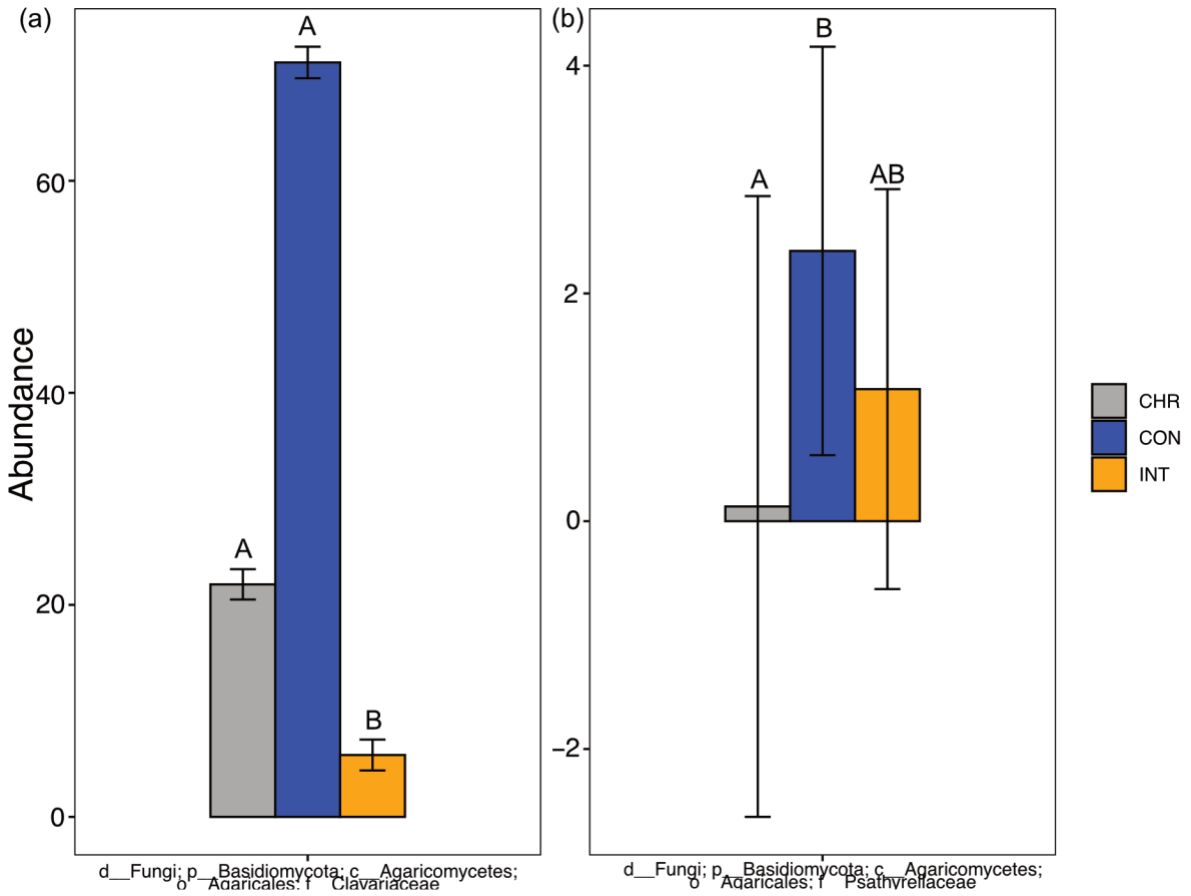
Appendix 3 Figure 7. The three significant orders for fungi in 2018. Standard error bars are shown above and letters indicate significance.



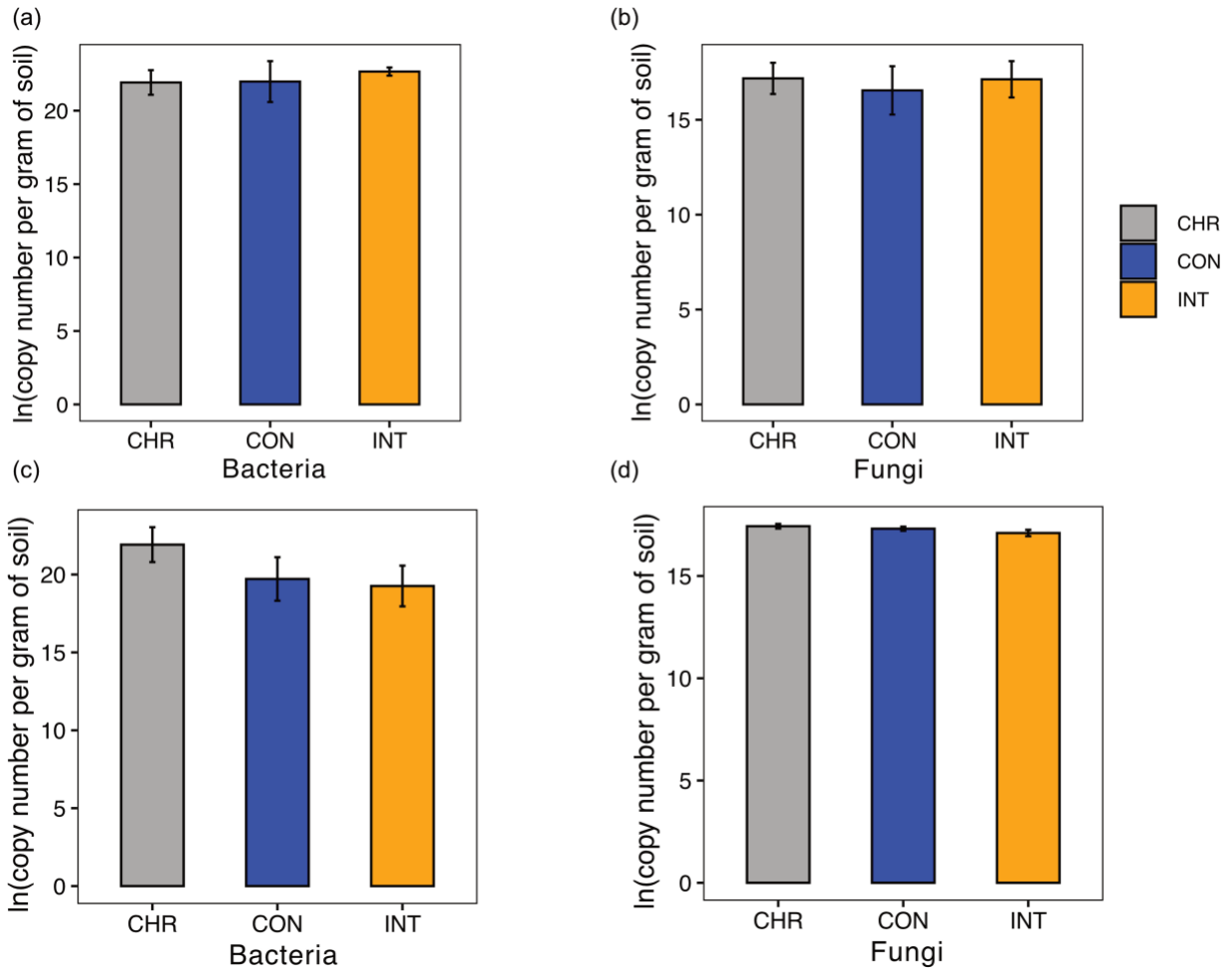
Appendix 3 Figure 8. The six significant families for fungi in 2018. Standard error bars are shown above and letters indicate significance.



Appendix 3 Figure 9. Fungal class (a) and order (b) differences in 2019. Standard error bars are shown and letters indicate significance.

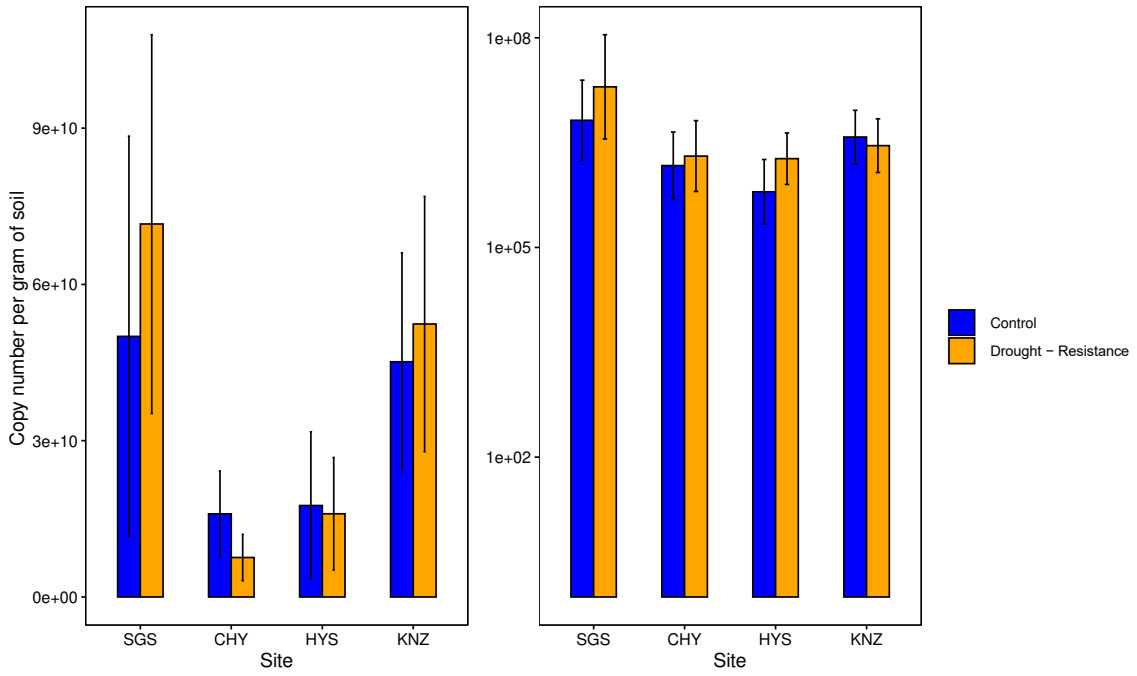


Appendix 3 Figure 10. Fungal families with significant differences. Standard error bars are shown and letters indicate significance.

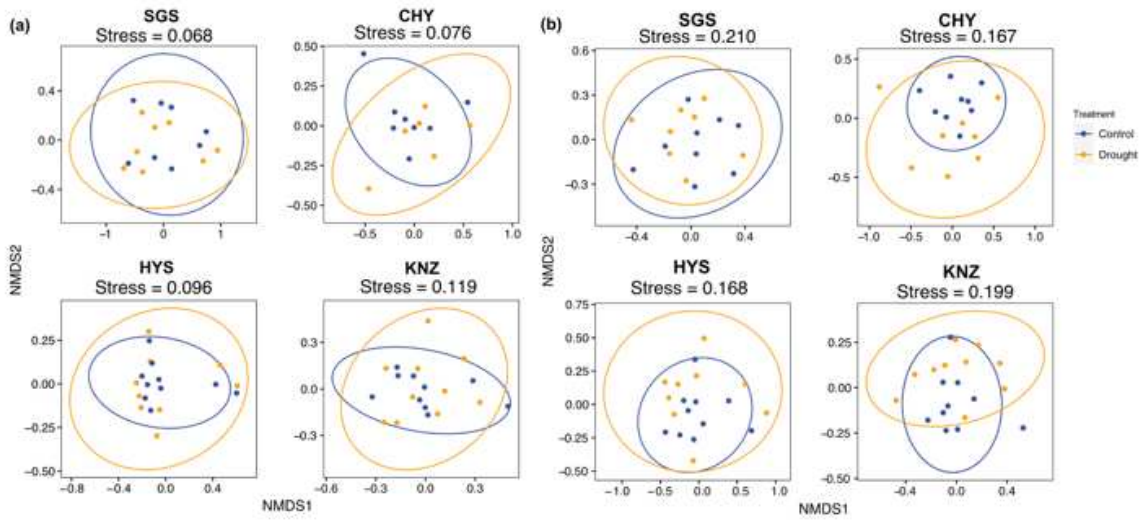


Appendix 3 Figure 11. qPCR of both bacteria (a,c) and fungi (b,d). The data is ln-transformed and standard error bars are shown. 2018 data is in the top row (a,b) and 2019 data is in the bottom row (c,d).

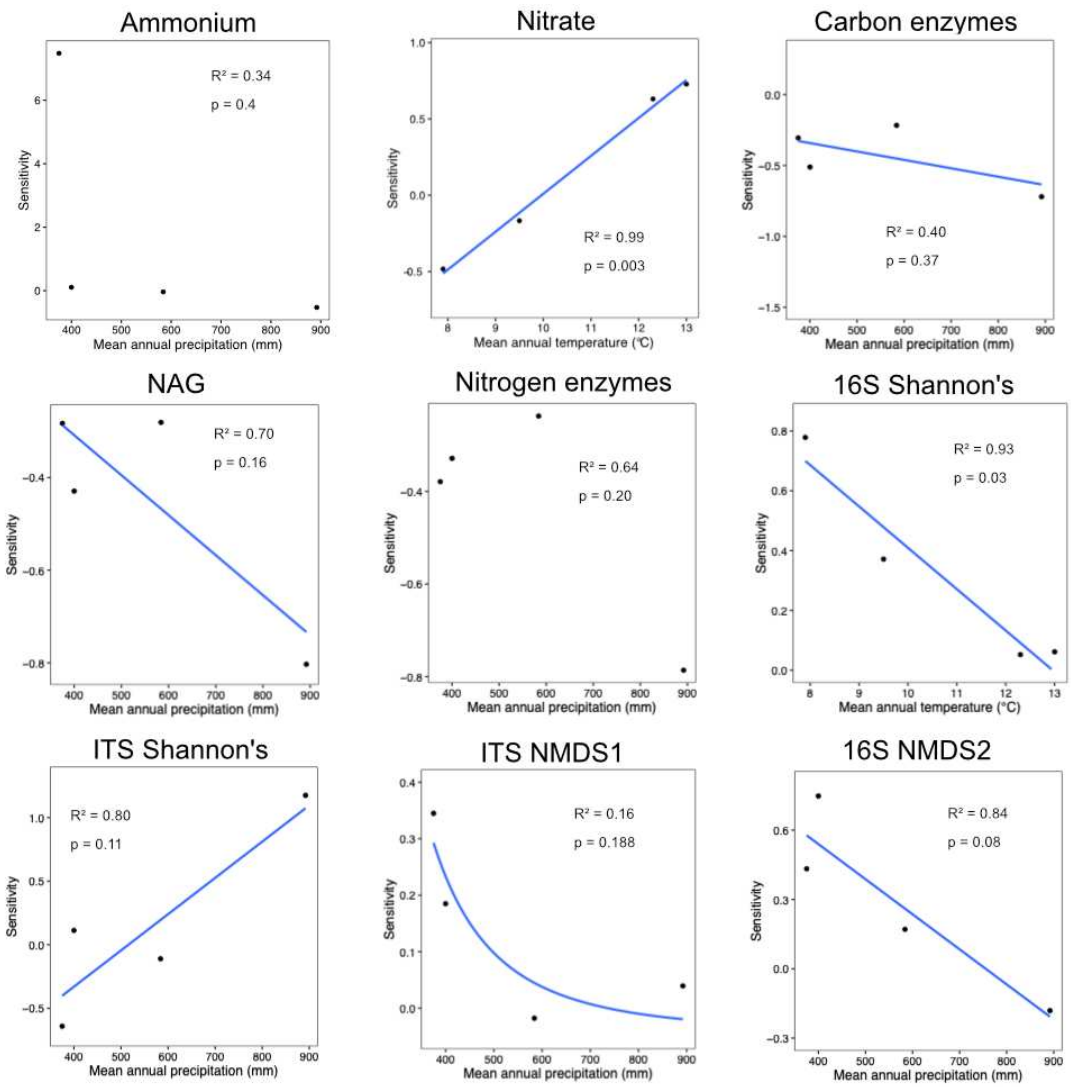
APPENDIX 4 CHAPTER 4



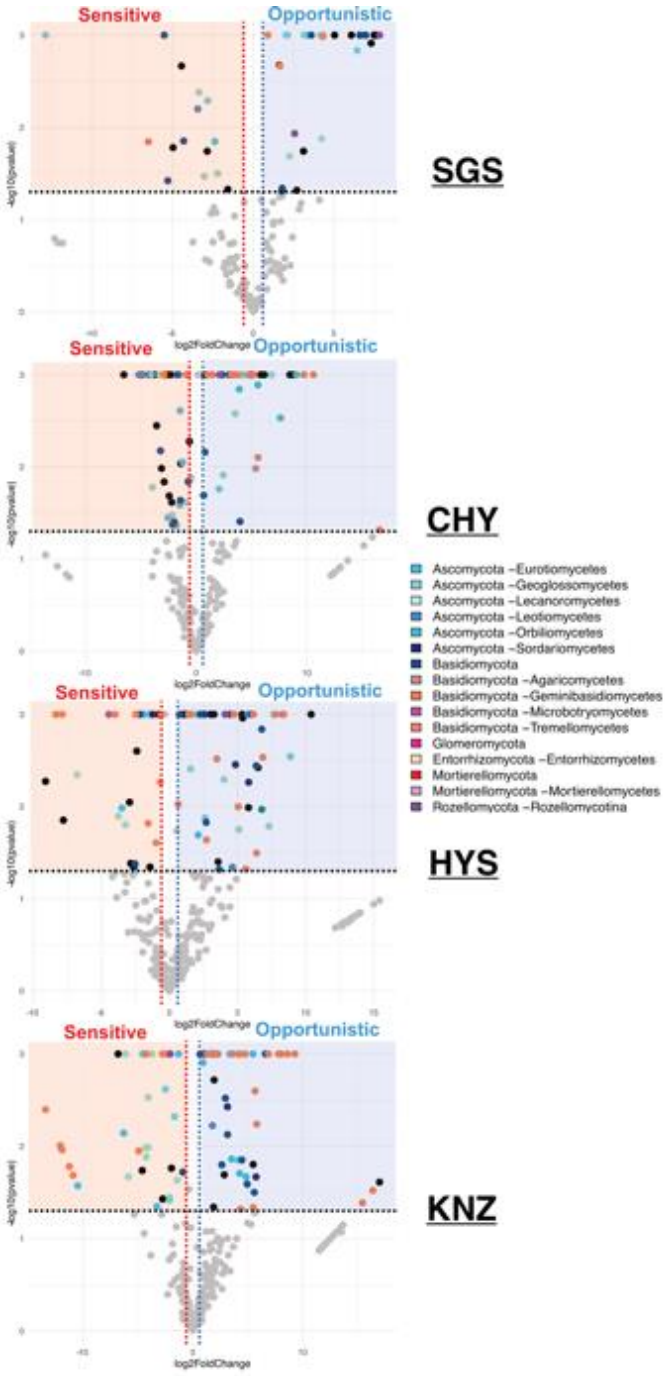
Appendix 4 Figure 1. qPCR of bacteria (a) and fungi (b).



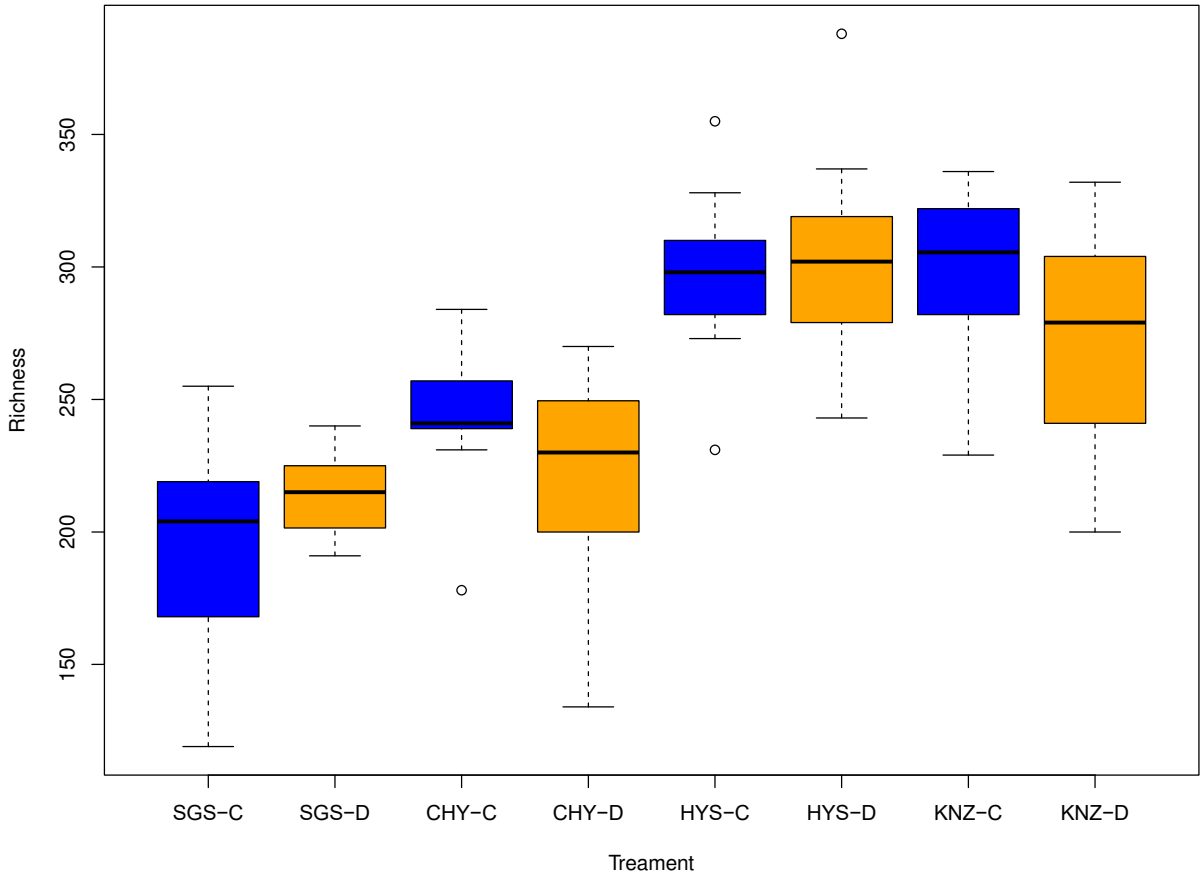
Appendix 4 Figure 2. NMDS plots for bacteria (a) and fungi (b).



Appendix 4 Figure 3. Compilation of sensitivity graphs. Sensitivity was calculated by percent change (drought – control)/control. MAP or MAT are shown on the x-axis. R^2 and p-values are shown on the graph.



Appendix 4 Figure 4. Plots of fungal OTUs indicating which are sensitive or opportunistic under drought conditions. Each OTU is colored by class. All OTUs above the black dotted line indicate OTUs that had significant p-values ($p<0.05$). p-values of less than 0.001 were changed to 0.001 for better visualization. We divided the OTU estimated abundance in the drought treatment and divided by the estimated abundance in the control treatment as the fold change from control to drought. The log2 of this fold change is shown above. OTUs that decreased in abundance under drought are highlighted in red, and OTUs that increased in abundance under drought are highlighted in blue.



Appendix 4 Figure 5. Richness of fungi for each site and treatment during the drought. Box plots are shown with median bars and outliers shown outside of the boxes.

Appendix 4 Table 1. Numbers of OTUs that were statistically sensitive or opportunistic to drought.

SITE	BACTERIA SENSITIVE	BACTERIA OPPORTUNISTIC	FUNGI SENSITIVE	FUNGI OPPORTUNISTIC
SGS	**11**	**31**	**15**	**28**
CHY	**13**	**25**	**40**	**43**
HYS	**77**	**92**	**30**	**56**
KNZ	**14**	**68**	**35**	**57**