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**Microbial Enzyme Activity as a Function of Ecosystem Type and Precipitation**

A thesis submitted in partial satisfaction of the requirements for the degree of

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with a Specialization in Hydrology and Terrestrial Ecosystems

by

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**Abstract**

A small change in the soil carbon pool can cause significant changes in our climate that can either exacerbate climate change or mitigate climate change. Therefore, it is important to evaluate feedbacks between the soil and the climate. This experiment investigates how the responses of litter microbial enzyme activity to climate change vary by ecosystem types. The study was conducted at the Loma Ridge Global Change Experiment, where the predominant vegetation types are a variant of chaparral known as coastal sage scrub (CSS) and a grassland dominated by exotic species. Precipitation was reduced by ~40% in half of the CSS and grassland plots under study. Results indicate that amounts of microbial enzymes (as indicated by Vmax) are unaffected by drought and remains constant between drought plots and ambient plots across both ecosystems. However, amounts of microbial enzymes β-glucosidase and cellobiohydrolase (both of which degrades cellulose) and N-acetyl-β-D-glucosaminidase (which mineralizes chitin, a nitrogen-containing component of fungal cell walls) are significantly higher in grassland litter than CSS due to higher cellulose proportions and lower lignin proportions in grasslands. These results indicate that microbial production of extracellular enzymes is unlikely to change due to changes in precipitation.

**Introduction**

Climate change can be viewed as humanity’s defining problem of the 21st century (UN). Due to the multi-faceted nature of the problem, tackling this issue involves multiple interdisciplinary approaches in terms of both solutions and effects of climate change. For one, the different feedbacks between climate and the rest of the Earth system needs to be considered in order to understand and project the pace at which the climate is changing (Bony et al. 2006). Multiple feedbacks are at play with some being positive feedbacks (Friedlingstein et al. 2003) while others are negative feedbacks (Matthews 2007). It is also worthwhile to compare the sizes of different reservoirs of carbon in the Earth system. As soil carbon is larger than either land plants or the atmosphere, a small change in this reservoir, depending on the direction of the change, can either greatly exacerbate or curb climate change (Gleixner 2013). Therefore, the stability of soil as a reservoir of carbon needs to be studied.

The flux of carbon from soils to the atmosphere is mediated primarily by microbes (Gleixner 2013). Historically, studies of decomposition primarily considered climatic abiotic factors – such as precipitation, temperature, evapotranspiration – and litter chemistry in studying decomposition while neglecting the role of microbial community composition and function (Adair et al. 2008; Currie et al. 2010; Meetenmeyer 1978). Only more recently, towards the beginning of the 21st century, did biogeochemists consider the role of soil microbes in carbon cycling in terrestrial ecosystems (Gleixner 2013; Schmidt et al. 2011). This bias was seen not just in empirical studies (Gleixner 2013; Schmidt et al. 2011) but also models that range from the ecosystem scale (Ťupek et al. 2019) to global scale ESMs (Hararuk et al. 2014). Even to this day, amongst the Earth system models from CMIP6, only one explicitly considers soil microbes in decomposition (Arora et al. 2020).

This study aims to fill some of the knowledge gaps regarding the role(s) of microbes in biogeochemistry and climate change. Responses of microbes based on their physiology and ecology will influence the nature of feedbacks between soils and climate (Gleixner 2013; Schmidt et al. 2011). As a result, studying the responses of microbes to the varying effects of climate change (e.g. from drought, rising temperatures, increasingly intense precipitation) is crucial in projecting future climate change.

The symptoms of climate change are varied and includes increasing drought (Haile et al. 2020), extreme precipitation (Madsen et al. 2014), and, amongst other symptoms, rising temperatures (Rapacciuolo et al. 2014). There have been empirical studies on the influence of the aforementioned changes on climate on either microbial community composition or function. For example, studies along a Mediterranean climate gradient in California indicated that microbial enzyme activity in cold alpine ecosystems will increase faster with temperature than warm semi-arid/arid ecosystems (Baker & Allison, 2017; Nisson & Allison, 2020). Specifically, Baker & Allison (2017) found that the amount of enzymes – measured by an enzyme’s Vmax – in wet seasons shows this temperature sensitivity, while only one enzyme – leucine aminopeptidase (LAP) – shows this temperature sensitivity during the dry season.

There have been a variety of empirical studies on the effects of changes in precipitation – and the interactions between precipitation and other factors such as vegetation type and nitrogen input – on soil microbes. Some results suggests that the responses of litter microbes to drought differs across vegetation type, with microbes in grasslands showing a decrease in expression of resource acquisition traits and an increase in the expression of stress tolerance traits while microbes from a chaparral ecosystem showing no significant changes in either classes of traits (Malik et al. 2020b). Other results involving transplants of litter and microbes found that leaf litter inoculated with microbes from drought environments show significantly less mass loss during the dry season of a Mediterranean climate, and that this can be attributed to the smaller microbial biomass of drought-derived microbes (Allison et al. 2013). These serve as powerful pieces of evidence of the role of soil microbes in regulating soil carbon while also showing that microbial responses to changes in precipitation – which is already being witnessed across the globe and is projected to continue with climate change – influences decomposition. However, a study in the same system as Allison et al found that while there is no significant change in litter mass loss between two time points of a dry season, there is a significant increase in the amount of enzymes in the latter time point, indicating a significant decoupling between amount of enzymes and mass loss (Alster et al. 2013). In contrast, studies in temperate woodlands and forests show that enzyme activity and mass loss are positively correlated (Sinsabaugh et al. 1993; Sinsabaugh et al. 2002). These varied characteristics of extracellular enzyme activity across ecosystems warrant further investigation.

A trait-based framework of microbial life history strategies was proposed and grouped microbes according to three strategies: (1) growth yield, (2) resource acquisition, and (3) stress tolerance (YAS; Malik et al. 2020a). There are tradeoffs between each class of traits depending on the amount of resources available as well as the stresses microbes are subjected to. For example, when in an environment with abundant resources (such as litter with high proportions of labile, soluble substrates), as the amount of environmental stress increases (e.g. as drought conditions become more frequent or severe), then microbes will divert resources from growth yield traits to stress tolerant traits. Microbes are grouped according to the class of traits they invest resources in, and this grouping can be applied at various scales, from the species or strain level (Alster et al. 2021) all the way up to the community level (Malik et al. 2020b). As this study investigates how microbial extracellular enzyme activity across different ecosystems/litter types vary under drought, this study investigates how resource acquisition traits vary depending on the amount of resources and stress. Specifically, the ecosystems are a variant of chaparral known as coastal sage scrub and a Californian Mediterranean grassland consisting of mostly exotic grasses.

Microbes decompose organic matter via the secretion of extracellular enzymes, which have been modeled by microbial ecologists using Michaelis-Menten kinetics (Wallenstein et al. 2011; see Tang & Riley 2013 for a different formulation of microbial enzyme kinetics). The Michaelis-Menten enzyme parameters are Vmax – defined as the maximum reaction velocity when the amount of substrates are abundant – and Km – Michaelis-Menten constant, defined as the ratio between the rate constants of the breakdown of the enzyme-substrate complex and the formation of the enzyme-substrate complex. Vmax, in the context of biogeochemistry and microbial ecology, is a proxy measurement of the amount of a particular enzyme where higher values indicate higher enzyme amounts (Wallenstein et al. 2011). This practical definition stems from the derivation for the Michaelis-Menten equation, where as a substrate becomes abundant and enzymes become fully saturated, reaction velocity is limited by the amount of enzymes rather than the amount of substrates (Wallenstein & Weintraub, 2008). Km, on the other hand, is used as a measure of the amount of *in situ* substrates (Wallenstein et al. 2011). This practical definition of Km stems from *in situ* substrates having been shown to be competitive inhibitors of laboratory substrates for the same enzyme such that *in situ* substrates and Km are positively correlated (Chróst 1992).

*Research questions & hypotheses*

This experiment investigates how microbial enzyme activity varies by ecosystem and precipitation. Specifically, it seeks to answer the following questions:

1. How will microbial investment of resources in enzyme production change under drought with climate change?
2. How will microbial responses to drought differ by ecosystems?

It is found that, compared to exotic grassland litter, coastal sage scrub litter has lower proportions of cellulose, hemicellulose, and nitrogen and higher proportions of lignin (Esch et al. 2019). In addition, precipitation was reduced for half of the plots to simulate drought as a stressor on litter microbes (Malik et al. 2020b). The following hypotheses were formulated in order to answer the questions above based on the amount of resources and level of stress.

H1: Litter chemistry controls enzyme activity such that microbes increase production of certain enzymes when subjected to litter with higher proportions of polymers targeted by these enzymes (Allison & Vitousek, 2005; Malik et al. 2020a) and lignin acts as a noncompetitive inhibitor of hydrolytic extracellular enzyme activity (Sewalt et al. 1997; Senior et al. 1991).

* It is expected that the Vmax of hydrolytic enzymes that degrade cellulose, hemicellulose, and organic nitrogen are higher in grassland litter than coastal sage scrub litter, while the Vmax of oxidative enzymes that degrade lignin are higher in coastal sage scrub litter.

H2: As hypothesized by the YAS framework, there are tradeoffs between resource acquisition traits and stress tolerant traits (Malik et al. 2020a; Wang & Allison in press).

* Microbes should decrease production of enzymes under the drought treatment to divert resources to stress tolerance traits. Vmax should be lower under the drought treatment than under ambient precipitation.

**Methods**

*Study site and field experiment*

The study was conducted at the Loma Ridge Global Change Experiment near Irvine, California (33°44’N, 117°42’W, 365 m elevation). The 2 ecosystem types at this site are (1) a variety of chaparral endemic to southern California and Baja California known as coastal sage scrub (hereafter, CSS) and (2) a grassland filled with exotic plants. The climate is Mediterranean, with a rainy season from November to March (which is also the growing season for plants) and a dry season for the rest of the year. The soil is remarkably coarse, with the fine earth fraction consisting of at least 80% sand across both vegetation types (Parker 2019). The site delineates plots of coastal sage scrub and grassland and manipulates precipitation to both vegetation types, with treatments for grassland plots having been in place since 2006 and treatments for CSS plots in place since 2008. The precipitation treatments are (1) plots where precipitation is reduced by being covered with clear polyethylene tarps during rainstorms, reducing mean annual precipitation by approximately 40% and simulating drought, and (2) plots with ambient, unmanipulated precipitation due to not being covered by polyethylene during rainstorms. Each treatment combination (vegetation x precipitation) has 4 replicate plots. Thus, there are a total of 16 plots involved in this experiment (2 vegetation types x 2 precipitation treatments x 4 replicate plots/treatment combination). In addition, the site was burned once over 2007. Therefore, the relevant treatments start in 2008.

The experiment took place from 2017 to 2019. Litter from all replicate plots of a treatment combination was sampled on August 30, 2017 and hand mixed while being kept separate from other treatment combinations. After hand mixing, 15 cm x 15 cm bags with 1 mm mesh were filled with 6 g of litter from a specific treatment combination. Bags were then deployed on September 12, 2017 in plots with the same treatment combination as litter inside the bags. Litter bags were then sampled in 4 time points: time point 1 litter bags were collected towards the end of November of 2017; time point 2 litter bags were collected on April 11, 2018; time point 3 bags were collected during November of 2018; and time point 4 bags were collected during February of 2019. Note the seasonality of the time points: time points 1 and 3 were at the beginning of their respective wet seasons; time point 2 was at the beginning of the dry season; and time point 4 was in the latter half of the wet season. In total, 64 bags were collected, with litter bags from the 16 plots being collected 4 times (16 plots per time point x 4 time points = 64 bags). Once collected, litter from each litter bag was grinded down in coffee grinders and then stored in freezers at -80°C for later laboratory analysis.

*Extracellular enzyme assays*

Extracellular enzyme assays were conducted as a way to measure the microbial resource acquisition trait of enzyme production (Malik et al. 2020a). The hydrolytic enzymes that were assayed are α-glucosidase (AG), (acid) phosphatase (AP), β-glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CBH), leucine aminopeptidase (LAP), and N-acetyl-β-D-glucosaminidase (NAG). The oxidative enzymes that were assayed are polyphenol oxidase (PPO) and peroxidase. The enzymes and the substrates they degrade are listed in Table 1.

Hydrolytic enzymes were assayed with fluorimetric methods described in Baker & Allison (2017) and German et al (2011). A homogenate suspension was made for each litter bag with ratios of 0.4 g litter per 150 mL of buffer, with the buffer solution being 25 mM maleate with pH 6. Homogenates were homogenized in 50 mL test tubes using a tissue tearor for four 30-second intervals, with at least 30 seconds of rest in between each interval. After homogenizing, a test tube and the tissue tearor is cleaned with more buffer until the homogenate is 150 mL in volume. In addition, serial dilutions were made of fluorogenic substrates that are either bonded with 4-methylumbilleffirone (MUB) – which assays for AG, AP, BG, BX, CBH, and NAG – or 7-amino-4-methylcoumarin (AMC) – which assays for LAP. Solutions of 62.5 µM AMC or 25 µM MUB were used as standards and were plated with homogenates and substrates in 96-well microplates, and microplates were left to incubate for 4 hours at room temperature in the dark before having their fluorescence read in a microplate reader. Each homogenate has three sets of controls: (1) a homogenate control in which only the inherent fluorescence of the homogenate is recorded; (2) a quench control in which the degree to which a homogenate decreases the fluorescence of either AMC or MUB is read; (3) and a substrate control in which the inherent fluorescence of a substrate is recorded (German et al. 2011). Each well has a volume of 250 µL, with 125 µL comprising of either the homogenate or plain buffer and the remaining 125 µL comprising of either the substrate, a standard solution, or water. Raw fluorescence values were then converted into enzyme activity using formulas described in German et al (2011) and enzyme activity was normalized by litter dry mass.

Oxidative enzymes were assayed with colorimetric methods as described in German et al (2011) and sharing many of the same steps as fluorimetric assays of hydrolytic enzymes described above. In this case, both PPO and peroxidase used pyrogallol as the substrate, while peroxidase also uses H2O2 as a second substrate (Bach et al. 2013). Pyrogallol serial dilutions were made from a stock solution of 1 mg pyrogallol per 7.9 mL of water. Homogenates were vacuum filtered, and the subsequent filtrates were plated with pyrogallol to assay for PPO or both pyrogallol and H2O2 to assay for total oxidase activity. Two sets of controls were used: (1) a homogenate control in which only the inherent absorbance of the filtrate is read and (2) a substrate control in which the inherent absorbance of pyrogallol or pyrogallol and H2O2 were read. Calculations of PPO and total oxidase activity were calculated using equations from German et al (2011), and peroxidase activity was calculated by subtracting PPO activity from total oxidase activity. Oxidase activities were also normalized by litter dry mass. However, because peroxidase activity is consistently negative, it is assumed that peroxidase activity is negligible in these two ecosystems and so peroxidase is not analyzed.

*Data analysis*

All analysis and calculations were conducted on Python. The activity of each enzyme from each litter bag is plotted against their respective substrate concentrations and judged visually for substrate inhibition or negative activity, with negative activity data points being set to 0. While Michaelis-Menten kinetics predict that reaction velocity reaches a maximum value at infinite substrate concentrations, the phenomenon of substrate inhibition is common in many enzymes (Reed et al. 2010; Steen & Ziervogal, 2012). As substrate concentrations increase, reaction velocity reaches a maximum level before decreasing again, contrary to Michaelis-Menten kinetics. This can lead to underestimates of Vmax, indicating lower enzyme amounts than there might actually be (Steen & Ziervogal, 2012). As a result, data points in which substrate inhibition is observed are removed. The final enzyme activity is then fitted against the Michaelis-Menten equation using the *curve\_fit()* function from the *optimization* module of the *scipy* package to obtain Michaelis-Menten parameters. Normality of these parameters were checked using the Shapiro-Wilk test, which was conducted using the *stats* module from *scipy*. Michaelis-Menten parameters were then log10 transformed to improve normality. While some of the data still shows a lack of normality under the Shapiro-Wilk test, the transformation overall greatly improved normality.

Further data analysis was then conducted with the transformed data were conducted using a factorial multivariate analysis of variance (MANOVA), factorial analyses of variance (ANOVAs), and followed by Tukey’s Honest Significant Difference (HSD) test as post-hoc tests of significant interactions from ANOVAs or significant main effects that are not part of significant interactions from ANOVAs. These subsequent forms of statistical analyses were conducted using the *statsmodels* package. Given that there were 8 enzymes under analysis (peroxidase was not analyzed due to consistently negative activity) and the Michaelis-Menten equation has 2 parameters, there were a total of 16 dependent variables under analysis.

A factorial MANOVA was ran as a form of exploratory data analysis with time points, vegetation, and precipitation treatments as “between-subjects” factors (i.e. independent variables) and with the dependent variables being the Vmax and Km of all enzymes. Afterwards, factorial ANOVAs were ran on each dependent variable using the same factors. Type III factorial ANOVAs were ran repeatedly on each dependent variable, with nonsignificant interactions removed after each run. When there are no significant interactions associated with a dependent variable, type II factorial ANOVAs were ran repeatedly on main effects with nonsignificant main effects removed after each iteration.

Tukey’s HSD were then conducted as post-hoc tests on dependent variables with significant interactions and significant main effects that were not part of significant interactions from ANOVAs. Interestingly, some significant interactions/main effects predicted by ANOVAs were then shown to be non-significant under Tukey post-hoc testing.

**Results**

Of particular note is the small influence that precipitation has on either Vmax or Km. MANOVA results, on the surface, indicate that precipitation is significant at α = 0.05 in three-way interactions and also as part of an interaction with vegetation. Diving deeper into ANOVAs and subsequent Tukey post-hoc comparisons reveal that the effect of precipitation on either Vmax or Km are all relatively minor, either as a main effect alone or as part of an interaction with either time, vegetation, or both. For example, precipitation only influenced Vmax as part of a three-way interaction for PPO and does not have an effect on any other enzyme’s Vmax either as part of an interaction with vegetation or time or either as a main effect (Table 2). In contrast, the effect of precipitation on Km is somewhat larger (Table 3); its interaction with vegetation is significant for AP, and its interaction with time is significant for CBH. As a main effect, precipitation only influences the Km of AP. Overall, these results show that enzyme amounts remain constant irregardless of precipitation, and so H2 is not supported.

In contrast, vegetation has more influence on V­max and Km. On Km, vegetation is part of a significant interaction with precipitation for AP, and its interaction with time is significant for AP, CBH, NAG, and PPO (Table 3). Its influence on Vmax is significant as an interaction with time on CBH and NAG and as a three-way interaction on PPO, while its influence as a main effect is significant for BG (Table 2). On closer inspection, the Vmax of cellulose-degrading enzymes (BG & CBH) and of the chitin-degrading enzyme NAG are higher in grassland litter than CSS litter (Figures 2, 3a, 4). These results would seem to validate H1, indicating that lower lignin proportions result in less “disabling” of cellulose-degrading enzymes and NAG and that higher proportions of cellulose cause higher microbial investment in cellulose degradation. However, H1 also predicts that amounts of the hemicellulose-degrading enzyme BX and LAP should be higher in grassland litter, which has higher hemicellulose and nitrogen content than CSS. In contrast to this hypothesis, enzyme amounts of BX and LAP are not significantly different between both ecosystems, with vegetation either as a main effect or as part of an interaction with time and/or precipitation (Table 2). Likewise, PPO results do not necessarily follow this hypothesis. According to this hypothesis, amounts of the enzyme PPO should be higher in CSS where there are higher lignin proportions. However, amounts of this enzyme tend to be higher – although not significantly – in grassland despite lower lignin proportions in grassland (Figure 6).

**Discussion**

Higher amounts of cellulose-degrading enzymes (BG & CBH) in grassland litter (Figures 2, 3a) validates H1 and seem to indicate that microbes primarily use cellulose as a carbon and energy source rather than hemicellulose or lignin. In contrast to H1, the increase in hemicellulose in grassland litter does not cause a significant change in production of BX (Table 2), indicating that this increase in hemicellulose does not incentivize microbes in acquiring more carbon or energy from hemicellulose. However, H1 is also validated due to results of the enzyme NAG. Production of NAG is notably higher in grassland plots across all time points than CSS plots (Figure 4), and grassland litter tends to have higher nitrogen content than CSS litter.

In addition, these results also validate the mechanism of lignin “disabling” hydrolytic enzymes as a noncompetitive inhibitor, as proposed by H1. Indeed, BG, CBH, and NAG have lower enzyme amounts in coastal sage scrub litter than grassland litter (Figures 2, 3a, 4), and CSS litter has higher lignin proportions than grassland litter. While many studies have found that lignin is negatively correlated with decomposition rates (Adair et al. 2008; Bontti et al. 2009; Cornwell et al. 2008), it is still relatively unknown *how* lignin might cause decreases in decomposition rates in the field of biogeochemistry. This study provides data that supports a mechanism to explain this link: that lignin acts as a noncompetitive inhibitor and removes enzymes from organic matter degradation. While this mechanism had been supported in previous laboratory studies (Sewalt et al. 1997; Senior et al. 1991), this study provides some of the first field results to support this mechanism.

Potential activities of LAP and NAG have been used to indicate the amount of resources devoted to nitrogen mineralization (Sinsabaugh et al. 2008). While amounts of LAP does not significantly differ between both ecosystems despite higher nitrogen proportions in grassland litter (Table 2), higher amounts of NAG in grassland litter than CSS (Figure 4) indicates that overall nitrogen mineralization rates are higher in grassland litter than CSS. This might provide grassland litter microbes enough nitrogen to fuel higher production of cellulose-degrading enzymes in grassland litter. These results reflect previous findings that found that BG activity increases only when soils are amended with nitrogen and phosphorus (Allison & Vitousek, 2005). In addition, these results also reflect a more recent study conducted in grassland vegetation at the same study site, which found that amounts of NAG is positively correlated with amounts of BG and CBH (Alster et al. 2021).

In addition to differences in litter chemistry and nitrogen mineralization explaining differences in BG, CBH, and NAG enzyme amounts between both ecosystems, there is a third explanation. LAP Km is significantly higher in CSS vegetation than grassland (Figure 5) despite vegetation not significantly altering LAP Vmax. This indicates that *in situ* LAP activity, rather than the amount of LAP enzymes, might be higher in CSS litter, resulting in more LAP degradation products in CSS litter. As a result, BG, CBH, and NAG enzymes in CSS litter might be more decomposed, resulting in lower enzyme amounts than grassland litter. These results mirror that of Baker & Allison (2017), which found that protein proportions in pine-oak and alpine ecosystems are low while LAP Km in these ecosystems are high. Altogether, this explanation, along with H1 and differences in nitrogen mineralization rates from NAG, explains amounts of BG, CBH, and NAG in grassland litter. In addition, these results do not support H1, as vegetation does not have the same effect on the amount of LAP enzyme (Table 2).

The higher rate of nitrogen mineralization by grassland litter microbes (via NAG) and the higher rate of carbon acquisition from cellulose (via BG and CBH) in grassland litter microbes might have led to slightly higher production of oxidative enzymes that degrade lignin in grassland liter microbes despite lower lignin proportions in grassland litter than CSS litter (Figure 6). Thus, with increasing drought due to climate change (Rapacciuolo et al. 2014), the “recalcitrance” of lignin in Mediterranean grasslands might decrease, potentially leading to faster decomposition in Mediterranean grasslands as lignin content decreases (Cornwell et al. 2008). However, note that the difference in production of PPO between CSS and grassland is, for the most part, insignificant by Tukey comparisons (Figure 6), and so this conclusion should be tempered.

In addition, time has relatively little effect on enzyme amounts of all enzymes. Time does not influence the amount of enzymes AG, BG, BX, and LAP (Table 2). In addition, amounts of the enzymes CBH and NAG in each ecosystem are relatively constant over time in both ecosystems (Figures 3a, 4). Time also does not have a clear effect on PPO amounts (Figure 6). While time does significantly affect AP (Table 2), there is no significant difference between the first three time points (Figure 1a), indicating that investment in phosphorus mineralization does not differ between the beginning or end of the dry season. While there is a significant difference between the 2nd and 4th time points (Figure 1a), which represent the beginning of the dry season and the latter half of the wet season, respectively, there are not enough time points to conclude a seasonality effect on resource investment in phosphorus mineralization. Overall, these results show that amounts of extracellular enzymes remain constant with time at least over the initial ~1.5 years of decomposition, regardless of seasonality. Investment of resource acquisition traits (either for carbon, phosphorus, or nitrogen) remain constant regardless of season.

Similar to seasonality, precipitation does not influence enzyme amounts of all enzymes across all ecosystems except for a three-way interaction with PPO. Precipitation still has some significance on product concentrations of some enzymes, such as an interaction with vegetation for AP and an interaction with time for CBH (Table 2). However, when CBH Km is visualized as a function of time and precipitation, the effect of precipitation becomes even more unclear (Figure 3b). On the other hand, when AP Km is visualized as a function of vegetation and precipitation, ambient CSS plots have higher Km than the other 3 combinations (drought CSS and both grassland precipitation combinations) (Figure 1b). Regardless, effects of precipitation on AP *in situ* substrates and phosphorus mineralization are unclear. Still, it is clear that *investment* of resources in phosphorus mineralization is unaffected by drought (Table 2).

The lack of an effect of precipitation on enzyme amounts (Table 2) and *in situ* substrates (Table 3) indicates that enzyme production in both ecosystems is robust to precipitation changes. Thus, enzyme production in semi-arid ecosystems might be constitutive. These results reflect some of the findings from recent studies conducted across a California climate gradient (Baker & Allison, 2017; Nisson & Allison, 2020) that found that enzymes from colder-and-wetter ecosystems tend to be more responsive to environmental change via temperature increases than warm, semi-arid ecosystems.

It is also worthwhile to compare this study to an earlier reciprocal transplant study conducted in the grassland ecosystem at the same site (Alster et al. 2013). Alster et al (2013) found that while enzyme amounts are significantly higher in drought plots than ambient plots, enzyme amounts are significantly lower in *litter* that originated from drought environments than control environments. They suggested that, over the relatively short amount of time of the study, drought environments have lower diffusion rates which cause microbes to temporarily produce more enzymes to obtain more resources and, thus, explaining the increase in enzyme amounts under drought environments (Alster et al. 2013). In contrast, litter that originated from drought environments have reduced proportions of cellulose and hemicellulose – thus explaining lower enzyme amounts in litter that originated from drought environments (Alster et al. 2013). These two competing mechanisms could be occurring in the drought treatment in this study. Instead of exerting osmotic stress, drought can reduce access to resources due to more limited diffusion, causing microbes to produce more enzymes to compensate (Malik et al. 2020a; Alster et al. 2013). However, drought also changes leaf chemical composition and, thus, litter chemistry by reducing proportions of cellulose, hemicellulose, or other polymers in litter (Allison et al. 2013), exerting an opposite force that reduces production of extracellular enzymes (Alster et al. 2013). These two processes can cancel each other, resulting in the lack of an effect of precipitation on enzyme amounts as observed in this study (Table 2).

*YAS framework implications*

The YAS framework predicts that microbes will invest more resources in resource acquisition traits in environments with limited resources (Malik et al. 2020a), such as environments with high concentrations of complex polymers (Allison & Vitousek, 2005). Our results validate this prediction from the framework, as amounts of cellulose-degrading enzymes and the nitrogen-cycling enzyme NAG increases in grassland litter, where there are more complex polymers such as cellulose and organic nitrogen.

However, our results show that investment in resource acquisition traits do not change even under drought, suggesting that there are few or no tradeoffs between resource acquisition traits and traits that tolerate environmental stressors such as drought. These results also reflect results from a more recent study (Alster et al. 2021). Specifically, Alster et al (2021) evaluates the entire YAS framework while this study only evaluates resource acquisition traits. While Alster et al (2021) found that individual fungal strains do not exhibit the tradeoffs postulated by the framework (Malik et al. 2020a; Wang & Allison in press), it should be noted that the study is only applied to fungal strains, not whole microbial communities.

This study should also be taken into context with another recent study at the same site (Malik et al. 2020b). Malik et al (2020b) found that in grassland litter, expression of stress tolerance traits tends to be negatively correlated with expression of growth traits, validating the tradeoff between stress tolerance and growth in the YAS framework. However, this study found that expression of resource acquisition traits does not change under stress, indicating that there is unlikely to be a tradeoff between resource acquisition and stress tolerance, unlike what was posited by the framework. Similar to Malik et al (2020b), this study validates some predictions by the framework. However, it is also similar to Alster et al (2021) for not supporting other predictions by the framework, namely tradeoffs between different classes of traits.

**Conclusion**

These results show that, at least for the initial ~1.5 years of litter drop, microbial production of extracellular enzymes remain constant regardless of season or precipitation. While these results indicate that production of extracellular enzymes might be constitutive, drought can exert opposing mechanisms that maintain constant production of extracellular enzymes (Alster et al., 2013). Vegetation significantly influences amounts of extracellular enzymes, with litter with high cellulose proportions and low lignin proportions resulting in higher microbial investment in enzymes that decompose cellulose and chitin. Thus, these results indicate that vegetation has a more significant influence on microbial resource acquisition than seasonality or precipitation in semiarid ecosystems, and that environmental change via drought has a relatively small effect on enzyme pools. These results reinforce previous studies that show that microbial enzyme amounts in semiarid ecosystems are robust to environmental change (Baker & Allison, 2017; Nisson & Allison, 2020). This can constrain enzyme pools in microbial-explicit models, which predict that enzyme pools are proportional to microbial biomass pools (Li et al. 2014) and so easily change as microbial biomass changes. In contrast, the results of this study show that enzyme pools, at least in semiarid ecosystems, are relatively robust to environmental changes, and the relatively rapid response of enzyme pools to environmental change predicted by microbial-explicit models (Li et al. 2014) might not accurately describe enzyme pools in semi-arid ecosystems. However, these results should not be taken to mean that decomposition rates will be constant regardless of precipitation change. This study only looks at amounts of enzymes, not *in situ* enzyme activity which is more representative of actual decomposition rates. Furthermore, while decomposition rates and enzyme activity are coupled in temperate forests (Sinsabaugh et al., 1993; Sinsabaugh et al., 2002) and are predicted by microbial-explicit models to be coupled (Li et al. 2014), decomposition rates and enzyme amounts at least in Mediterranean grasslands have been shown to not be coupled (Alster et al., 2013), indicating that decomposition dynamics in semi-arid ecosystems are still poorly understood.

**Figures and tables**

***Figure 1.*** AP enzyme amounts (a) and Km (b). Letters above each boxplot are Tukey labels where boxes with the same labels are similar to each other. Note that Tukey labels only apply to groups within the same subplot, not between subplots. The x-axis in (a) are labeled with time points. Treatment combinations on the x-axis in (b) are written in the order of (vegetation, precipitation) where precipitation treatments are described by their initials (“A” = “Ambient”, “D” = “Drought”).

Chart, box and whisker chart

Description automatically generated

(a)

Chart, box and whisker chart

Description automatically generated

(b)

***Figure 2.*** BG enzyme amount (Vmax) as a function of vegetation. Letters above each box plot represents Tukey labels where groups that share the same letter are similar to each other by Tukey post-hoc comparisons.

Chart, box and whisker chart

Description automatically generated

***Figure 3.*** CBH enzyme amounts (a) and Km (b) over time. Letters above each boxplot represents Tukey labels where boxplots that share the same letter within the same subplot are similar to each other. Note that Tukey labels only describe similarities/differences in the same plot. Treatment combinations on the x-axis in (a) are written in the order of (time, vegetation) while treatment combinations on the x-axis in (b) are written in the order of (time, precipitation) where precipitation treatments are described by their initial (“A” = “Ambient”, “D” = “Drought”).

Chart, box and whisker chart

Description automatically generated

(a)

Diagram

Description automatically generated

(b)

***Figure 4.*** NAG enzyme amounts as a function of time and vegetation. Letters above each boxplot are Tukey labels where boxplots that share the same letter(s) are similar to each other. Treatment combinations are written in the order of (time, vegetation).Chart, box and whisker chart

Description automatically generated

***Chart, box and whisker chart

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***Figure 5.*** LAP Km as a function of vegetation. The letter above each boxplot represents Tukey labels where boxplots with the same letter are similar to each other by Tukey HSD’s test.

Chart, box and whisker chart

Description automatically generated

***Figure 6.*** PPO enzyme amount (Vmax) as a function of time, vegetation, and precipitation. The hatch type represents the precipitation treatment while color represents vegetation type. Letters above each box plot represents Tukey labels where groups with the same labels are similar to each other by Tukey HSD’s test. Treatment combinations on the x-axis are written in the order of (time, vegetation, precipitation) with time being written in ascending order from left to right and precipitation treatments are described by their initial (“A” = “Ambient”, “D” = “Drought”).

Chart, box and whisker chart

Description automatically generated

***Table 1.*** Enzymes under analysis and the substrates they degrade. Based on Romaro-Olivares et al (2017) and German et al (2011).

|  |  |  |  |
| --- | --- | --- | --- |
| **Enzyme** | **Acronym** | **Substrate** | **Nutrient cycled** |
| α-glucosidase | AG | Starch | Carbon |
| (acid) phosphatase | AP | Organic phosphorus | Phosphorus |
| β-glucosidase | BG | Cellulose | Carbon |
| β-xylosidase | BX | Hemicellulose | Carbon |
| cellobiohydrolase | CBH | Cellulose | Carbon |
| leucine aminopeptidase | LAP | Proteins | Nitrogen |
| N-acetyl-β-D-glucosaminidase | NAG | Chitin | Nitrogen |
| polyphenol oxidase | PPO | Lignin | - |

***Table 2***. MANOVA and ANOVA results of Vmax, updated with non-significant interactions and main effects from Tukey post-hoc comparisons. (blank cells indicate lack of significance; \* 0.01 ≤ p < 0.05; \*\* 0.001 ≤ p < 0.01; \*\*\* p < 0.001).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Enzyme** | Time | Vegetation | Precipitation | Time x Precipitation | Time x Vegetation | Vegetation x Precipitation | Three-way |
| MANOVA | \*\*\* | \*\*\* | \* |  | \*\*\* | \*\* | \* |
| AG |  |  |  |  |  |  |  |
| AP | \*\* |  |  |  |  |  |  |
| BG |  | \*\*\* |  |  |  |  |  |
| BX |  |  |  |  |  |  |  |
| CBH | \*\*\* | \*\*\* |  |  | \*\*\* |  |  |
| LAP |  |  |  |  |  |  |  |
| NAG | \*\*\* | \*\*\* |  |  | \*\* |  |  |
| PPO |  | \*\* |  |  |  |  | \* |

***Table 3.*** MANOVA and ANOVA results of Km, updated with non-significant interactions and main effects from Tukey post-hoc comparisons. (blank cells indicate lack of significance; \* 0.01 ≤ p < 0.05; \*\* 0.001 ≤ p < 0.01; \*\*\* p < 0.001).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Enzyme** | Time | Vegetation | Precipitation | Time x Precipitation | Time x Vegetation | Vegetation x Precipitation | Three-way |
| MANOVA | \*\*\* | \*\*\* | \* |  | \*\*\* | \*\* | \* |
| AG |  |  |  |  |  |  |  |
| AP | \* | \*\*\* | \* |  | \* | \* |  |
| BG |  |  |  |  |  |  |  |
| BX |  | \* |  |  |  |  |  |
| CBH | \*\*\* | \*\*\* |  | \* | \*\*\* |  |  |
| LAP |  | \*\*\* |  |  |  |  |  |
| NAG | \* | \*\*\* |  |  | \*\*\* |  |  |
| PPO | \*\* | \*\* |  |  | \* |  |  |

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