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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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in

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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 – 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), 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 of these 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 cellulose or other polysaccharides), 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 at the species or strain level (Alster et al. 2021) or scale 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 (Wallenstein et al. 2011), 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 enzymatic products (Wallenstein et al. 2011). This practical definition of Km stems from products having been shown to be competitive inhibitors of substrates for the same enzyme such that high product concentrations correlate with high Km, and that high Km is correlated with low amounts of substrates (Chróst 1990; Chróst 1992). Thus, Km is a function of two processes: (1) the production of products and (2) microbial uptake of enzymatic products.

*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?
3. How will changes in amount of enzymes due to drought responses affect amounts of products?

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). Thus, it is expected that coastal sage scrub litter is the resource-limited environment. In addition, precipitation is reduced over half of the plots under study to simulate drought. Therefore, this drought treatment serves as a stress for half of the plots. The following hypotheses were formulated in order to answer the questions above based on the amount of resources and level of stress. (new hypotheses, rewrite the hypotheses cited in the results & discussion)

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 such that Vmax is 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 was sampled from each treatment combination on August 30, 2017 and hand mixed, with litter from each treatment combination being kept separate from other 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 and 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 microplate wells and 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 reaction velocity using formulas described in German et al (2011).

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. 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.

*Description of enzymes*

Oxidative EEs (in this case, the only relevant enzyme is PPO) uses polyphenols – e.g. lignin and tannins – as their substrate. In this case, PPO activity is used to primarily gauge the degradation of lignin rather than other polyphenols. Long-term decomposition studies have found that lignin as a proportion of the remaining mass in litter increases with time (Schlesinger 1985; Schlesinger and Hasey 1981), and lignin content has been found to be negatively correlated with decomposition rates in many field studies (Adair et al. 2008; Bontti et al. 2009; Cornwell et al. 2008).

AG primarily catalyzes the hydrolysis of starch (German et al. 2011). The hydrolysis of cellulose – a key structural component in plant cells – is conducted by BG and CBH, with CBH hydrolyzing 1, 4-β-D glucosidic linkages in cellulose while BG hydrolyzing terminal glycosidic linkages in the latter steps of cellulose degradation (German et al. 2011; Romero-Olivares et al. 2017). BX catalyzes the breakdown of hemicellulose (German et al. 2011; Romero-Olivares et al. 2017), which is not a major structural component of plant cells but is still a major polymer in the chemical composition of litter. Altogether, AG, BG, CBH, and BX breaks down carbon containing compounds and, therefore, serve as carbon sources for microbes, and BG activity had been used as a measure of the rate of carbon acquisition by soil microbes (Allison and Vitousek 2005; Sinsabaugh et al. 2008).

AP mineralizes organic phosphorus, and its activity had been used to measure the rate of phosphorus immobilization by soil microbes (Allison and Vitousek 2005; Sinsabaugh et al. 2008). In addition, its activity has been linked to the level of phosphorus in soils, with activity increasing in soils with low levels of phosphorus and when exposed to high concentrations of organic carbon and organic phosphorus (Allison and Vitousek 2005; Ali et al. 2019; Sinsabaugh et al. 2008). LAP catalyzes the hydrolysis of amino acids from the N-terminus of polypeptides (German et al. 2011). Its activity has been shown to be negatively correlated with the amount of other hydrolytic enzymes in the field (Baker and Allison 2017), suggesting that LAP also catalyzes the breakdown of other enzymes. NAG mineralizes chitin, a structural component of fungi cell walls. Taken together, AP, LAP, and NAG mineralizes organic nutrient sources, with AP mineralizing organic sources of phosphorus and LAP and NAG mineralizing nitrogen. NAG mineralizes fungal sources of nitrogen while LAP mineralizes proteins, which can be of plant origin (if proteins originated from the litter itself) or of microbial origin (e.g. if proteins are extracellular enzymes secreted by microbes). Activities of LAP and NAG had been used as a measure of the rate of nitrogen immobilization by soil microbes (Sinsabaugh et al. 2008).

*Hypotheses*

In trying to answer the three questions that motivate this experiment, the following hypotheses were formulated and will be tested.

H1: Enzyme amount controls product concentrations in such a way that they are positively correlated with each other.

* If this hypothesis is true, then Vmax and Km of an enzyme should be positively correlated in a linear regression. In addition, the treatments should have similar effects for both Vmax and Km.

H2: Microbes will increase investment of a particular enzyme when exposed to higher concentrations of substrates for that enzyme (Allison et al. 2005?).

* Predictions for this hypothesis are based on initial litter chemistry from Esch et al (2019). Litter from the coastal sage scrub ecosystem is of “lower quality” (citation needed) due to lower nitrogen content, higher lignin content, and lower cellulose and hemicellulose content (Esch et al. 2019) and so should see lower Vmax of enzymes that degrade cellulose (BG and CBH), hemicellulose (BX), and organic nitrogen (LAP, NAG) and higher PPO Vmax.

H3: Lignin acts as a noncompetitive inhibitor of hydrolytic enzymes, decreasing the actual amount of hydrolytic enzymes (Sewalt et al. 1997; Senior et al. 1991).

* Coastal sage scrub leaf litter, which has higher lignin, should see lower Vmax of hydrolytic enzymes and higher Vmax of polyphenol oxidase.

H4: In a high resource environment, there is a tradeoff between resources and energy between resource acquisition traits and stress tolerance traits where increased investment of resources in one class (e.g. stress tolerance) will lead to decreased investment of resources in another (e.g. resource acquisition) (Malik et al. 2020a; Wang and Allison in press).

* This hypothesis is based on the YAS framework (Malik et al. 2020a) that frames microbial life history in terms of 3 strategies (the relevant ones in this hypothesis are stress tolerance and resource acquisition). The stress in this experiment is the reduced precipitation treatment. Grassland litter is viewed as of “higher quality” (Cornwell et al) due to lower C:N ratio and lower lignin content (Esch et al. 2019). In this litter type, compared to ambient precipitation, microbes will decrease investment in enzymes (as indicated by lower Vmax) when under drought.

H5: In a low-resource environment, enzyme production is primarily constitutive (citation needed).

* Vmax does not significantly vary across the two precipitation treatments in coastal sage scrub.

*Data analysis*

All analysis and calculations were conducted on Python. After being converted from raw fluorescence or absorbance readings into enzyme activity, the activity of each enzyme from each litter sample is plotted against their respective substrate concentrations and judged visually for substrate inhibition or negative activity. 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; response to German et al. 2011). 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 (response to German et al. 2011). As a result, data points in which substrate inhibition is observed are removed. The final enzyme activity, after having negative activity set to 0 and substrate inhibition data points removed, are 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.

In order to test hypothesis 1, linear regression was conducted between transformed Vmax and Km of all enzymes using *scipy*’s *stats* module, and the significance of each regression was analyzed using Wald’s test. Further testing of hypothesis 1 and all other hypotheses were conducted using a factorial multivariate analysis of variance (MANOVA), factorial analysis of variance (ANOVA)s, 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 of these dependent variables using the same factors as the MANOVA. 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 showed to be non-significant under pairwise comparisons of the same interactions/main effects by 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. These results do not support H2.

In contrast, vegetation has more influence on V­max and Km than precipitation. 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 2). 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 3, 4, 6). 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 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 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 the difference tends to not be significant – in grassland despite lower lignin proportions in grassland (Figure 9).

**Discussion**

Higher amounts of cellulose-degrading enzymes in grassland litter (BG & CBH; Figures 3, 4) validates H1 and seem to indicate that microbes primarily use cellulose as a carbon and energy source rather than hemicellulose or lignin, and that changes in hemicellulose might not affect microbial community composition or functioning. The increase in hemicellulose in grassland litter does not cause a significant change in production of BX, indicating that this increase in hemicellulose does not incentivize microbes in acquiring more carbon or energy from hemicellulose. In addition, 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 6). This higher production of NAG is also accompanied by higher NAG substrates (i.e. chitin) – although in a mostly non-significant manner – in grassland plots across all time points than CSS plots (Figure 7). This correlation between NAG Vmax and Km is especially strong (Figure 1), indicating that chitin is readily mineralized and that chitin is a key source – if not the primary source – of nitrogen for soil and litter microbes rather than proteins.

This increased nitrogen immobilization might also provide enough nitrogen to fuel higher production of cellulose-degrading enzymes (BG & CBH) in grassland litter compared to CSS litter. These results reflect previous findings that found that BG activity increases only when soils are amended with nitrogen and phosphorus (Allison and Vitousek 2005). In addition, these results also reflect a more recent study conducted in grassland vegetation at the same study site, which found that the amount of NAG is positively correlated with BG and CBH (Alster et al. 2021).

In addition, the results of this experiment also validates H3. Indeed, the production of BG, CBH, and NAG are higher in grassland litter where there are lower amounts of lignin (Figures 3, 4, 6). Thus, there is less lignin to serve as a noncompetitive inhibitor to lower actual amounts of BG, CBH, and NAG in grassland plots. This provides a missing link into how lignin deters decomposition. 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 hypothesis to explain this link: that lignin acts as a noncompetitive inhibitor and removes enzymes from organic matter degradation. While this hypothesis 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 hypothesis.

There is a third explanation of higher amounts of BG, CBH, and NAG in grassland litter. LAP Km is significantly higher in CSS vegetation than grassland (Figure 8) despite vegetation not significantly altering LAP Vmax. This indicates that *in situ* LAP activity, rather than the amount of LAP enzyme, 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 decomposed due to this higher *in situ* LAP activity, resulting in higher amounts of BG, CBH, and NAG in grassland litter. These results mirror that of Baker and Allison (2017). Altogether, this explanation, along with hypotheses H2 and H3, explain higher BG, CBH, and NAG activity 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 1).

The higher rate of nitrogen immobilization 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 9). 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 and higher heterotrophic respiration 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 9), and so this conclusion should be tempered.

Noteworthy is the lack of an effect of precipitation on enzyme production 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 in 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 5). 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 2). This observation yields two possible corollaries: (1) *in situ* AP activity is significantly higher in ambient CSS plots than the other vegetation x precipitation combinations, (2) rates of AP reaction products uptake are higher in grassland litter than CSS litter. If the first corollary is true, then this indicates that phosphorus concentrations do not control and limit microbial decomposition, as BG, CBH, and NAG production is significantly higher in grassland litter than CSS. However, if the second corollary is true, then this indicates that phosphorus immobilization, along with nitrogen, helps to increase amounts of cellulose-degrading enzymes and the chitin-degrading enzyme NAG in grassland litter compared to CSS litter. As it is unclear which of these corollaries are true, the role that phosphorus plays in organic matter decomposition is unclear. Either way, the lack of an effect of precipitation on the production of AP is evident (Table 2), and the differential effects of precipitation on reaction products and enzyme amounts of AP is more evidence that do not support H1.

In addition, the lack of an effect of precipitation on enzyme production (Table 2) and reaction product concentrations (Table 3) is present across both ecosystem types and does not support H5. H5 predicts that enzyme production is primarily constitutive in low-resources environment (in this case, coastal sage scrub). However, the results indicate that enzyme production in *both* coastal sage scrub and grasslands might be constitutive and relatively robust to environmental change in the form of precipitation. 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, semiarid ecosystems. Thus, while these results indicate that lignin in Mediterranean grasslands might become less recalcitrant with increasing drought, changes in decomposition will be negligible, and soils and litter carbon pools in Mediterranean ecosystems will remain relatively stable even with increased drought.

*YAS framework implications*

These results also reflect results from a more recent study (Alster et al. 2021) that do not follow predictions from the YAS framework of microbial life history strategies (Malik et al. 2020a; Wang and Allison, in press). 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, it should be noted that the study is only applied to fungal strains, not whole microbial communities. In contrast, the results of this study applies to microbes at the community level.

This study should also be taken into context with another recent study at the same site (Malik et al. 2020b). According to the YAS framework, there are tradeoffs between resource acquisition traits, stress tolerance traits, and growth traits, and so increases in expression of one class of traits should also cause decreases in expressions of another class of traits. Malik et al (2020b) found that in grassland litter, expression of stress tolerance traits are 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 do not change even under stress, indicating that there is unlikely to be a tradeoff between resource acquisition and other classes of traits, unlike what was posited by the framework. Taken with Alster et al (2021), these results indicate that the YAS framework might not be an accurate description of microbial life history strategies, although I do not think that the framework should be entirely refuted as Alster et al (2021) only investigated individual fungal strains while this study only investigated resource acquisition.

**Figures and tables**

Chart, scatter chart

Description automatically generated

***Figure 1.*** Linear regressions of log10-transformed Vmax and Km of all enzymes.

Chart, box and whisker chart

Description automatically generated

***Figure 2.*** AP reaction products (Km) as a function of vegetation and precipitation. The hatch type indicates the precipitation treatment and the color indicates the vegetation type. Letters above each boxplot represents Tukey labels where boxes (treatment combinations) that share the same labels are similar to each other by the Tukey HSD test. Treatment combinations on the x-axis are written in the order of (vegetation, precipitation).

Chart, box and whisker chart

Description automatically generated

***Figure 3.*** BG enzyme amount (Vmax)as a function of vegetation. Color indicates vegetation type. 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 4.*** Amount of CBH enzymes (Vmax)as a function of time and vegetation. Color indicates vegetation type. Letters above each box plot are Tukey labels where box plots that share the same letter(s) are similar to each other by Tukey HSD post-hoc comparisons. Treatment combinations on the x-axis are written in the order of (time, vegetation).

Diagram, schematic

Description automatically generated

***Figure 5.*** CBH reaction products (Km) as a function of time & precipitation. The hatch type indicates the precipitation treatment. Letters above each box plot are Tukey labels where groups that share the same letter(s) are similar to each other by Tukey HSD post-hoc comparisons. Treatment combinations on the x-axis are written in the order of (time, precipitation).

Chart, box and whisker chart

Description automatically generated

***Figure 6.*** Amount of NAG enzymes (Vmax) as a function of time & vegetation. Colors indicate vegetation type. Letters above each boxplot represents Tukey labels where boxplots that share the same letter(s) are similar to each other based on Tukey’s post-hoc comparisons. Treatment combinations on the x-axis are written in the order of (time, vegetation).

Chart, box and whisker chart

Description automatically generated

***Figure 7.*** NAG reaction products (Km) as a function of time & vegetation. Color indicates vegetation type. Letters above each boxplot represents Tukey labels where boxplots with the same letter(s) are similar to each other by Tukey HSD’s test. Treatment combinations written on the x-axis are written in the order of (time, vegetation).

Chart, box and whisker chart

Description automatically generated

***Figure 8.*** LAP reaction products (Km) as a function of vegetation. Colors represent vegetation type. 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

Description automatically generated

***Figure 9.*** Amount of PPO enzymes 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.

***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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