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Microbial community diversity, function, and succession in California's Mediterranean habitats



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# Microbial community diversity, function, and succession in California's Mediterranean habitats

A dissertation submitted in partial satisfaction

of the requirements for the degree

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by

**Emily Elizabeth Curd**

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ABSTRACT OF THE DISSERTATION

Microbial community diversity, function, and succession in California's Mediterranean habitats

by

Emily Elizabeth Curd

Doctor of Philosophy in Biology

University of California, Los Angeles, 2016

Professor Thomas Bates Smith, Co-Chair

Professor Huiying Li, Co-Chair

We live on a predominantly microbial planet. I it is estimated that more than a billion microorganisms can live in a gram of soil. Microorganisms comprise the largest pool of genetic diversity on the planet and drive global biogeochemical cycles. Since microbial ecology is intimately associated with environment, changes in environmental conditions can have profound effects on the microbial diversity and function of microbial communities. In this dissertation I study; 1) the relationship between environmental heterogeneity and microbial diversity, 2) the relationship between the environment, microbial diversity, and microbial functional traits, and 3) microbial secession related to changing environmental conditions during anaerobic decomposition.

**Chapter 1**

Annual grassland invasions can increase environmental heterogeneity and reduce the biological diversity of plants and animals. There is a generally positive relationship between environmental heterogeneity and biodiversity, and more specifically, soil heterogeneity is known

to influence plant diversity. Here I test if the diversity of soil microorganisms, like that of plants, displays a positive relationship with soil environmental heterogeneity. Specifically, I test to see if invasive annual grasses lead to reductions in soil heterogeneity and microbial alpha- and beta-diversity. I sampled the soil profile across invasive annual grassland, oak woodland, and coastal sage scrub habitat and characterized environmental heterogeneity (soil percent carbon, nitrogen, water content, total dissolved solids, and pH in addition to litter percent carbon, nitrogen and C:N), alpha and beta diversity. I found that invasive annual grassland habitat has greater soil environmental homogeneity than native woody habitats throughout the soil profile. Annual grassland communities have lower alpha-, but not beta-, diversity than native woody species. Patterns of alpha diversity with depth differ between grassland and woody habitat, and although not significant, woody habitats have higher community heterogeneity. Alpha diversity and beta diversity show positive relationships with several measures of environmental heterogeneity, suggesting that like plants, soil microbial diversity increases with environmental heterogeneity. Annual grassland invasions into native woody habitats reduce soil microbial diversity. This is particularly true in deep soil communities.

**Chapter 2**

Plant invasions frequently alter ecosystem processes in part because they modify soil microbial communities. These communities decompose the bulk of terrestrial organic matter by producing and releasing extracellular enzymes. California's native Mediterranean habitats (e.g. Oak woodland and coastal sage scrub) are invaded by annual grasses and are converted to invasive annual grasslands. I investigated the relationship between extracellular enzyme activities and microbial community composition in these habitats by examining 1) how

extracellular enzyme activities differed between native and invasive habitats, 2) whether changes in microbial community correlate with changes in extracellular activity, and 3) if the composition of bacterial phyla that contain genes for extracellular enzymes differ between habitats. I found that annual grassland enzyme activities are much different from those of woody habitat, and the differences in enzyme activities between habitats generally declined with depth as did enzyme activities. There was also a strong correlation between community composition and extracellular enzyme activity. This correlation was not influenced by soil environmental variables. The relative abundance of phyla with genes for extracellular enzymes were similar between habitats and those genes are contained in distinct assemblages of phyla. Habitat change through annual grassland invasion modifies soil communities and their functions thought out the soil profile. Future studies on the effects of annual grassland invasion on ecosystem processes in deep soil are needed to fully understand the consequences of these invasions.

**Chapter 3.**

Natural tar seeps are the source of millions of fossils from animals that became entrapped, died and were decomposed over the millennia. The microbial communities responsible for the anaerobic decomposition of these entrapped animals are not known. However, microbial communities likely play a role in the rapid time to skeletonization of animal components submerged in tar. I hypothesized that high energy animal tissue would support fast growing taxa and support lower microbial diversity, and that microbial succession across different locations in the tar environment and animal tissue decay would resemble known patterns of microbial decomposition in similar habits. I sampled different locations in a tar seep and also bobcat limbs that were experimentally submerged in the seep and left to decay until

skeletonization. Microbial communities were characterized using 16S rDNA sequencing of the V4 region. I found that decay communities had lower diversity than tar environment communities and that microbial succession proceeded similarly to that in analogous habitats. The addition of animal tissue into this tar seep appeared to lead to rapid microbial community succession. This microbial succession likely affected the rate of decomposition of this tissue. Future experiments are required to understand the role of microbial succession in determining the rate of decomposition and time to skeletonization in tar environments.

This dissertation of Emily Elizabeth Curd is approved.

Jennifer B.H. Martiny

Priyanga Amarasekare

Philip W. Rundel

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University of California, Los Angeles

2016

To Dad.

Thank you for standing by me, and for your skills with pen and shovel.

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CHAPTER 1

# Invasive annual grasslands reduce environmental heterogeneity: implications for soil microbial diversity and community composition

## Introduction

Biological invasions frequently result in reduced biological diversity and increased spatial similarity in community composition (Elton 1958; Gilbert and Levine 2013). This is particularly the case for invasions of annual grasses that may alter the above- and below- ground distribution of resources (Dickens et al. 2013; Lindsay and Cunningham 2012; Wilcox et al. 2012; Molinari and D'Antonio 2014). These modifications can render habitat unsuitable for native species and result in a reduction in plant and animal diversity (Vaness, Wilson, and MacDougall 2014; Gilbert and Levine 2013) while promoting further invasion and establishment of nonnative species (Alofs and Fowler 2013; Cox and Allen 2011; Eliason and Allen 1997; Wood et al. 2006). Less is known about how such invasions influence microbial diversity and composition (Weber 2015; Dickens et al. 2013).

A common characteristic of ecological communities is the positive relationship between environmental heterogeneity and biological diversity. Typically, more heterogeneous habitats contain more niche space, which can support a greater species richness (MacArthur and MacArthur 1961) leading to a positive relationship between environmental heterogeneity and biodiversity (Stein, Gerstner, and Kreft 2014), but see (Seiferling, Proulx, and Wirth 2014). This can also lead to high rates of beta-diversity, or variation in community composition (Whittaker 1960; Heino, Melo, and Bini 2015; Chase et al. 2003). Plant alpha and beta diversity is

positively correlated to soil heterogeneity in observational studies ((reviewed in (Lundholm 2009); (Freestone and Inouye 2006)), and also in experimental manipulations (Williams and Houseman 2014; Questad and Foster 2008).

In contrast to larger organisms, few studies have directly investigated the relationship between soil heterogeneity and soil microbial diversity (specifically alpha diversity). There are positive taxa area relationships for microbes driven by environmental heterogeneity (Griffiths et al. 2011; Horner-Devine et al. 2004; Ranjard et al. 2013). Environmental drivers promote plant and microbial beta diversity (Prosser 2015) and plant diversity alone can promote soil beta diversity (Barberán et al. 2015). It has been proposed that plants that promote soil environmental heterogeneity could promote greater soil microbial diversity (Hooper et al. 2000; Wardle 2006), and conversely plants that reduce soil heterogeneity, like invasive annual grasses, should reduce soil biodiversity.

California's Mediterranean habitats have had a long history invasion by nonnative annual grasses (Bartolome, Klukkert, and Barry 1986; Heady 1977; Minnich and Dezzani 1998; Minnich 1988). These invasive grasslands typically occur as habitat mosaics with native woody habitat: oak woodland and coastal sage scrub. Relative to soils under native woody habitats, invasive annual grassland soils should be more homogeneous both vertically and horizontally. The relatively short root system of grasses is very dense and homogenous relative to native woody species (Pinno and Wilson 2013; Goldstein and Suding 2014; Ward, Wiegand, and Getzin 2013) and results in more homogenous soil resource distributions (Parker, Seabloom, and Schimel 2012; Dickens et al. 2013). Across a depth profile, annual grass plants primarily affect shallow soils, whereas native woody species affect shallow and deep soils. Woody species have heterogeneous distributions of deep roots and vertical stratification of soil resources under reflect

the differences in root architecture between plant species (Koteen, Raz-Yaseef, and Baldocchi 2015; Moody and Jones 2000).

In this study I investigate the relationship between soil environmental heterogeneity and microbial diversity, horizontally and vertically, in soils in invasive annual grassland and native oak woodland and coastal sage scrub. I test if the diversity of soil microorganisms, like that of plants, displays a positive relationship with soil environmental heterogeneity. Specifically, I ask if invasive annual grasses lead to reductions in soil heterogeneity and microbial alpha- and beta-diversity. I expect that microbial communities in soils of invasive annual grasslands will have lower diversity than those in soils of oak woodlands and coastal sage scrub. In addition, I expect the differences in soil microbial diversity between habitats to persist through the depth profile due to vertical differences between the root architecture of invasive and native species.

## Materials and Methods

*Study location and sampling design*

To study the long term consequences of habitat conversion from native woody to invasive annual grassland on soil biodiversity and function, I sampled soils at Sedgwick Reserve in the Santa Ynez Valley (Figure 1, Table 1), which is part of the University of California Natural Reserve System (NRS). The reserve contains mosaics coastal sage scrub (dominated by *Salvia leucophylla* and *Artemisia californica*), oak woodlands (dominated by *Quercus agrifolia)* and annual grasslands (dominated by *Avena sp*. and *Bromus sp*.) which is typical of this ecosystem (Wells 1962), and soils of the reserve (Kirkpatrick and Hutchinson 1980). I sampled the Shedd silty clay loams (the Shedd soil series is a calcareous, fine-silty, thermic Typic Xerorthents) of Sedgwick (Shipman 1972; Gessler et al. 2000). These soils are shallow and are

not predicted to be strongly divided by horizons to a depth of 1 m, the predicted boundary with bedrock (Soil Survey Staff). This soil supports native woody and nonnative annual grassland habitats that are well established and have persisted for at least 80 years (Gabet, Fierer, and Chadwick 2005). The boundaries between habitat types at Sedgwick have been maintained through cattle grazing and other ranching activities (Callaway and Davis 1993), which are ongoing.

I sampled soils from three annual grassland sites, three coastal sage scrub sites, and three oak woodland sites within the reserve (Figure 1, Table 1). Sampling pits were a maximum of 5 km apart to minimize differences in precipitation and temperature. The study site has a summer average high of 37.78 C and a winter average low temperature of -5 C and mean annual precipitation is 38.1 cm (NRS Staff).

Plant litter was collected prior to excavation, dried and stored at room temperature. Soils were extracted from five depths (5, 20, 45, 60 and 80 cm), and collected from two faces within each soil pit (60-100 cm apart) for all depths. In total, nine litter and ninety soil samples were collected between May 21 and June 10, 2011. Soil samples were divided into 15 mL (for genetic work) and 200 mL (for edaphic characterization) sub-samples, while maintaining soil structure as much as possible. Genetic samples were kept in liquid nitrogen in the field and stored at -80 C. Samples for edaphic characterization were stored at ambient temperature.

*Characterization of Soil Resources and Litter Quality*

I measured five soil resources, and characterized plant litter carbon and nitrogen. All soils were sieved at 2 mm prior to analyses. I gravimetrically assayed soils for moisture content (M) by water loss after 24-hour oven drying at 105$^{o}$C. Soil samples were tested for moisture content

within 10 days of sample collection. I determined the percent of soil total carbon and nitrogen from air dried and ground soils using an ECS 4010 CHNSO Analyzer (Costech Analytical Technologies, Inc., Valencia, CA). I measured soil solution pH and total dissolved solids in a 1:1 soil to distilled water slurry using an Extech EC400 EXSTIK Conductivity Meter (Nashua, NH). Because this soil series is calcareous, I also tested for the presence of carbonates by a drop of 1N HCl. Effervescence was observed if a sample contained carbonates. Litter carbon and nitrogen were determined using the same method for analyzing soil C and N.

*Characterization of Soil Bacteria and Archaea*

I extracted total soil DNA in triplicate from frozen samples (stored at -80 C) and amplified the V4 region of the 16S rDNA (Caporaso et al. 2012). For each subsample, 0.25 g of soil was physically lysed in 0.12 M Sodium Phosphate Buffer (pH 8) with 1.75% SDS by bead beating for 5 minutes with 0.25 g of 0.1 mm glass beads and 0.25 g of 0.5 mm zirconia/silica beads. The lysate was precipitated in 1.6 M NaCl with 30% PEG 8000 for two hours at room temperature, pelleted, washed twice with 70% ethanol, and re-suspended in water. The DNA was purified by gel electrophoresis (2% agarose gel), and gel extraction with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Triplicates were pooled and stored at -20$^{o}$C.

I amplified the V4 region of the 16S rRNA gene using the 515F/806R primer pair (Caporaso et al. 2012) but with the addition of an oligo sequence that is recognized by Nextera Indexing Primers (Nextera® Index Kit, Illumina, San Diego, CA). PCR was performed in triplicate using Platinum® Taq DNA Polymerase (Life Technologies, Grand Island, NY, USA) and 1 uM of each primer under the following conditions: Hold at 95$^{o}$C for 3 min; 30 cycles of

95°C for 45 secs, 50°C for 60 secs, and 72°C for 90 secs; and a hold at 72°C for 10 min and a final hold at 10°C. PCR products were verified with 2% agarose gel electrophoresis, and purified by agarose gel extraction using the Zymoclean™ Gel DNA Recovery Kit. Amplicon libraries were then amplified with Nextera Indexing Primers (1.25 uL of each primer) to add indices using the KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Woburn, MA, USA) under the following PCR conditions: Hold at 95°C for 5 min; 5 cycles of 98°C for 20 secs, 56°C for 30 secs, and 72°C for 3 min; and a hold at 72°C for 5 min and a final hold at 8°C. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). Amplicons were quantified by qPCR sing the Library Quantification Kit – Illumina for the LightCycler® 480 (Roche, Indianapolis, Indiana, USA). Amplicons were adjusted to an equimolar concentration, pooled and loaded onto a MiSeq Personal Sequencer using a MiSeq Reagent Kits v1 or v2 and paired end sequenced with either 150 or 250 bp read lengths.

The resulting sequences were characterized using the following pipeline: 1) MiSeq reads were quality filtered and the ends were trimmed to a minimum quality score of 30 and length of greater than 75 bp using Galaxy (Goecks et al. 2010; Giardine et al. 2005; Blankenberg et al. 2001). 2) Sequences were classified using the Qiime (Caporaso et al. 2010) pick_open_reference_otus.py script at 97% similarity (Edgar 2010). OTU's with five or fewer occurrences across all samples were removed from the analysis. 3) Samples were then rarefied to 1000 sequences for subsequent analyses. Four samples were dropped from the data set due to low sequence read number ($n < 1000$).

*Diversity and Statistical Analysis*

Alpha diversity was measured by the number of observed operational taxonomic units (OTUs), Shannon Entropy, and Faith's phylogenetic diversity (Caporaso et al. 2010). I report these metrics using the average of 10 replicated rarefactions (to 1000 sequences) of the sequence dataset (Rodrigues et al. 2013). Beta diversity was calculated as the weighted Unifrac distance (Lozupone and Knight 2005). Weighted UniFrac distances are also the averages of 10 replicated rarefactions to 1000 sequences.

I used linear mixed models (Moroney and Rundel 2013; Bates et al. 2014) to analyze how edaphic factors (M, C, N, C:N, pH, $EC_{1:1}$) changed by habitat and depth. These models included sample location as random variables and habitat and depth as predictor variables. Initial models included all variables, but only the best models were retained (Kuznetsova, Brockhoff, and Christensen 2013). Linear models were used to analyze differences in litter variables (C, N, C:N) with habitat type as the predictor variable (Venables and Ripley 2002). For all models, predictor variable significance was determined by ANOVA. Tukey's post hoc methods were used for all post-hoc comparisons. I explored soil resource heterogeneity by calculating the coefficients of variation for samples grouped by habitat and depth, and by using Principal Components Analysis (PCA) on the edaphic factors. The value for edaphic factors were centered and scaled prior to PCA. Heterogeneity between sample groups was calculated from the PCA as dispersion around the centroid of each group using betadisper and the amount of variation explained by habitat and depth was calculated using the adonis function PERMANOVA in vegan (Dixon 2003).

I used linear mixed models with a quadratic term to investigate habitat and depth related differences in alpha diversity. These models included sample location as a random variable and habitat and depth as predictor variables. Initial models included all variables, but only the best

models were retained (Kuznetsova, Brockhoff, and Christensen 2013). To explore the relationships between alpha diversity and environmental variables I ran Pearson's correlations between alpha diversity and the soil and litter variables (Harrell 2014). Linear models were run to assess the relationships between sample groups for the total alpha diversity and the coefficients of variation and the median distance to the centroid for all environmental variables. I explored community composition and turnover using Non-metric Multidimensional Scaling (NMDS) on weighted Unifrac distance (Dixon 2003). Soil and litter variables were correlated with community composition using envfit function in vegan. Community heterogeneity was also determined by the betadisper function in vegan, and the amount a variation explained by habitat and depth was also calculated using the adonis function PERMANOVA in vegan (Dixon 2003). I identified individual environmental variables associated with community turnover by running linear models between the median distance to the centroid for all microbial communities by sample group and the coefficients of variation for each soil and litter variable. To assess the similarities between environmental heterogeneity and community heterogeneity, I used Procrustes rotation analyses on the environmental and community composition ordinations and a Mantel test between the Euclidean distance of environmental variables and the weighted Unifrac distances between communities.

## Results

### *Environmental Heterogeneity: Soil Variables*

The measured soil variables differed 1) by depth and the interaction between depth and habitat (percent total carbon, percent soil moisture, and soil pH), 2) by habitat and the interaction between depth and habitat (total dissolved solids), or 3) by depth alone (percent total N) (Figure

2). The overall variation in these variables differed by habitat type (Table 2) for percent carbon ($p = 0.00067$), percent nitrogen ($p = 0.00067$), and pH ($p = 0.003397$). In each case oak woodland (for percent carbon $p = 0.0005$, for percent nitrogen $p < 0.0001$, for pH $p = 0.0196$) and coastal sage scrub (for percent carbon $p = 0.036$, for percent nitrogen $p < 0.0001$, for pH $p = 0.0034$) had larger CVs than grassland habitat. The CV for soil varaibles did not vary significanlty by depth.

To investigate soil heterogeneity overall, I combined the five soil variables in a PCA analysis (Figure 3a.). Most of the variation in this composite measure could be explained by the first two principle coordinates (PC 1: 47% and PC 2: 37%; Figure 3A). Percent C and N and pH loaded on PC1, and TDs, percent M and pH loaded on PC2. Only a small amount of the total difference in soil resources across samples could be explained by habitat type (PERMANOVA; $r^2 = 0.222$, $p > 0.0001$), depth ($r^2 = 0.161$, $p > 0.0001$), and the interaction between habitat type and depth ($r^2 = 0.043$, $p > 0.0032$). This suggests that depth and habitat are not the sole driver of soil resource differences across soils (Parker, Seabloom, and Schimel 2012). To quantify variation in soil resource variability for each habitat and depth combination, I measured the dispersion around the centroid for each group (Figure 3B). There were marginally significant differences in variability in the composite of soil variables by habitat ($p = 0.08729$), but not depth. Pairwise comparisons between habitats showed differences in heterogeneity between grassland and coastal sage scrub ($p = 0.04695$) and grass and oak woodland ($p =0.0419$), but not between oak woodland and coastal sage scrub.

*Environmental Heterogeneity: Litter Variables*

I collected litter from each sampling location and determined the percent litter carbon and nitrogen, and the ratio of litter C:N (Figure 4). The average percent litter carbon did not change by habitat, but there were large differences in the heterogeneity of litter carbon between habitats (Table 3). Woody habitats had higher variability than annual grasslands. Litter percent N and the C:N ratio differed by habitat. Oak woodlands had the highest litter N concentration and grassland had the lowest, and the opposite was true of the litter C:N ratio. Woody habitats had higher variability in litter N than annual grasslands, but for litter C:N ratios coastal sage scrub had higher variability than annual grassland and oak woodland.

*Alpha Diversity*

The alpha diversity of soil microorganisms was calculated using Faith's Phylogenetic Diversity, Shannon Entropy, and the number of observed OTUs. All alpha diversity metrics were strongly correlated (minimum $r^2 = 0.91$, $p < 0.001$), so only Faith's Phylogenetic Diversity is shown (Figure 5). Depth has both a linear ($p = 0.001$) and non-linear ($p = 0.004$) effect on Phylogenetic Diversity, as does habitat ($p = 0.053$) and the interactions between habitat and the linear effect of depth ($p = 0.022$) and habitat the non-linear effect of depth ($p =0.014$). Alpha diversity declined with depth in annual grassland, but oak woodland and coastal sage scrub alpha diversity showed a curvlinear realtinship with depth. Woody habitat alpha diversity declined to 45 cm and then increased with greater depth.

*Environmental Heterogeneity and Alpha Diversity*

To examine if resource levels or spatial heterogeneity in resources influenced microbial alpha diversity, I first examined correlations between alpha diversity and soil and litter variables.

Alpha diversity was weakly correlated with soil moisture (Pearson's R = -0.24, p = 0.028; multiple $r^2$= 0.055, p = 0.0287), pH (Pearson's R = -0.31, p = 0.004; multiple $r^2$= 0.095, p = 0.003), litter N (Pearson's R = 0.33, p = 0.002; multiple $r^2$= 0.108, p = 0.002), and litter C:N (Pearson's R = -0.35, p = 0.001; multiple $r^2$= 0.123, p = 0.001). I then compared the total alpha diversity within each sample group with the coefficients of variation for environmental variables found in those groups. Total Phylogenetic diversity within a sample group was positively related to the CV of percent soil carbon (multiple $r^2$ = 0.566, p = 0.0012), percent soil nitrogen (multiple $r^2$ = 0.541, p = 0.0017), pH (multiple $r^2$ = 0.3499, p = 0.0202), litter nitrogen (multiple $r^2$ = 0.4274, p = 0.0082), litter carbon (multiple $r^2$ = 0.6538, p = 0.0003), and all soil variables calculated from the group distance to the centroid in the PCA (Figure 3B) (multiple $r^2$ = 0.566, p = 0.0012) (Figure 6).

*Beta Diversity*

I characterized microbial beta diversity by habitat and depth using weighted Unifrac distance and visualized differences in community composition using NMDS with k = 3 (Figure 7A). A small amount of the difference in community composition could be explained by habitat type (PERMANOVA: r2 = 0.116, p > 0.0001), depth (r2 = 0.1588, *P* > 0.0001), and habitat type by depth interactions (r2 = 0.0376, *P* = 0.004). Pairwise comparisons between habitats showed that slightly less of the difference in community composition can be explained by habitat type for oak woodland and coastal sage scrub (r2 = 0.067, p = 0.0004) than oak woodland and grassland (r2 = 0.107, p < 0.0001) or coastal sage scrub and grassland (r2 = 0.094, p < 0.0001). These results suggest that woody habitats are more similar to each other than to annual grasslands. Variation in community variability did not differ across habitat and depth groups, as calculated from the

NMDS using the dispersion to the centroid of each group (Figure 7B). Although dispersion from the centroid is greater in woody rather than grassland habitats, there were no significant differences for pairwise comparisons of dispersion between habitat types.

*Environmental Heterogeneity and Beta Diversity*

Community composition had strong correlations ($r^2 > 0.5$) with soil pH, litter nitrogen and litter C:N, and moderate correlations ($r^2$ between 0.25 and 0.5) for soil carbon, nitrogen, and total dissolved solids (Figure 7A). The ordinations of environmental variables and community composition were significantly associated (Correlation in a symmetric Procrustes rotation = 0.5148, $p = 0.001$). This result was supported by a positive correlation between the similarity in resources and the similarity in microbial composition between pairs of soil samples (Mantel statistic r: 0.352, $p < 0.001$). Variation in microbial community composition variability (calculated from the group distance to the centroid in the NMDS (Figure 7B)) positively associated with variation in soil percent nitrogen and total dissolved solids as well as litter nitrogen and C:N (Figure 8.) by habitat and depth.

**Discussion**

In this study I tested the relationship between microbial diversity and environmental heterogeneity in invasive annual grassland and native woody habitats. Invasive annual grassland habitat has greater soil environmental homogeneity than native woody habitats. This difference in spatial heterogeneity across the habitats persisted throughout the soil profile. In addition, microbial communities in annual grassland have lower alpha diversity than native woody species and patterns of alpha diversity with depth differ between grassland and woody habitat. In contrast, beta diversity did not change significantly by habitat type or depth, but woody habitats

have higher heterogeneity in their microbial communities. Alpha- and beta- diversity increased with several measures of environmental heterogeneity suggesting that, like plants, soil microbial diversity increases with environmental heterogeneity.

*Environmental Heterogeneity*

In this study, grasslands soils were less heterogeneous than the soils in woody habitats. This is consistent with previous studies of annual grassland invasion into coastal sage scrub (Dickens et al. 2013) and other habitats (Lindsay and Cunningham 2012), and woody encroachment into grasslands (Kleb and Wilson 1997; Pärtel and Helm 2007). Woody plants tend to be resource islands where resources accumulate heterogeneously in soils under the canopy (Kuiters, 1990; Kraus et al., 2003), and resource accumulation can vary spatially based on plant age and distance between the drip line and tree trunk (Koteen, Raz-Yaseef, and Baldocchi 2015; Moody and Jones 2000). Nutrients from litter enter soils through decomposition and the leaching of nutrients. Annual grasses form a uniform thatch of litter but woody species litter fall varies spatially relative to the canopy of plants (Aponte et al. 2014; Ushio, Kitayama, and Balser 2010). Differences in litter fall between plant species also contribute to soil environmental heterogeneity. I did not see significant changes in soil environmental heterogeneity with depth suggesting that these habitat effects do not diminish with depth. This is consistent with differences in root morphology between plants in each habitat. The shorter roots of grasses are dense and homogenous relative to woody species, which have more heterogeneous distributions of roots that grow thought-out the soil profile (Pinno and Wilson 2013; Goldstein and Suding 2014; Ward, Wiegand, and Getzin 2013). Above and below ground differences between annual grasses and native woody species contribute to the differences in soil heterogeneity between habitats.

There were moderate to strong relationships between total alpha diversity and the coefficient of variation for soil C, N, and pH, litter N and C, and heterogeneity across all soil variables, and weak to moderate relationships between heterogeneity in community composition and the coefficient of variation for soil N, and TDS, litter N and C:N. These environmental variables are known to correlate with soil microbial diversity. Litter quantity and quality and soil C and N are often coupled (Wolkovich et al. 2010; Ball, Carrillo, and Molina 2014), and changes in the diversity or chemical composition of litter can alter the composition of a soil microbial community (Chapman and Newman 2010). Soil pH is also an important driver of microbial diversity (Prober et al. 2015; Rousk et al. 2010; Griffiths et al. 2011; Fierer and Jackson 2006). Total dissolved solids, a measure of soluble inorganic salts and organic matter soils (e.g. $Na^{+}$, $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, carbonate, bicarbonate, etc.), is also a major driver of microbial community composition across soil and aquatic habitats (Lozupone and Knight 2007).

Many of these variables were more heterogeneous in woody habitats. Dickens et al. (2013) also found that grasslands invading coastal sage scrub show less spatial variation in total carbon and nitrogen. Similarly, soils under oaks show different C and N distribution than soils under grasses (Aponte et al. 2014). Soil pH can be affected by plant characteristics (Sayer 2006). For example, plant tannins can decrease soil pH and other factors like tree species (Thoms et al. 2010), the distance from the trunk, and soil depth can affect soil pH (Ushio, Kitayama, and Balser 2010).

*Alpha Diversity*

Soil alpha diversity differed between grassland and woody habitats. Alpha diversity also differed by depth but in habitat specific patterns. Alpha diversity declined with depth in

grasslands but showed a curvilinear relationship with depth under woody habitats. Previous studies in grasslands (LaMontagne, Schimel, and Holden 2003; Will et al. 2010) and a forested watershed (Eilers et al. 2012) report declines in alpha diversity with depth, which is consistent with the trend I observed in annual grassland samples. The trend curvilinear trend in diversity with depth observed in woody habitat is interesting for two reasons. The first is that woody communities with the lowest alpha diversity are located near 45 cm, which is near the transition point between soil horizons (between A and C). The second is that 45 cm should be beyond the lowest reaches the surface feeding roots of woody species (Jackson et al. 1988). Roots promote soil community diversity through mutualistic interactions (Hooper et al. 2000). At 45 cm, soils may be shifting from rhizosphere to increasingly bulk soil. This would reduce the surface soil mutualistic plant interactions with bacteria and soil nutrients, and depletion of certain nutrients adjacent to roots and accumulation of others not related to roots (Jobbágy and Jackson 2001). With increasing depth, there may be increased more microbial interactions with deep roots (Egerton-Warburton, Graham, and Hubbert 2003; Bornyasz, Graham, and Allen 2005), differences in hydrology, or patchiness in soil micronutrients driving the uptick in diversity. The rhizosphere of deep soils constitute hotspot of nutrients (Uksa et al. 2014), and 45 cm in our soils may be in between deep soil and surface hotspots of nutrients. The lack of deep roots in grasslands and declining alpha diversity with depth supports the hypothesis that deep roots are related to increases in deep soil biodiversity. Because deep grass samples lie below the fine rooting zone of grasses (Jackson, Mooney, and Schulze 1997), there are large declines in fresh organic matter additions into the subsoil (Fontaine et al. 2007). The microbes in deep grassland subsoil likely persist on much older pools of organic resources (Kramer and Gleixner 2008) that

are cycled through microbial biomass (Agnelli et al. 2004). This less diverse subsoil resource pool could ultimately support less diverse microbial communities.

This study shows that variability in environmental variables by habitat and depth contribute to patterns of alpha diversity. This suggest that increases in resource heterogeneity leads to greater niche diversity (Eisenhauer et al. 2011) which supports more soil biodiversity. The co-occurrence patterns of microbial taxa in soils and other terrestrial and aquatic habitats show that niche differentiation is a strong driver of microbial diversity (Barberan et al. 2012; Stegen et al. 2012; Wang et al. 2013). In addition, microbial diversity in this system follows the generally observed positive relationship between environmental heterogeneity and biodiversity as seen in larger organisms (Stein, Gerstner, and Kreft 2014). Landscapes with more habitat types can support more plant and animal species (Kallimanis et al. 2008; Hortal et al. 2009), here I find that at smaller scales plant driven heterogeneity in soil resources can support greater microbial diversity. Although our measures do not completely capture changes in patterns of biodiversity, which could be due to other factors that were not measured (e.g. plant diversity, micronutrients, productivity, and energy) but may be important (Kerr and Packer 1997; Eisenhauer, Reich, and Isbell 2012; Hooper et al. 2000), there is strong evidence that environmental heterogeneity is important in driving microbial biodiversity. Due to the low sample size and the scale of sampling it is likely that I under sampled environmental heterogeneity (Stein, Gerstner, and Kreft 2014; van Rensburg, Chown, and Gaston 2002).

*Beta Diversity*

In addition to influencing alpha diversity, changes in soil environmental heterogeneity can affect the spatial variability of microbial communities. Low spatial environmental

heterogeneity with in a habitat can lead to spatially similar communities across sites of the same habitat. The general separation between grasslands and oak woodland visualized by the NMDS is consistent with previous microbial community studies in these habitats (Waldrop and Firestone 2006; Fierer, Schimel, and Holden 2003a). Coastal sage scrub communities did not always group by habitat type, but rather these communities associated with oak woodland or grassland communities. In a study of surface soils in annual grass invaded, restored, and pristine coastal sage scrub habitat, specific bacterial groups varied but no major changes in microbial community composition were observed (Dickens et al. 2013). This is consistent with my observations of coastal sage scrub microbial communities clustering with grassland communities but it does not explain why sage communities also cluster with oak woodland communities. My results are in contrast with a study of microbial community turnover across a shrub encroachment gradient found distinct grass, shrub and tree communities (Yannarell, Menning, and Beck 2014).

A possible explanation for the beta diversity patterns observed for sage samples is that plant effects on microbial communities do not appear to extend into the bulk soil, but rather dominate near the rhizosphere (Uksa et al. 2014) and communities under woody species are more distinct the closer they are to the trunk of the plant (Ushio, Kitayama, and Balser 2010). It is possible that the coastal sage scrub samples that resembled grassland communities were sampled closer to the bare patches between plants rather than near the trunk of the sage plants. Distinct changes in soil communities turnover with depth have been observed in many other studies (Thoms et al. 2010; Steenwerth et al. 2008; Ushio, Kitayama, and Balser 2010; Uksa et al. 2014; Huang et al. 2013; Fierer, Schimel, and Holden 2003b). Habitats and depth influence microbial community turnover in this system.

Beta diversity was not significantly more variable by habitat or by depth, however, there was a trend towards more heterogeneous subsurface soil communities in woody habitats. I hypothesized that woody habitats would be more heterogeneous based on studies of soil communities in grassland and woody habitats. Recently converted pasture land microbial communities are spatially more similar than those of native tropical forests (Rodrigues et al. 2013), and in sage brush steep ecosystems invasive cheat grass support more vertically homogenous soil communities (Weber 2015). In this study there were trends towards greater community turnover in woody habitats, particularly in deeper soils. Deep soil environmental heterogeneity due to patchiness in bulk soil and deep rhizosphere resources may be important drivers of beta diversity. It is also possible that surface soils are experiencing more disturbance (e.g. temperature, animal activity, etc.) than deep soils. This in turn could select for surface soils with similar disturbance resistant communities (Steenwerth et al. 2008). Because the trends between variation in soil microbial beta-diversity and habitat and depth are not significant, additional sampling is needed to verify if this relationship is being obscured by low sample size or the scale of sampling (Martiny et al. 2011).

**Future Directions**

One of the underappreciated consequences of annual grassland invasions into native woody habitats is the alteration of deep soil communities. Although deep soils do not contain as much organic matter as surface soils, there are considerable pools of organic C and N (Jobbágy and Jackson 2001) that are effected by invasions (Rau et al. 2011). Deep soil communities have less biomass meaning that individual microbes likely have a larger effect on biogeochemical cycling in these soils (Stone, DeForest, and Plante 2014; Blume et al. 2002; Fierer, Schimel, and

Holden 2003b; Taylor et al. 2002). Invasive annual grassland soils have lower diversity than those of native woody habitats, and habitats affect diversity throughout the soil profile. Microbes are key players in carbon and nitrogen cycles, and shifts in microbial biodiversity could alter ecosystem functioning. Grassland invasions into woody habitat are known to alter carbon and nitrogen cycling (Hawkes et al. 2005; Parker and Schimel 2010) and soil storage in surface soils (Dickens et al. 2013). Because changes in microbial community can result in functional changes in ecosystems (Reed and Martiny 2013), future research on the ecological impact of grassland invasion on ecosystem processes should consider the vertical consequences of invasion.

**Figures**

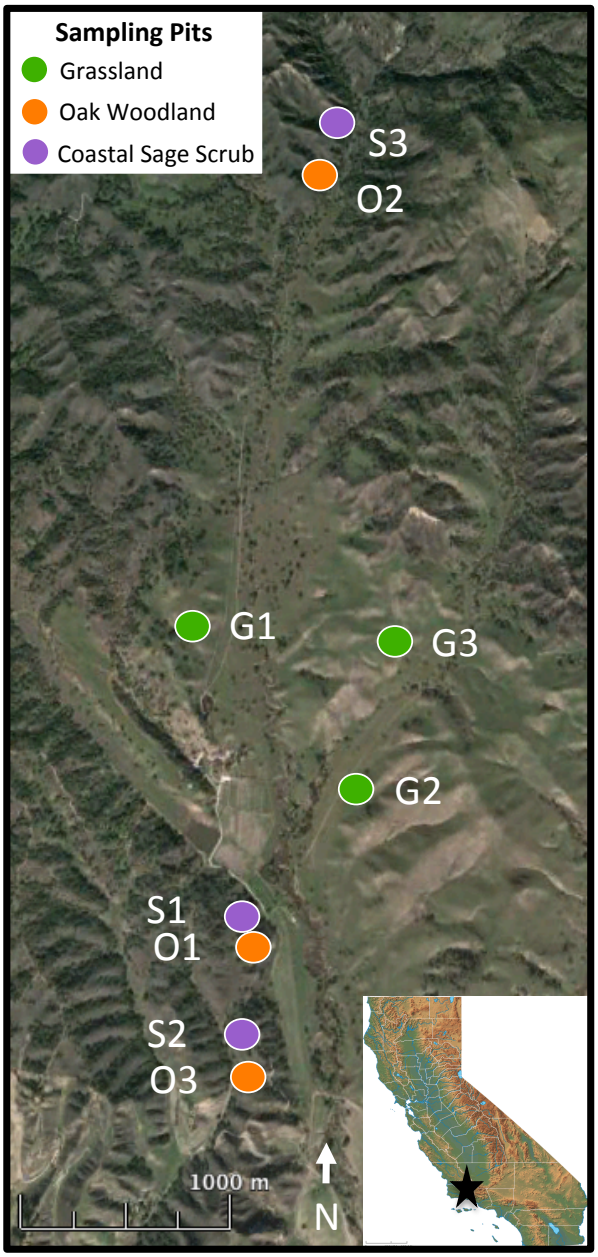


**Figure 1**. Sampling site locations at Sedgwick Reserve in the Santa Ynez Valley, California.

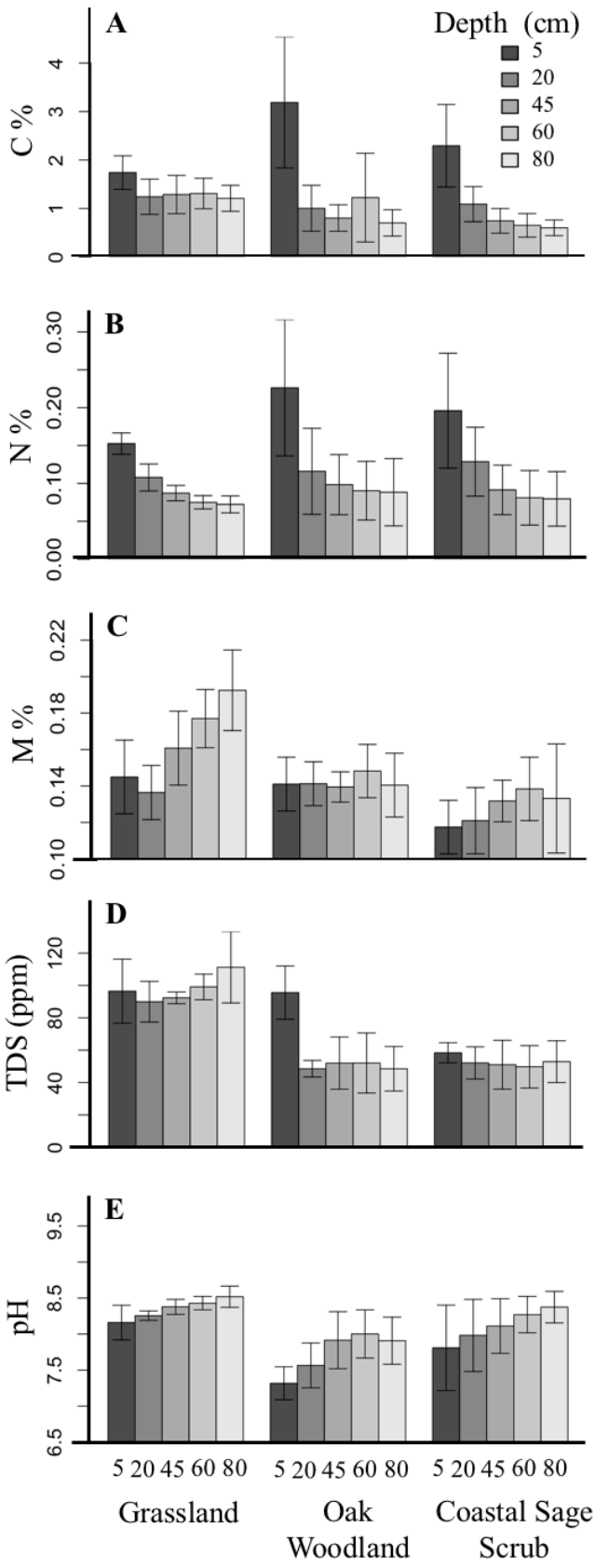
A
Depth (cm)
5
20
45
60
80
C %
0
1
2
3
4
B
N %
0.00
0.10
0.20
0.30
C
M %
0.10
0.14
0.18
0.22
D
TDS (ppm)
0
40
80
120
E
pH
6.5
7.5
8.5
9.5
5 20 45 60 80
5 20 45 60 80
5 20 45 60 80
Grassland
Oak Woodland
Coastal Sage Scrub

**Figure 2.** Differences in soil variables by habitat and depth. All soil variables had significant effects of sampling pit. A) The percent total carbon (C %) decreased with depth ($p = 4.875e-08$) and differed by the interaction between depth and habitat ($p= 0.015$). B) The percent total carbon (N%) also decreased with increasing depth ($p = 4.441e-16$). C) Soil moisture (M %) increased with depth ($p = 0.092$) and differed by the interaction between depth and habitat ($p= 0.00027$). D) Total dissolved solids (TDS) differed by habitat type ($p = 0.021$) and the interaction between depth and habitat ($p = 0.001$). Soil pH increased with depth ($p = 1.887e-14$) and differed by the interaction between depth and habitat ($p= 0.035$).

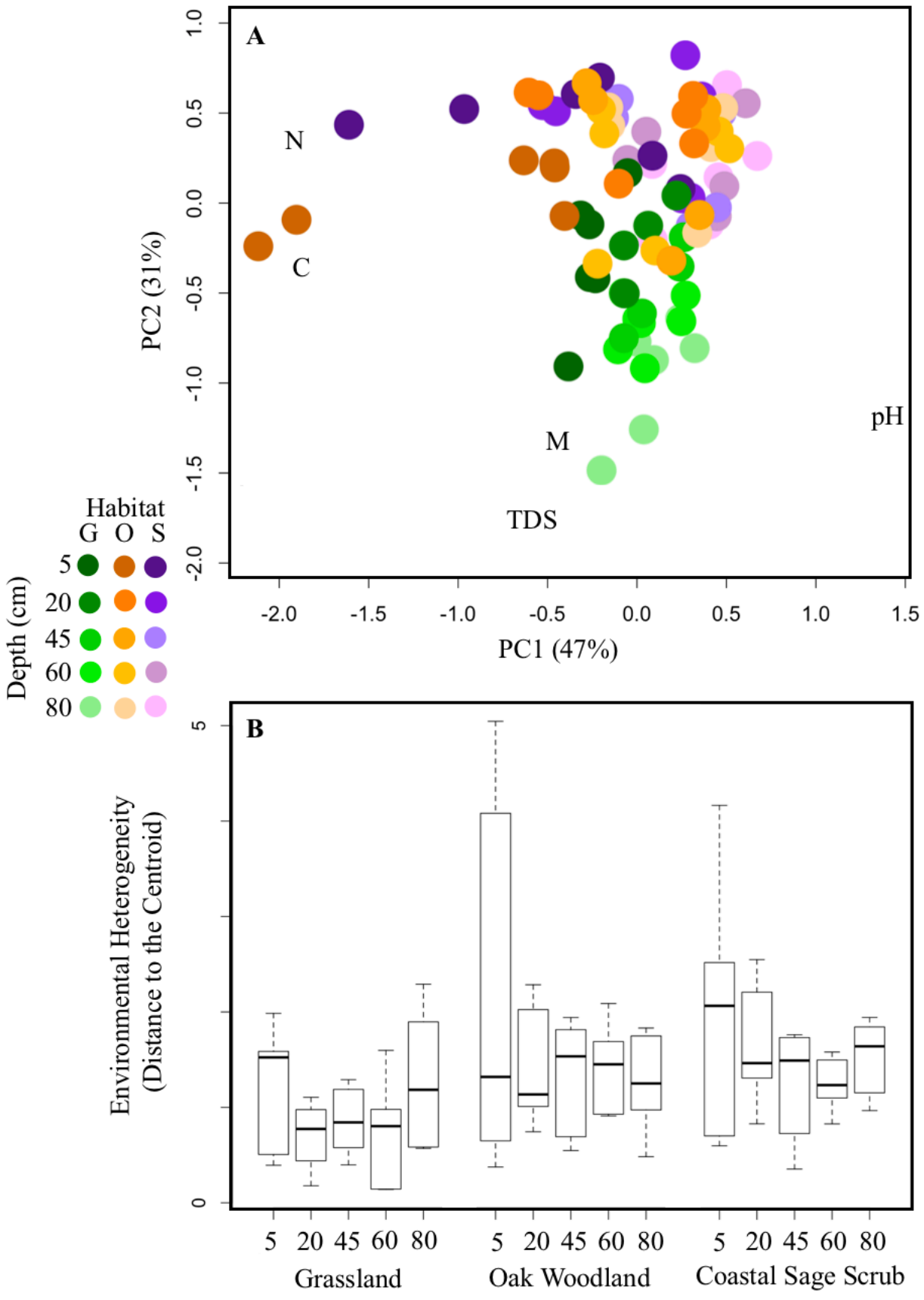
A
N
C
M
pH
TDS
PC2 (31%)
PC1 (47%)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Habitat
G O S
Depth (cm)
5
20
45
60
80
B
Environmental Heterogeneity
(Distance to the Centroid)
5
0
5 20 45 60 80
5 20 45 60 80
5 20 45 60 80
Grassland
Oak Woodland
Coastal Sage Scrub

**Figure 3.** Relationships between the combined soil variables and habitat and depth. A) The principal components analysis of scaled and centered soil variables. B) The distance to the centroid of samples grouped by habitat and depth. Across all sample groups, there were differences in heterogeneity by habitat type ($p = 0.08729$), but not depth. Between pairs of habitats, there are differences in heterogeneity between grassland and coastal sage scrub ($p = 0.04695$) and grass and oak woodland ($p = 0.0419$), but not between oak woodland and costal sage scrub.

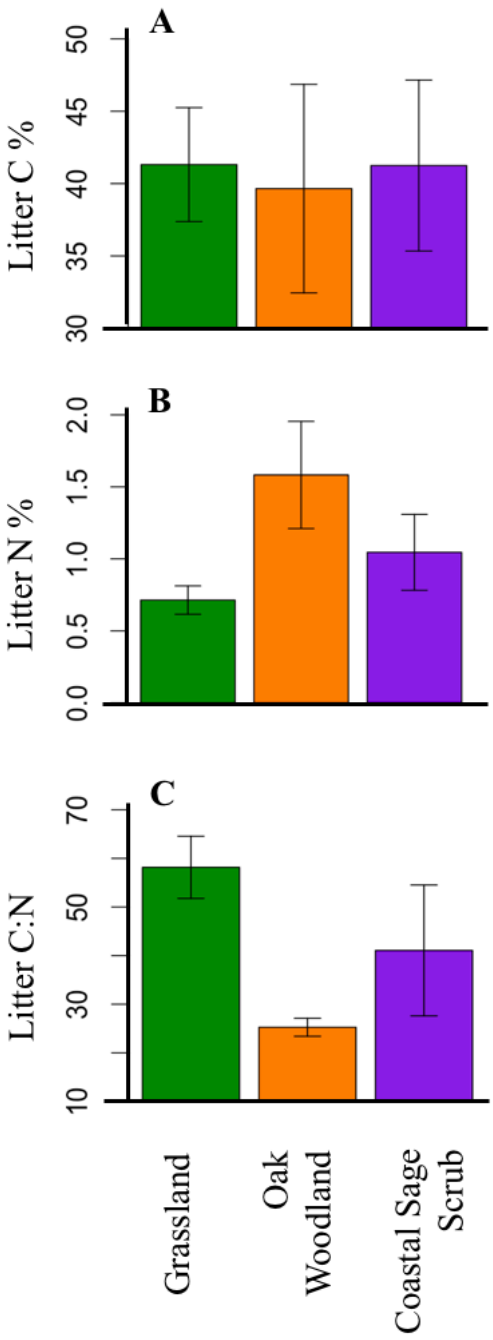


**Figure 4.** Differences in litter resources by habitat. A) The percent of litter carbon did not change with habitat ($p = 0.906$). B) The percent of litter N differed between habitats ($p = 0.012$) and was higher in oak woodland than grassland ($p = 0.010$) or coastal sage scrub ($p = 0.073$). C)

The ratio of litter carbon and nitrogen differed by habitats ($p = 0.006$) and was greater in grassland than oak woodland ($p = 0.005$) or coastal sage scrub ($p = 0.076$).

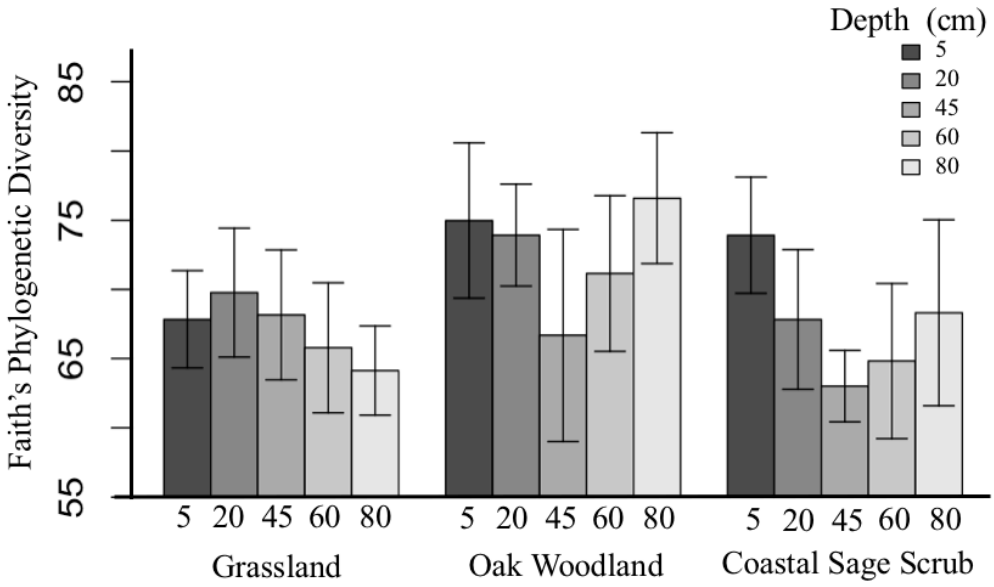


**Figure 5.** Habitat by depth differences in Species diversity measured by Faith's Phylogenetic Diversity. Two other diversity metrics were explored; Shannon Entropy and the number of observed species. All metrics were strongly correlated (minimum $r^2 = 0.91$, $p < 0.001$) and only Faith's Phylogenetic Diversity is shown here.

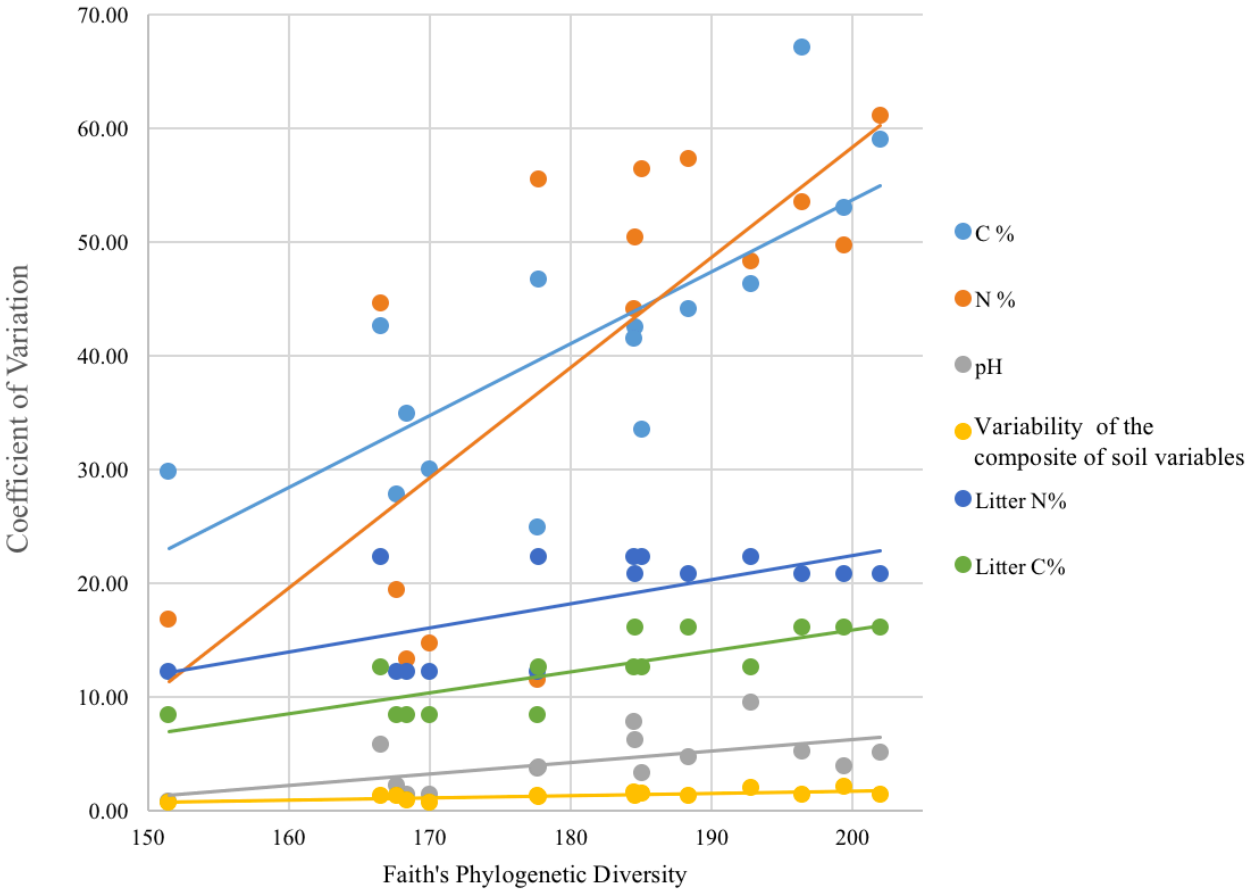


**Figure 6.** Comparisons between the average of Faith's Phylogenetic Diversity and the coefficient of variation (CV) for individual environmental and litter variables by habitat and depth. There is a positive relationship between the total Phylogenetic Diversity within soil groups and the coefficient of variation within groups for percent soil carbon (multiple $r^2 = 0.566$, $p = 0.0012$), percent soil nitrogen (multiple $r^2 = 0.541$, $p = 0.0017$), pH (multiple $r^2 = 0.3499$, $p = 0.0202$), the variability of the composite of soil variables calculated from median distance to the centroid in the PCA of soil variables (multiple $r^2 = 0.566$, $p = 0.0012$), the CV of litter nitrogen (multiple $r^2 = 0.4274$, $p = 0.0082$), and the CV of litter carbon (multiple $r^2 = 0.6538$, $p = 0.0003$). The CVs of the variables showed no relationship with phylogenetic diversity (data not shown).

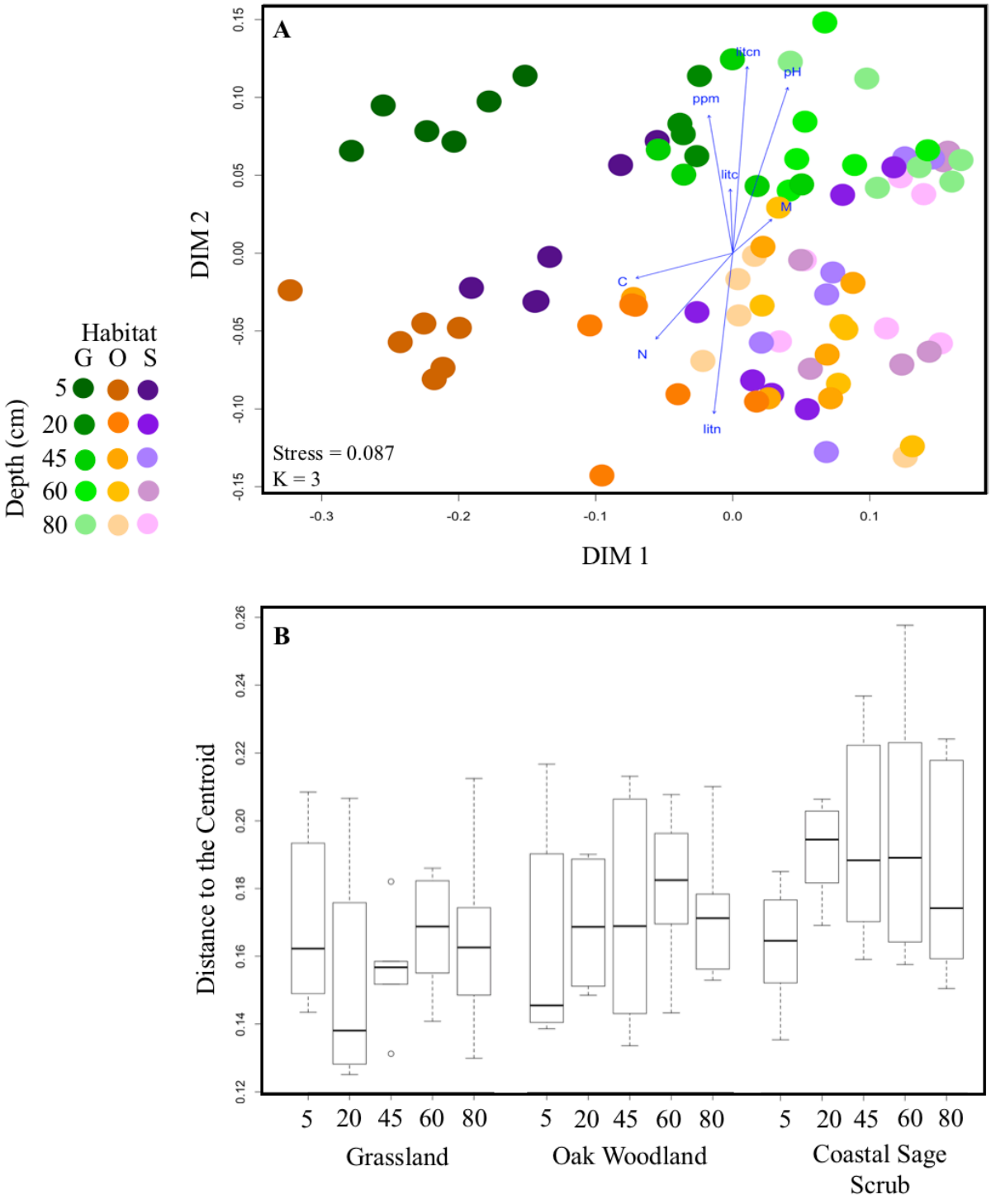


**Figure 7.** Microbial community composition by habitat and depth. A) The NMDS of weighted Unifrac distances between communities. Vectors for soil and litter variables indicate maximum correlations with community composition; percent soil carbon (C) r2 = 0.263, $p < 0.001$; percent

soil nitrogen (N) $r^2 = 0.311$, $p < 0.001$; soil pH $r^2 = 0.650$, $p < 0.001$; total dissolved solids (ppm) $r^2 = 0.409$, $p < 0.001$; percent soil moisture (M) $r^2 = 0.0065$, $p = 0.061$; percent litter nitrogen (litn) $r^2 = 0.543$, $p < 0.001$; percent litter carbon (litc) $r^2 = 0.082$, $p < 0.029$; litter carbon: nitrogen (litcn) $r^2 = 0.727$, $p < 0.001$. B) The distance to the centroid of communities grouped by habitat and depth.

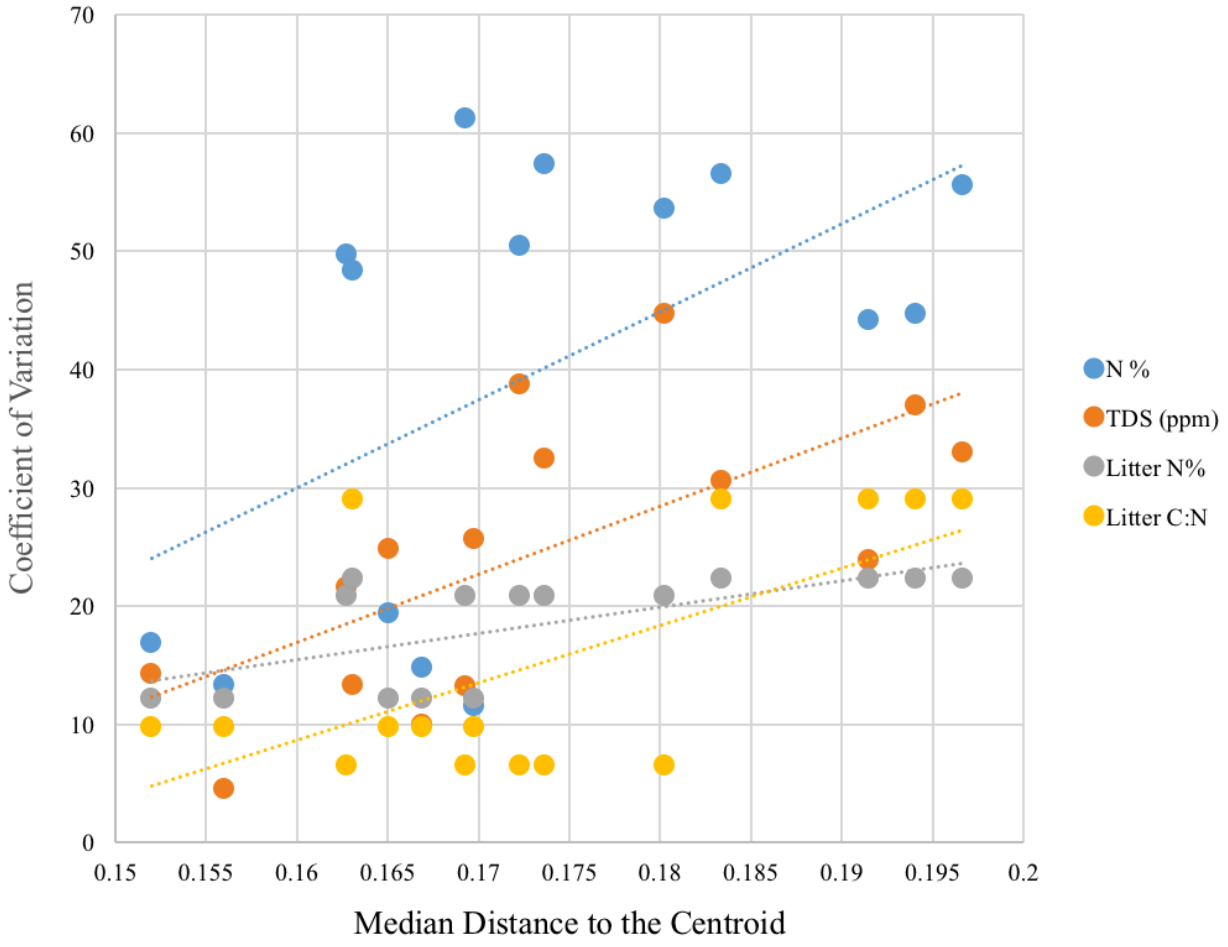


**Figure 8.** Comparisons by habitat and depth between the variation in microbial community compositional variability and the coefficient of variation (CV) for individual environmental and litter variables by habitat and depth. The variation in microbial community compositional variability was calculated from the median group distance to the centroid in the NMDS of the weighted Unifrac distances between communities. There was a positive relationship between the CV and community heterogeneity for soil nitrogen (multiple $r^2 = 0.290$, $p = 0.0382$) and TDS (multiple $r^2 = 0.445$, $p = 0.0066$), and the CV of litter C:N (multiple $r^2 = 0.404$, $p = 0.0108$) and N (multiple $r^2 = 0.4331$, $p = 0.007$). The other CVs of the variables showed no relationship with median distance to the median (data not shown).

**Table 1. Sampling site information**

| Site Name | Habitat | Plant Species | Plant litter | Date | Depth (cm) | Carbonates | Latitude | Longitude | Elevation (m) | % Slope | Slope Faces | Misc |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G01** | Non-native | *Bromus madritensis* | grass thatch | 5/21/11 | 5 | + | 34°41'45.99"N | 120° 2'33.76"W | 373 | 30-45 | East | No recent grazing |
| | annual | *Bromus diandrus* | 1 - 2 cm | | 20 | + | | | | | | Coastal sage scrub |
| | grassland | *Gnaphalium* | | | 45 | + | | | | | | < 300 ft |
| | | *Sillene galica* | | | 60 | + | | | | | | Oak trees |
| | | | | | 80 | + | | | | | | < 100 ft |
| **G02** | Non-native | *Avena spp.* | grass thatch | 5/23/11 | 5 | + | 34°41'32.91"N | 120° 1'56.21"W | 359 | 30-45 | North | Recent grazing |
| | annual | *Bromus madritensis* | 1 - 2 cm | | 20 | + | | | | | West | Oak trees |
| | grassland | *Bromus hordeaceus* | | | 45 | + | | | | | | < 100 ft |
| | with native | *Erodium spp.* | | | 60 | + | | | | | | |
| | component | *Hazardia spp.* | | | 80 | + | | | | | | |
| **G03** | Non-native | *Avena spp.* | grass sparse | 5/23/11 | 5 | - | 34°41'56.75"N | 120° 1'53.61"W | 378 | 15-30 | South | Recent grazing |
| | annual | *Medicago polymorpha* | < 1cm | | 20 | - | | | | | East | Coastal sage scrub |
| | grassland | *Bromus hordeaceus* | | | 45 | + | | | | | | < 250 ft |
| | | *Brassica spp.* | | | 60 | + | | | | | | Coastal sage scrub |
| | | | | | 80 | + | | | | | | < 250 ft |
| **O01** | Coastal live | *Quercus agrifolia* | oak dominated | 6/5/11 | 5 | - | 34°41'6.95"N | 120° 2'17.47"W | 353 | 30-75 | North | |
| | oak woodland | *Carduus pycnocephalus* | 1 - 2 cm | | 20 | - | | | | | East | |
| | | | | | 45 | - | | | | | | |
| | | | | | 60 | - | | | | | | |
| | | | | | 80 | - | | | | | | |
| **O02** | Coastal live | *Quercus agrifolia* | oak dominated | 6/10/11 | 5 | - | 34°43'11.48"N | 120° 2'12.84"W | 389 | 15-30 | South | |
| | oak woodland | Yucca whipplei | > 3 cm | | 20 | - | | | | | East | |
| | | *Bromus hordeaceus* | | | 45 | - | | | | | | |
| | | | | | 60 | - | | | | | | |
| | | | | | 80 | - | | | | | | |
| **O03** | Coastal live | *Quercus agrifolia* | oak dominated | 6/10/11 | 5 | - | 34°40'44.77"N | 120° 2'16.63"W | 369 | 30-75 | North | |
| | oak woodland | *native grass* | 1 - 2 cm | | 20 | - | | | | | West | |
| | | | | | 45 | + | | | | | | |
| | | | | | 60 | + | | | | | | |
| | | | | | 80 | + | | | | | | |



**Tables**

**Table 1 (cont). Sampling site information**

| Site Name | Habitat | Plant Species | Plant litter | Date | Depth (cm) | Carbonates | Latitude | Longitude | Elevation (m) | % Slope | Slope Faces | Misc |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **S01** | Coastal | *Salvia leucophylla* | *S. lucophylla* | 6/5/11 | 5 | - | 34°41'8.13"N | 120° 2'18.33"W | 346 | 30-75 | South | |
| | sage scrub | *Artemisia californica* | A. californica | | 20 | - | | | | | West | |
| | | *Pentagramma triangulari* | < 1cm | | 45 | - | | | | | | |
| | | moss sp | | | 60 | - | | | | | | |
| | | | | | 80 | - | | | | | | |
| **S02** | Coastal | *Salvia leucophylla* | *S. lucophylla* | 6/5/11 | 5 | + | 34°40'53.14"N | 120° 2'16.80"W | 372 | 30-75 | South | Soil contained |
| | sage scrub | *Artemisia californica* | < 1cm | | 20 | + | | | | | West | numerous rocks |
| | | | | | 45 | + | | | | | | |
| | | | | | 60 | + | | | | | | |
| | | | | | 80 | + | | | | | | |
| **S03** | Coastal | *Salvia leucophylla* | *S. lucophylla* | 6/10/11 | 5 | - | 34°43'18.49"N | 120° 2'11.13"W | 402 | 30-75 | South | |
| | sage scrub | nonnative grass | old branches | | 20 | - | | | | | West | |
| | | | <1cm | | 45 | - | | | | | | |
| | | | | | 60 | - | | | | | | |
| | | | | | 80 | - | | | | | | |

**Table 2**. Coefficients of Variation for Soil Variables by Habitat and Depth

| | | Coefficient of Variation | | | | |
|---|---|---|---|---|---|---|
| Habitat | Depth | C % | N % | M % | TDS (ppm) | pH |
| Grassland | 5 | 24.88 | 11.47 | 17.43 | 25.60 | 3.67 |
| | 20 | 29.82 | 16.83 | 11.09 | 14.26 | 0.81 |
| | 45 | 34.96 | 13.27 | 14.38 | 4.46 | 1.42 |
| | 60 | 30.06 | 14.72 | 11.27 | 9.98 | 1.37 |
| | 80 | 27.78 | 19.36 | 14.34 | 24.73 | 2.16 |
| Oak Woodland | 5 | 52.99 | 49.68 | 13.08 | 21.49 | 3.93 |
| | 20 | 58.97 | 61.16 | 10.66 | 13.17 | 5.13 |
| | 45 | 42.56 | 50.39 | 7.45 | 38.73 | 6.24 |
| | 60 | 67.12 | 53.52 | 12.32 | 44.61 | 5.22 |
| | 80 | 44.16 | 57.36 | 14.21 | 32.39 | 4.71 |
| Coastal Sage Scrub | 5 | 46.32 | 48.31 | 15.63 | 13.27 | 9.49 |
| | 20 | 41.47 | 44.11 | 18.76 | 23.86 | 7.84 |
| | 45 | 42.66 | 44.66 | 10.82 | 36.93 | 5.85 |
| | 60 | 46.75 | 55.54 | 15.70 | 32.91 | 3.81 |
| | 80 | 33.56 | 56.46 | 28.07 | 30.51 | 3.26 |

**Table 3** Coefficients of Variation for Plant Litter by Habitat and Depth

| | Coefficient of Variation | | |
|---|---|---|---|
| Habitat | Litter C% | Litter N % | Litter C:N |
| Grassland | 8.42 | 12.18 | 9.72 |
| Oak Woodland | 16.07 | 20.78 | 6.47 |
| Coastal Sage Scrub | 12.65 | 22.26 | 28.98 |

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CHAPTER 2

# The relationship between soil enzyme activity and microbial composition in invasive annual grassland and native woody habitats in California's Mediterranean ecosystem

## Introduction

Plant invasions frequently alter ecosystem processes (Cardinale 2012) by changing above and below ground communities of organisms (Bohlen 2006; D'Antonio and Vitousek 1992). Plants exert control over soil microbial community composition and function in soils through the addition of plant litter (Ball, Carrillo, and Molina 2014), alteration of soil resources (Dickens et al. 2013), and root interactions with microbial communities (Ladygina and Hedlund 2010). Plant invasions that lead to changes in litter and soil resource distributions have significant consequences for decomposition and nutrient cycling (Gessner et al. 2010) in part because they modify soil microbial communities.

Soil microbial communities decompose the bulk of terrestrial organic matter and are important drivers of nutrient cycling. Microorganisms participate in organic matter decomposition by producing and releasing extracellular enzymes. These enzymes break down complex organic molecules into simple molecules that are then assimilated into microbial cells (Burns 1978; Sinsabaugh 1992). The amount of extracellular enzymes found in soils is related to several factors. Microorganisms, and in particular bacteria and fungi, produce enzymes in response to the abundance and type of organic material present soils (Allison and Vitousek 2005; Shackle, Freeman, and Reynolds 2000; Sinsabaugh et al. 2008). Plants influence extracellular activity through root exudates (Brzostek et al. 2013; Yin, Wheeler, and Phillips 2014; Cheng et al. 2014), as does microbial demand for nutrients (Allison and Vitousek 2005) and soil properties

(Sinsabaugh and Follstad Shah 2012). Plants exert control over organic matter and soil properties and thus, plants directly and indirectly influence enzyme activity.

Invasions of annual grasses into native habitat are occurring across California's Mediterranean ecosystem (Underwood et al. 2009; D'Antonio and Vitousek 1992), and climate change is predicted to exacerbate this trend (Pfeifer-Meister et al. 2016). These invasions often result in nonnative grassland habitats with shallower and denser roots (Pinno and Wilson 2013; Goldstein and Suding 2014; Ward, Wiegand, and Getzin 2013) that produce more and lower quality litter (D'Antonio and Vitousek 1992; Dickens et al. 2013). These changes can lead to differences in the vertical stratification of organic matter and physical conditions (Rau et al. 2011; Goldstein and Suding 2014).

At the same time, previous studies suggest that conversion to annual grassland also alters the soil microbial community (Chapter 1, (Waldrop and Firestone 2006; Fierer, Schimel, and Holden 2003)). Extracellular enzyme activity in the surface soil of these habitats differ (Waldrop and Firestone 2006). Across soils, extracellular enzyme activity generally declines significantly with soil depth (Sardans and Peñuelas 2005; Stone, DeForest, and Plante 2014; Taylor et al. 2002; Venkatesan and Senthurpandian 2006; Waring, Weintraub, and Sinsabaugh 2013) as does microbial biomass (Eilers et al. 2012; Fierer, Schimel, and Holden 2003; Taylor et al. 2002). However, because fewer microorganisms produce enzymes in deep soil, individual microorganisms could have a greater influence over organic matter decomposition relative to those in surface soils (Stone, DeForest, and Plante 2014). Microbial community changes in deep soil could greatly influence microbial community function. Understanding how the relationship between habitat type and soil community composition influences the function of soil

communities (Schimel and Schaeffer 2012; Prosser 2012; Martiny et al. 2015) is important in understanding how grassland invasions alter below ground processes throughout the soil profile.

Here I investigate the relationship between extracellular enzyme activity and microbial community composition in three Mediterranean ecosystems in California; invasive annual grasslands, oak woodlands, and coastal sage scrub. The objectives of this study are three fold: 1) to examine differences in extracellular enzyme activities between annual grasslands oak woodlands, and coastal sage scrub; 2) to investigate whether bacterial and archaeal community composition correlates with extracellular enzyme activities and 3) to determine the composition of microorganisms with traits for extracellular enzyme production changes between invasive grasslands and native woody habitat. Because soil resources and litter in annual grasslands differ more with woody habitats than they do between woody habitats (see Chapter 1), I hypothesized that annual grasslands will have different extracellular enzyme depth profiles than the native woody habitats throughout the soil profile (5, 20, 45, 60, 80 cm). I also hypothesized that extracellular enzyme activity and soil community composition would be weakly correlated, because soil communities and the distribution of soil resources show different relationships with habitat (see Chapter 1). Finally, I expected that the communities of bacteria and archaea with traits for extracellular enzyme production would be similar between habitats despite differences in community composition and possible differences in extracellular enzyme activities. Similar groups of microorganisms can assimilate the relatively simple molecules derived from extracellular enzyme decomposition in grassland and oak woodland soils (Schimel and Gulledge 1998; Waldrop and Firestone 2004), and genes for extracellular enzymes are broadly, but non-randomly, distributed across the genomes of microbial phyla (Berlemont and Martiny 2013; Berlemont and Martiny 2015; Zimmerman, Martiny, and Allison 2013).

## Materials and Methods

For sampling design and characterization of the soil environmental and microbial communities, please see the materials and methods section in Chapter 1.

*Extracellular Enzyme Assays*

Microbial community function was determined based on the activities of six hydrolytic extracellular enzymes. I assayed soils for enzyme activity related to the degradation of: 1) carbohydrates by α-1,4-glucosidase (AG), 2) cellulose by cellobiohydrolase (CBH), 3) cellobiose by β-1,4-glucosidase (BG); 4) hemicellulose by β-1,4-xylosidase (BX), 5) chitin and peptidoglycan by β-N-acetylglocosaminidase (NAG), and 6) organic phosphates (phospholipids and phosphosaccharides) by alkaline phosphatase (AP). Extracellular enzyme activities were determined fluorometrically using 4-methylumbelliferyl (4-Mub) hydrochloride-linked substrates (DeForest 2009). See Table 1 for additional details about the extracellular enzymes.

Assays were conducted as follows. One gram of soil was homogenized in 50 mM acetate buffer. 100 uL of the homogenate was added to 50 uL of 200uM substrate and 50 uL of 50 mM acetate buffer in the wells of a 96 well plate. Each soil sample assay was replicated eight times. Assays were incubated for 30 min (NAG, AP), or 2 hours (AG, BG, CBH, BX) at room temperature. Prior to measuring florescence (Excitation 360 nm, Emission 465 nm) on a GENios Plus Multifunction florescence microplate reader (Tecan, Grodig, Austria), 10 uL of 1N NaOH was added to each well. Standards of 4-Mub (0-10 uM) were included on each plate and used to determine the concentration of substrate cleaved during the assay. All EEA were normalized by soil weight and are expressed in units of nmol $hr^{-1}$ g $soil^{-1}$.

*Metagenomic Shotgun Sequencing*

I identified the taxa that contain genes involved in extracellular enzyme production, using metagenome shotgun sequencing for three top soil (5 cm) microbial communities; one from each habitat. Metagenomic sequencing reads were generated on a Roche 454 using GS FLX+ chemistry (Roche 454, Branford, CT, USA). After reads were generated, they were demultiplexed and quality filtered to a minimum quality score of 30, using Galaxy (Goecks et al. 2010; Giardine et al. 2005; Blankenberg et al. 2001). Sequences were uploaded to the MG-Rast metagenomics analysis server (Meyer et al. 2008) where they were annotated and analyzed for the relative abundance of phyla (by total sequence content for all reads). In addition to identifying genes responsible for extracellular enzyme production (see Table 1 for Enzyme Classification numbers), I identified three highly conserved genes chosen from the hypothetical minimum bacterial gene set (Gil et al. 2004) to serve as control genes: cysS or cysteine-tRNA ligase (EC 6.1.1.16), pepA or leucyl aminopeptidase (EC 3.4.11.1), and dnaE or DNA polymerase III, subunit (EC 2.7.7.7). Sequencing reads identified as genes of interest based on the UniProt database were mapped to the NCBI nr database by BLAST using the nucleotide megablast default parameters. Reads were retained if they were 50 bp or longer and had a BLAST expected value of 1E-20 or better. The best hit for each read was identified to phylum.

*Statistical Analysis*

Extracellular Enzyme Activities by Habitat and Depth. I analyzed the extracellular enzyme activities only in samples that had been characterized for microbial community composition as described in Chapter 1 (n = 86). Individual extracellular enzyme activities were log transformed prior to data analysis to increase symmetry in the data set. I used linear mixed models with a

quadratic term to investigate habitat and depth related differences in extracellular enzyme activity. These models included sampling location as a random variable and habitat and depth as predictor variables. Initial models included all variables, but only the best models were retained (Kuznetsova, Brockhoff, and Christensen 2013). I compared ratios of enzyme activities in order to determine if microorganisms were expending resources to acquire organic C relative to organic N ln(BG:NAG) or P ln(BG:AP), and organic N relative to organic P ln(NAG:AP) (Sinsabaugh et al. 2008). Differences between ratios by habitat and depth were determined as detailed above. I investigated differences in total extracellular enzyme activities by running a Principal Components Analysis (PCA) on the log transformed enzyme data. The amount of variation in total enzyme activities explained by habitat and depth was calculated using the ADONIS function PERMANOVA in vegan (Dixon 2003).

Relationship Between Community Composition and Extracellular Enzyme Activities. To assess the relationship between community composition and total extracellular enzyme activity, I ran Mantel tests in vegan. Community composition was based on weighted Unifrac distances for microbial sequences at 97% identity. The environment can contribute to extracellular enzyme activity and community composition, therefore, I also ran a partial Mantel test (Dixon 2003) that controlled for the effect soil environmental variables on the relationship between enzyme activity and community composition. The Partial mantel test was preformed using a Euclidean distance for scaled values of soil moisture content, percent carbon and nitrogen, pH, total dissolved solids (TDS) on the relationship between enzyme activity and community composition. I also determined environmental correlates of extracellular enzyme activity using Pearson's correlations between individual activities and environmental variables (soil moisture content,

percent carbon and nitrogen, pH, total dissolved solids (TDS) and litter percent carbon, percent nitrogen, and C:N) and patterns of extracellular activates and environmental variables using envfit function in vegan on the PCA of enzyme activity.

Microorganisms with Traits for Extracellular Enzyme Production. I identified the relative abundances of phyla that contain gene reads for extracellular enzymes and control genes, and generated a Hellinger transformed distance for the relative abundance of phyla by habitat and gene. Differences in phyla between habitat and gene combinations were visualize using PCA with standard error ellipses for each gene. I used ANOSIM in vegan (Dixon 2003) to determine differences in phyla by gene and habitat. I excluded the sequence data for total MG-Rast reads because the distribution of dissimilarities for this gene, as determined using betadisper in vegan, was significantly different from those of the other genes. I also ran linear models on the relative abundance data to assess the associations between phylum, gene and habitat.

**Results**

*Extracellular Enzyme Activities by Habitat and Depth*

Soil communities release extracellular enzymes into the environment where they breakdown large organic molecules into smaller pieces that can be absorbed and metabolized. In this study, I measured the activity of six hydrolytic extracellular enzymes. Summing over all enzymes and controlling for significant pit effects, depth had a linear ($p < 2e\text{-}16$) and a nonlinear ($p = 7.05e\text{-}10$) effect on total enzyme activity as did habitat type ($p = 0.005$). Grasslands had the highest total enzyme activity and oak woodlands and coastal sage scrub had similar amounts of enzyme activities. Enzyme activity decreased with depth in a sigmoidal manner.

I next investigated activities for individual extracellular enzymes. Enzyme extracellular enzyme activities were highly correlated, with the strongest correlations between for organic carbon cycling enzymes ($r = 0.92$ to 0.96, P value $<0.001$), but also between C and N ($r = 0.78$ to 0.86, P value $<0.001$), C and P ($r = 0.69$ to 0.79, P value $<0.001$) and N and P enzymes ($r = 0.75$, P value $<0.001$). Enzyme activities for all enzymes differed, after controlling for significant sampling pit effects, with habitat type, depth (with linear and nonlinear effects), or the interaction between habitat and depth (Figure 1).

Grassland soils typically had higher extracellular enzyme activities than oak woodland or coastal sage scrub. In the top 5 cm soil, grassland enzyme activity was between 3 and 6.8 fold more for carbon cycling enzymes than oak woodland, and between 4.5 and 7.7 fold more than for coastal sage scrub. The difference for the same enzyme activities between oak woodland and coastal sage scrub is less than 1.8 fold, where oak woodland had the highest activity. For NAG and AP, grassland and oak woodland had similar enzyme activities (equivalent for NAG, and 1.08 fold for AP where oak woodland had a higher activity). Coastal sage scrub activities were about 2.5 fold less for NAG and 1.8 fold less for AP than the other habitats. At 80 cm soil, grassland activities were the greatest of all of the six extracellular enzyme activities measured, between 1.6 - 3.7 fold more than oak woodland, and between 2.2 - 4.8 fold more than coastal sage scrub. At 80 cm, oak woodland enzyme activities were between 1.1 - 1.7 fold greater than for those of coastal sage scrub.

In grassland the C cycling enzymes activity declined more than 10 fold from 5 to 80 cm (between 10.9 - 18.7 fold), and for NAG enzyme activity declined by a factor of 2.4 from 5 to 80 cm. AP in grassland is the exception to the trend, where there was little change in average enzyme activity with between 5 and 80 cm (1.09 – 1.4 fold decrease). The declines in activity in

oak woodland and coastal sage scrub were more similar in magnitude. For C cycling enzymes there was typically less than a 10 fold decrease between 5 and 80 cm (between 5.4 - 11.7 in oak woodland, and 5.2 - 9.5 in coastal sage scrub). For NAG and AP, both woody habitats had a larger fold decrease with depth relative to grassland (NAG decreased 5.1 fold in oak woodland and 3.4 fold in coastal sage scrub; AP decreased 3.2 fold in oak woodland and 2.7 fold in coastal sage scrub).

Ratio of Extracellular Activity by Habitat and Depth. Although extracellular enzyme activities may differ across sample types, it is still possible that the nutrient acquisition strategy between samples is similar. For example, a soil at one site may have low activity relative to another site, but they may both show relatively similar ratios of C and N acquiring enzyme activates. These strategies can indicate the availability or nutritional demand for a specific resource (Sinsabaugh et al. 2008). Here I investigated the relative activities of enzymes for organic C, N and P (Figure 2) across habitats and depths. The acquisition strategy by habitat type and depth varied across enzyme ratio (Figure 2). There were nonlinear differences in ln(BG:NAG) by depth that varied by habitat type, and depended on sampling pit. Samples closer to the surface had higher BG than NAG activities for all habitats, but to a greater depth for grassland (5 – 45 cm), followed by oak woodland (5 and 20 cm) and coastal sage scrub (5 cm). Deep grassland and oak woodland (60 and 80 cm), and coastal sage scrub (>5cm) had relatively equivalent BG and NAG activity. There were also nonlinear differences in ln(BG:AP) with depth and random effects of sampling location and habitats differed. Only one community type, grassland at 5 cm, had higher activity for BG than AP. With the exception of oak woodland, the ratio of ln(BG:AP) declined monotonically with depth. The ratio of ln(NAG:AP) differed only by depth. None of the

community types had higher activities of NAG relative to AP. In grassland this ratio declined after 5 cm, and samples from 20 – 80 cm had similar values. Coastal sage scrub ratios declined after 45 cm, but 5 – 45 cm were similar and 60 and 80 cm were also similar. The ratio in oak woodland declined after 5 cm, and the ratio at 20 cm was similar to those at 45 – 80 cm. There was considerable overlap in ln(NAG:AP) across habitats at similar depths.

Microbial Community Extracellular Enzyme Profile. I used PCA to explore patterns of total extracellular enzyme activity across microbial communities by habitats and depths (Figure 3). The ordination shows that most of the variation in extracellular enzyme activity is explained by PC 1 (89.59%) and much less of the variation is explained by PC 2 (4.71%). Using ADONIS, much of the variation in extracellular enzyme activity between samples could be explained by habitat ($r^2$= 0.315, p < 1e-04) and depth ($r^2$= 0.406, p < 1e-04). When comparing pairwise differences between habitats, I found that very little of the variation between oak woodland and coastal sage scrub could explained by habitat differences (r2 = 0.06221, p < 0.0042). However, a larger amount of the variation between grassland and oak woodland ($r^2$= 0.260, p < 0.0001) or coastal sage scrub ($r^2$=0.400, p = 0.0001) could be explained by habitat.

Environmental correlates of extracellular enzyme activity. Because extracellular enzyme activity can be influenced by soil and litter characteristics, I looked for correlations between the two (Table 2). All carbon-degrading enzyme activity, except BG, increase as litter N decreases (r = -0.26 to r = -0.42). All extracellular enzyme activity increased as litter C:N (r = 0.24 to r = 0.47), electrical conductivity (r = 0.44 to r = 0.56), Soil C (r = 0.35 to r = 0.52), and soil N (with the exception of AP, r = 0.31 to r = 0.45) increase. AP and soil M increase (r = 0.28) concomitantly.

Several environmental variables correlated with the PCA of extracellular enzyme activity as determined by envfit (Figure 3); soil percent carbon, nitrogen, moisture content, total dissolved solids, litter percent nitrogen, and litter C:N.

*Relationship Between Microbial Community Composition and Extracellular Enzyme Activities*

The relationships between in microbial community extracellular enzyme activity and habitat and depth are much stronger to those seen for microbial community composition. In chapter 1 I found using PERMANOVA that a small amount of the variation in community composition can be explained by habitat type ($r2 = 0.116$, $p > 0.0001$), depth ($r2 = 0.1588$, $P > 0.0001$), and habitat type by depth interactions ($r2 = 0.0376$, $P = 0.004$). Pairwise comparisons between habitats showed that slightly less variation in community composition can be explained by habitat type for oak woodland and coastal sage scrub ($r2 = 0.067$, $p = 0.0004$), than oak woodland and grassland ($r2 = 0.107$, $p < 0.0001$) or coastal sage scrub and grassland ($r2 = 0.094$, $p < 0.0001$).

I explored the relationships between microbial community composition and extracellular enzyme activity. Extracellular enzyme activity had a strong relationship with community composition (Mantel statistic $r = 0.5416$, $p < 0.0001$). Because both extracellular enzyme activity and microbial community composition are sensitive to soil variables (for extracellular enzyme activity, Mantel statistic $r = 0.1841$, $p = 0.012$; for microbial community composition Mantel statistic $r = 0.3516$, $p < 0.0001$), I ran a partial mantel tests remove the influence of the soil environmental variables on the correlation between community composition and extracellular enzyme. Soil variables had little effect on the correlation between community composition and extracellular enzyme activity (Mantel statistic $r = 0.5183$, $p < 0.0001$).

*Microorganisms with Traits for Extracellular Enzyme Production*

The microbial community is responsible for producing many of the extracellular enzymes found in soils, and I identified extracellular enzyme genes in soil communities using genomic DNA shotgun sequencing. In total, 1,174,351 bp of DNA were sequenced from three top soil communities; one from each habitat type (Figure 3). Three percent of the reads were removed after quality control, and the total average sequencing read length was 490 bp long (Table 3). The total number of reads that matched the target genes are provided in Table 3. I identified phylum level relative abundance for reads identified as a putative match for each of the extracellular enzyme and control genes (Table 1). This data set did not contain any reads for CBH. This enzyme is likely produced primarily by fungi, although activity to cellobiose is documented for bacterial isolates (Woo et al. 2014). I had a very low prevalence of fungal reads (1.7% in both grassland and oak woodland and 1.9% in coastal sage scrub samples) in our metagenomes, so the lack of CBH in our data set is not entirely unexpected.

I explored the relationships between habitat, extracellular enzyme gene and the relative abundance of phyla that contained reads extracellular enzyme genes. The relative abundance of each phylum by gene and habitat can be found in Figure 4, and the relationships between gene community subsets using a PCA of relative abundance of phyla for each gene (Figure 5) where the standard error is indicated by ellipses. I used ANOSIM to determine if gene type or habitat best explained variation in phylum relative abundance. Significant differences in phyla were observed for gene identity (ANOSIM statistic = 0.832, $r < 1e\text{-}05$) but not habitat. I found that gene communities clustered independently of habitat types, and habitat type did not significantly associate with PC 1 or PC 2. PC 1 explained the majority of variation (35.5%) in gene

communities and was associated with gene identity ($p < 0.001$). Tukey's post hoc comparisons of differences among genes along PC 1 showed that AG is different (minimum P value < 0.01) from all genes but BG; AP is different (minimum P value < 0.01) from all genes but BX; BG is also different from BX (P value < 0.001), MG and cysS (P value < 0.05); BX is also different from cysS (P value < 0.05) and NAG (P value < 0.001); NAG is not significantly different from the control genes or reads consensus which are not significantly different from each other. This indicates that the total microbial community composition is very different from those that contain extracellular enzyme traits, with the exception of NAG.

The phyla that had the greatest loading on PC 1, Actinobacteria and Proteobacteria, also reflect differences in the relative abundance of phyla between genes; Actinobacteria reads are most prevalent in AG and and BG communities, whereas Proteobacteria are most prevalent in BX and AP gene communities. PC 2 explains less variation (21.6%) in gene communities, and gene identity is also associated with this axis (P value < 0.001). Post hoc comparisons show that only NAG differs from the other genes (minimum P value < 0.01) and Planctomycetes, which have reads that are abundant in NAG gene communities, and the greatest loading along PC 2. I also found that the relative abundance of phylum reads across all genes differed significantly by between genes but not by habitat, with the exception of the Actinobacteria which are significantly less abundant in oak woodland than in coastal sage scrub ($p < 0.04$). Within each phylum, post hoc tests that showed the reads did not differ significantly across communities identified by control genes. This contrast with reads for communities identified by genes for extracellular enzymes, which in general, were different from the total community and from other extracellular enzyme communities.

## Discussion

In this study I examined if habitat change due to annual grassland invasions alters soil community extracellular enzyme activity though the soil profile, how the differences in enzyme activity relate to microbial community composition, and whether the microorganisms that contain extracellular enzyme genes differ by habitat. I found support for our first hypothesis and found that annual grassland enzyme activities are much different from those of woody habitat. Although I found that with depth the differences in enzyme activity between habitats generally declined, I also found similar habitat differences for the ratios of ln(BG/NAG), ln(BG/AP), and ln(NAG/AP). Grassland samples show the most difference from woody habitats in surface soils but with depth enzyme ratios between habitats are similar. Contrary to our second hypothesis, there is a strong correlation between community composition and extracellular enzyme activity, and this relationship does not change when I control for soil environmental variables. I also found support for our third hypothesis were the relative abundance of phyla with genes for extracellular enzyme production are similar between habitats and individual genes have distinct relative abundances of phyla that contain those genes.

### *Extracellular Enzyme Activities by Habitat and Depth*

Of the habitats, annual grassland had the highest rates of total extracellular enzyme activity, followed by oak woodland and then coastal sage scrub. Although I expected differences between habitats, I did not expect grassland to produce the most enzyme. Previously, Waldrop et al (2004) found higher activity in oak woodlands rather than grassland surface soils across several of seasons. Enzyme activity for their June sampling showed the most similar activities habitats, but also the least activity relative to other time points. In the current study, there were

few if any grasses present under oak canopies, however grasses were present under oak canopies in the Waldrop et al. 2004 study. It is possible that this could generate additive or synergistic effects on enzyme activity due to mixed litter (Gartner and Cardon 2004) and biomass inputs, and potentially explain the difference in results between studies.

Litter type is very influential on microbial activity in topsoil (Kramer et al. 2013; Ball, Carrillo, and Molina 2014), and potentially throughout the soil profile. The sampling in this study coincided with the senescence of the majority annual grassland plants. All grass net primary productivity enters the litter at this time point (Wolkovich et al. 2010), which should increase available soil resources. By contrast oak woodlands contribute the most to litter in late winter to early spring (Mooney 1973), and so the litter collected for this study was relatively older and potentially already leached most of the easily accessed organic molecules into the soil. Coastal sage scrub has a summer leaf fall that begins around June (Gray 1982). Each year sage litter accounts for of roughly 60-76% of coastal sage scrub NPP (Gray and Schlesinger 1981; Wolkovich et al. 2010; Gray 1982). The time of sampling sage litter consisted primarily of sticks and twigs suggesting that the litter material was not from recent litter fall, and was probably poor in extractable compounds (Voriskova and Baldrian 2013). Also consistent with the idea that large amounts of fresh litter are driving the high activity in grasses relative to woody habitat, extracellular enzyme activity can be induced by the availability of a resource (Whitehead 1995; Barker et al. 1966; Baba, Shinke, and Nanmori 1994; Schönert, Buder, and Dahl 1999). For example, additions of simple carbohydrates, like Glucose, can increase the activity of AG and BG (Hernandez and Hobbie 2010). Fresh litter contains higher carbohydrate and starch content than old litter (Voriskova and Baldrian 2013), and grass leaves have higher amounts of hemicelluloses relative to oak and sage species (Schadel et al. 2010). This in

combination with a precipitation that occurred within a week of sampling the grassland soils, where late season precipitation in grasslands can increase carbon respiration (Chou et al. 2008), could also explain high activity of C cycling enzymes. Litter addition studies and also seasonal sampling of theses habitats would provide support for the explanations of these observations.

In addition to elevated activity of C cycling enzymes in grassland, there is also higher activity for N and P cycling enzymes. The nitrogen cycling enzyme, NAG, examined in this study is involved in degrading chitin and peptidoglycan. Chitin degrading activity is correlated with fungal biomass and activity (Andersson, Kjoller, and Struwe 2004), and could indicate different fungal bacterial ratios across habitat types. Grass litter has high C:N which may favor fungi (de Vries et al. 2006; Strickland and Rousk 2010). Invaded coastal sage scrub communities with high levels of annual grass litter had higher F:B ratios than undivided coastal sage scrub (Wolkovich et al. 2010). Larger proportions of fungi in grasslands would increase the availability of substrates NAG (Hernandez and Hobbie 2010), and explain the high NAG activity. Phosphatase activity is high across all habitats. High investment in P enzymes suggest P limitation (Cleveland and Liptzin 2007; Waring, Weintraub, and Sinsabaugh 2013), however, the availability of substrates can induce Phosphatase (Hernandez and Hobbie 2010). Because, I did not measure phosphate in soils, or test for the effects of phosphate addition it is not clear what is driving the relatively high AP activity.

In addition to the habitat specific effects on enzyme activity, I found that extracellular enzyme activity also declined with depth for all enzymes, with the exception of AP in grasslands, and the decline was habitat specific. This general decline of activity with depth is consistent with previous observations of decreasing extracellular enzyme with depth (Gittel, Barta, Kohoutova, Mikutta, et al. 2014; Gittel, Barta, Kohoutova, Schnecker, et al. 2014; Kramer et al.

2013; Stone, DeForest, and Plante 2014; Waring, Weintraub, and Sinsabaugh 2013). In addition, depth had a stronger effect on extracellular enzyme activity than habitat, which was also found across a Siberian soil transact (Schnecker et al. 2015). Microbial biomass declines sharply with depth (Blume et al. 2002; Stone, DeForest, and Plante 2014; Taylor et al. 2002), and the per cell effort in producing extracellular enzyme activity increases with depth (Stone, DeForest, and Plante 2014). Because this study does not include biomass measurements and I cannot assess the activity per cell, it may be more informative to interpret patterns of extracellular activities with depth as ratios of activities between enzymes. For example, AP had the highest activity across all enzymes for most habitat and depth combinations. In grassland samples the average level of activity did not decline with depth. However, ratios of ln(BG:AP), ln(BG:NAG), and ln(NAG:AP) all show that deep soils have fairly similar nutrient acquisition strategies.

Overall these ratios would suggest that microorganisms in most communities spend more resources producing AP enzymes than NAG and BG enzymes. Most communities, except grassland at 5 cm, AP activity is increased relative to BG, but no community has higher NAG activity relative to AP. This patterns of increasing effort to produce phosphatase at depth is consistent with findings in tropical (Waring, Weintraub, and Sinsabaugh 2013) and agricultural (Taylor et al. 2002) soils. However, there are several possible reasons for this trend (outlined in (Stone, DeForest, and Plante 2014) including phosphate limitation (Cleveland and Liptzin 2007; Waring, Weintraub, and Sinsabaugh 2013), chemical stabilization of enzyme molecules on minerals (Allison 2006; Eusterhues et al. 2003), and or the use of phosphatases to acquire organic carbon (Spohn and Kuzyakov 2013; Steenbergh et al. 2011). Decreases in Fungal: Bacterial Ratios (Fierer, Schimel, and Holden 2003; Taylor, Parkinson, and Parsons 1989) with depth could also be driving these patterns.

*Relationship Between Microbial Community Composition and Extracellular Enzyme Activities*

The relationship between the microbial community and extracellular enzyme activity, indicates that a considerable amount of extracellular enzyme activity is due to microbial community composition independent of the soil environment. I expected that microbial community composition would be weakly correlated with extracellular enzyme activity because of observations, from chapter 1 of different patterns of variation in soil variables and microbial community composition. Extracellular enzyme activities reflect microbial nutritional needs (Burns et al. 2013), but many other factors can influence enzyme activity. Extracellular enzymes can saturate in soils or they can be constituently produced at low levels (Sinsabaugh and Follstad Shah 2012) and not reflect nutrient demands or the presence of an enzymatic substrates (but see (Moorhead et al. 2013)). Organisms not included in the soil microbial community analyses (e.g. fungi and plants) also produce and secrete extracellular enzymes (Schneider et al. 2012) and can obscure the relationship between the microbial community and enzyme activity. Soil characteristics can also uncouple enzyme activities from biological nutrient demand by influencing the kinetics of extracellular enzymes in soils (Sinsabaugh and Follstad Shah 2012), and active soil enzymes can bind on soil minerals (Burns et al. 2013) and persist long after the death of a microbial cell (Blankinship et al. 2014). All of which could contribute to weak correlations between microbial communities and extracellular enzymes.

Instead there were relatively strong correlations between community composition extracellular enzymes, and weak relationships between extracellular enzyme activity and soil variables. Many previous studies have found relationships between soil microbial community and enzyme activity in surface soils (Waldrop and Firestone 2006; Sinsabaugh et al. 2008; Berg

and Smalla 2009; Fierer and Jackson 2006; Gittel, Barta, Kohoutova, Schnecker, et al. 2014) and through the soil profile (Gittel, Barta, Kohoutova, Schnecker, et al. 2014; Schnecker et al. 2014; Schnecker et al. 2015; Stone, DeForest, and Plante 2014) , although the magnitude of the relationship varies considerably across studies. In this study, habitats that were sampled on the same soil series and physiochemical interactions between soils and free enzymes may have been of similar magnitude across samples. It is also possible that I did not measure environmental variables that are important drivers of extracellular enzyme activities in these soils.

*Microorganisms with Traits for Extracellular Enzyme Production*

The community metagenomic data consisted primarily of bacterial reads. There were few fungal reads in the total data set and none of those mapped to extracellular enzyme genes, similar to a recent metagenomic study of leaf litter decomposition (Berlemont et al. 2014). The reads that were homologous to extracellular enzyme genes were identified to the level of Phyla, due to low read coverage and limited genomic information for many soil microorganisms. Although Phylum level resolution may be useful in studying the functional potential of microbial communities (Philippot et al. 2010), it may be too broad for looking a fine scale and functionally important differences across communities. Microbial groups that decompose simple molecules are conserved at finer scale taxonomic resolution and are dispersed across Phyla (Martiny, Treseder, and Pusch 2013; Zimmerman, Martiny, and Allison 2013; Berlemont and Martiny 2015). For example, at finer resolutions there are correlations between the relative abundances of genera that contain cellulose producers and the proportion of cellulose functional genes in a community (Berlemont et al. 2014).

The relative abundances of microbial Phyla with genes for extracellular enzyme

production were similar in top soil communities from grassland, oak woodland, and coastal sage scrub. The relative abundance of phyla in the extracellular enzyme genes communities differed mostly from those of the control genes, and did not differ based on habitat type. This is consistent with c13 labeled carbon substrates enrichment studies that found simple carbon molecules were assimilated by similar groups of organisms in grassland and oak woodland soil microbial communities (Waldrop and Firestone 2004).

The relative abundance of phyla containing AP and BX overlapped as did AG and BG. The AP and BX groups were enriched in Proteobacteria, and the AG and BG groups were enriched with Actinobacteria. The Proteobacteria and Actinobacteria are the dominate phyla in these soils, and soil communities enriched with labile carbon increased in the proportions of Proteobacteria and Actinobacteria (Goldfarb et al. 2011). Genes for AP are more prevalent in the genomes of Proteobacteria relative to Actinobacteria, consistent with this study. The increased prevalence of AG and BG in Actinobacteria is consistent with the higher proportion of potential cellulose degraders in this group relative to Proteobacteria, which typically have 2x many BG genes (Berlemont and Martiny 2013). The presence of NAG genes in sequenced genomes is highly correlated with the presence of AP genes (Zimmerman, Martiny, and Allison 2013), yet in the current study there was low overlap between NAG and AP trait communities. The NAG trait group was enriched with genes from the Planctomycetes. Planctomycetes were implicated in NAG degradation in lake water (Tada and Grossart 2014), and NAG is frequently used as a substrate for isolating strains of Planctomycetes (Lage and Bondoso 2012). Although microbial communities had similar suits of taxa with traits for extracellular activity, the activity measured across habitats was very different.

Although microbial phyla with genes for extracellular enzymes did not vary by habitat, there was considerable variation in extracellular enzyme production between habitats. There are many reasons for this disconnect. Although an organism may possess a trait, the organism may not choose to express it (Martiny et al. 2015; Amend et al. 2016); activity depends not only on the functional capability of a group of organisms but also their ecological context. In addition, the activity of enzymes produced by an isolate can vary widely (Woo et al. 2014; Eichlerová et al. 2015), and identifying genes by sequence homology is no guarantee of enzyme production. Many putative extracellular enzyme genes may not actually produce enzymes (Nyyssönen et al. 2013). Lastly, there are numerous methodological complications and considerations when generating and interpreting metagenomic data (Prosser 2015), that may obscure the relationships between gene content and the actual function of a microbial community.

**Conclusions**

Invasive annual grassland differs in extracellular enzyme activity relative to native woody habitats, and these differences persist through the soil profile although the differences between habitats diminish with depth. Extracellular enzyme activities are a surrogate of organic matter decomposition, and based on the observations in this study habitat conversion to invasive annual grassland appears to change decomposition throughout the soil prolife. However, since extracellular enzymes are measured in laboratories rather than the field, they may not reflect microbial decomposition in situ and further studies of soil function throughout the soil profile are required to support these enzyme data. In addition, microbial communities and enzyme activity and community composition were strongly correlated across habitats and depth and similar organisms possessed genes for extracellular enzymes between habitats. This suggests high

functional redundancy within Phyla for extracellular enzyme production and or considerable phenotypic plasticity for enzyme production.

**Figures**

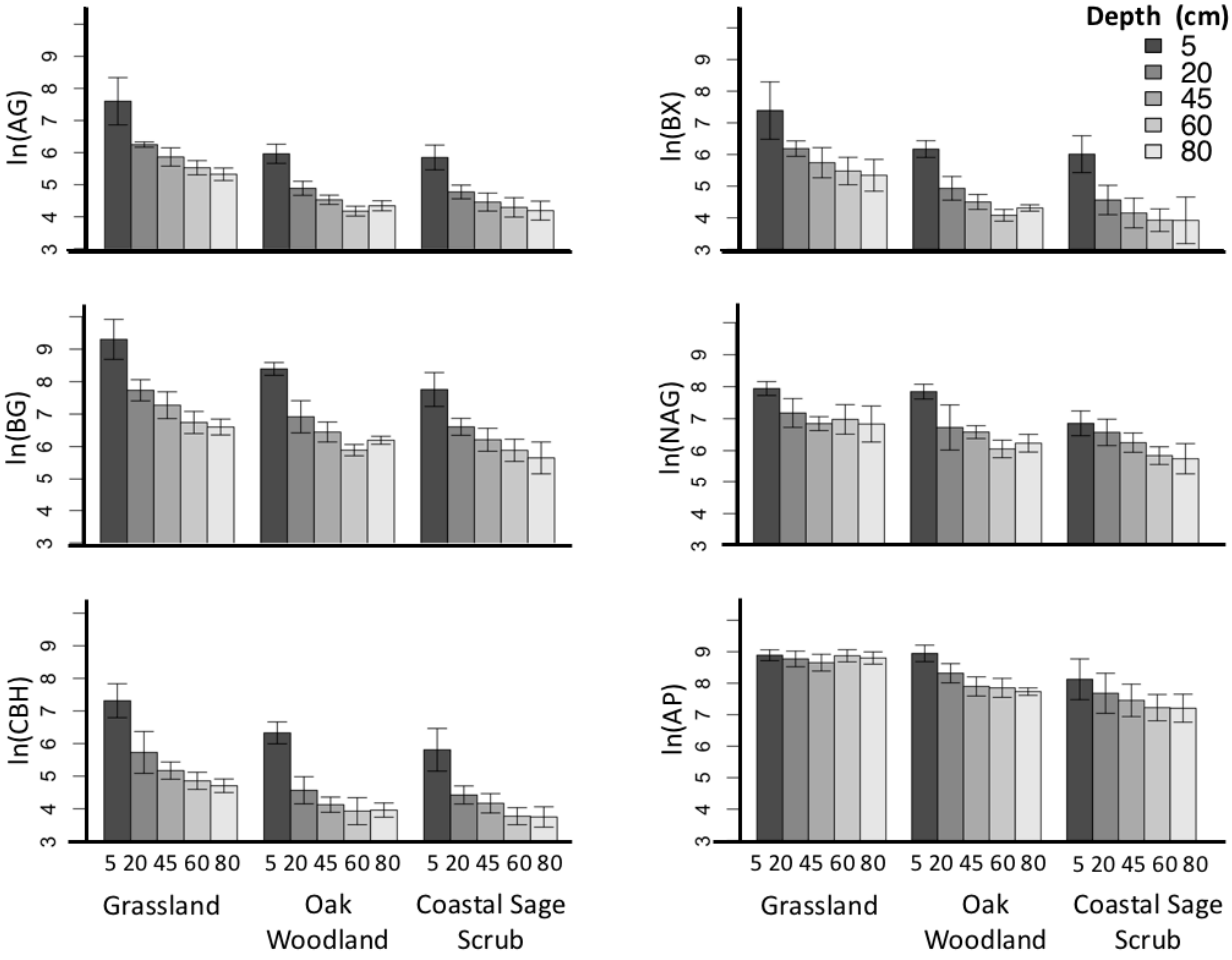


**Figure 1.** Differences in extracellular enzyme activity by habitat and depth. All extracellular activities had significant effects of sampling pit. AG differed between habtitat (p = 1.288e-05), by depth with a linear (p <2.2e-16) and a nonlinear (p = 1.283e-09) effect, and the interaction between habitat and depth (p = 0.0254). BG differed between type (p = 0.0139), and depth with a linear (p < 2.2e-16) and a nonlinear (p = 3.571e-09) effect. CBH differed between type (p = 0.00558) and depth with a linear (p < 2.2e-16) and a nonlinear (p = 1.467e-10) effect. BX differed between type (p = 0.006302) and depth with a linear (p = 3.879e-13) and a nonlinear (p = 1.281e-07) effect. NAG differed between type (p = 0.020039) and depth with a linear (p = 7.245e-07) and a nonlinear (p = 0.001923) effect. AP differed by depth with linear (p <7.427e-

08) and a nonlinear ($p = 0.000311$) effect, and also by the nitration between habitat and depth ($p = 1.049e-07$).

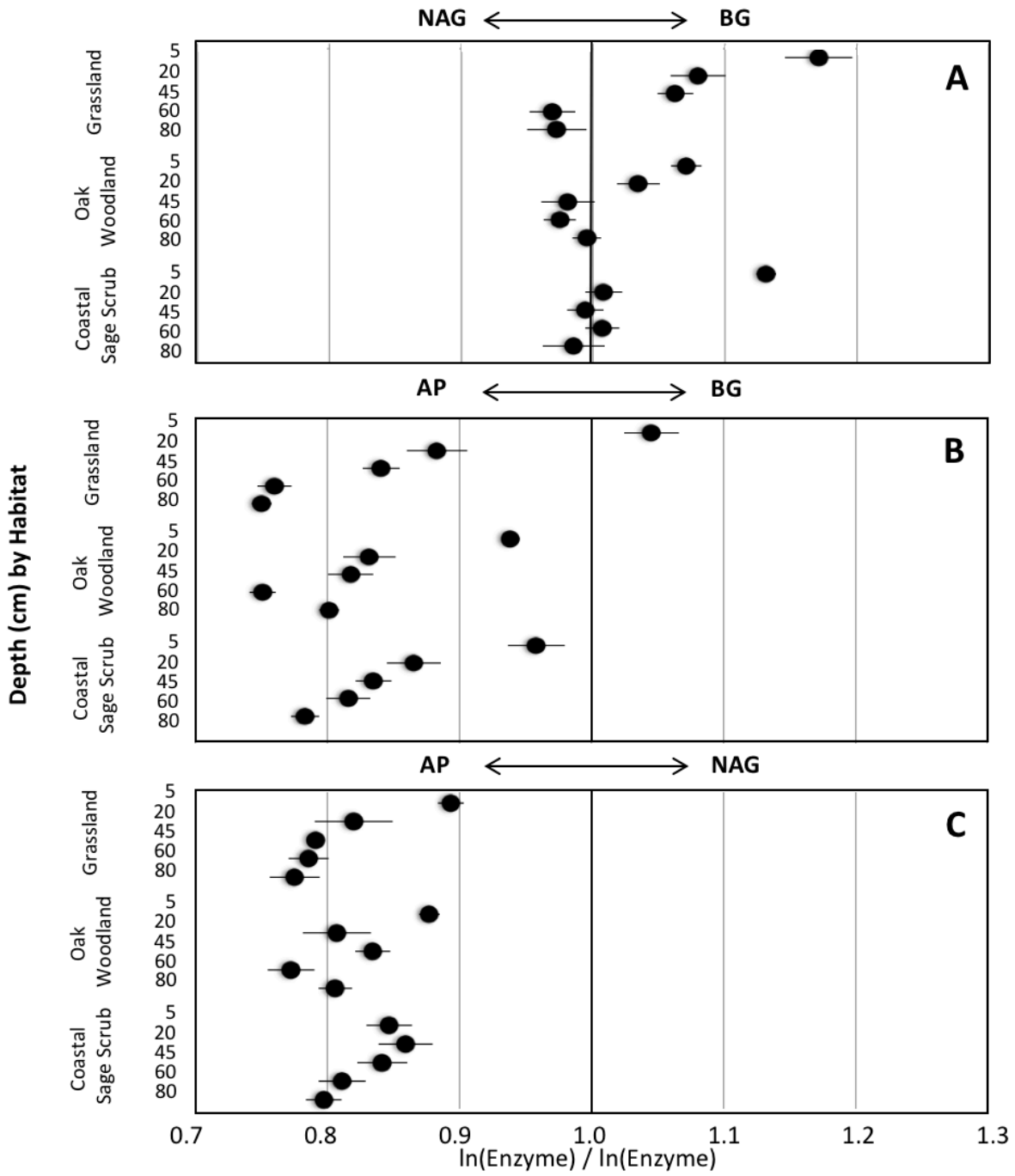


**Figure 2.** Ratio of extracellular enzyme activity by habitat and depth, the mean and 95% confidence intervals are shown. A) The ratio of the ln(BG:NAG) differed between by depth with linear ($p = 1.545e-11$) and nonlinear ($p = 0.002168$) effects and differed by the nonlinear interaction between depth and habitat ($p = 0.017563$). B) The ratio of the ln(BG:AP) had

significant effects of sampling pit and differed between type ($p = 0.01337$) and by depth with linear ($p = 5.342e-12$) and nonlinear ($p = 5.219e-06$) effects. C) The ratio of the ln(AG:AP) had significant effects of sampling pit and differed by depth ($p = 2.533e-06$).

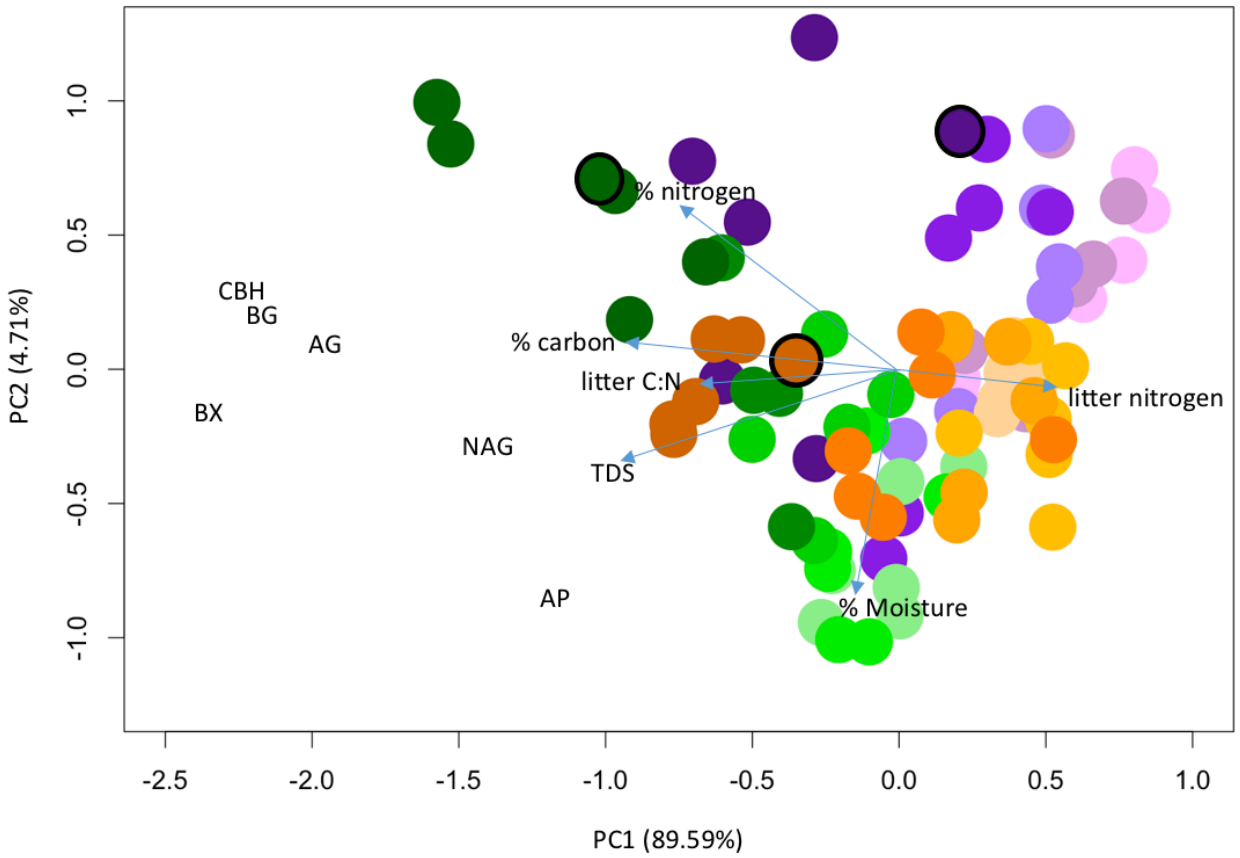


**Figure 3.** Microbial community turnover by habitat and depth. A) The principal components analysis of log transformed extracellular enzyme activity. Loadings for extracellular enzymes are indicated. Vectors for soil and litter variables indicate maximum correlations with community composition, only significant correlations are shown; soil moisture (r2 = 0.217, p = 0.001), soil % carbon (r2 = 0.2504, p = 0.002), soil % nitrogen (r2 = 0.213 p = 0.002), total dissolved solids (r2 = 0.2959, p = 0.001), litter nitrogen (r2 = 0.0805, p = 0.029), and litter C:N (r2 = 0.1349, p = 0.003). Extracellular enzyme gene content was determined for communities indicated with black circles.

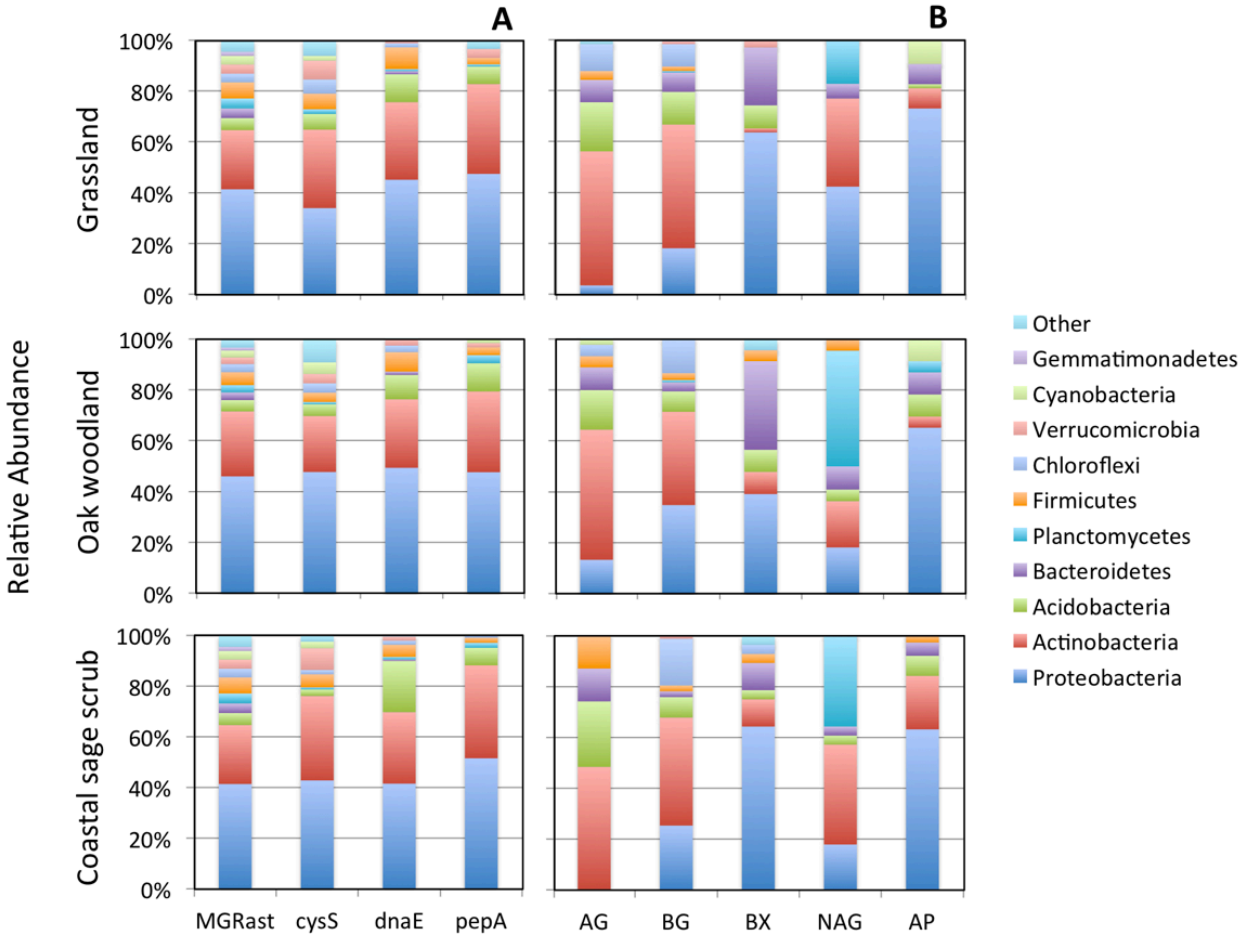


**Figure 4.** Relative abundance of sequencing reads by Phylum. A) The relative abundance of control gene reads per phyla for each habitat type; the consensus for all sequencing reads (MGRast), cysS (EC= 6.1.1.16), dnaE (EC:2.7.7.7), and pepA (EC 3.4.11.1). B) shows the relative abundance of reads per phyla that map to extracellular enzyme producing genes for AG, BG, BX, NAG, AP. No CBH reads were detected. The relative abundance of the following Phyla differs by gene but not habitat; Acidobacteria ($p < 0.001$), Bacteroidetes ($p < 0.001$), Chloroflexi ($p < 0.001$), Cyanobacteria ($p = 0.008$), Firmicutes ($p = 0.019$), Planctomycetes ($p < 0.001$), Proteobacteria ($p < 0.001$), Verrucomicrobia ($p < 0.001$). Only Actinobacteria differs by habitat ($p = 0.047$) and gene ($p < 0.001$).

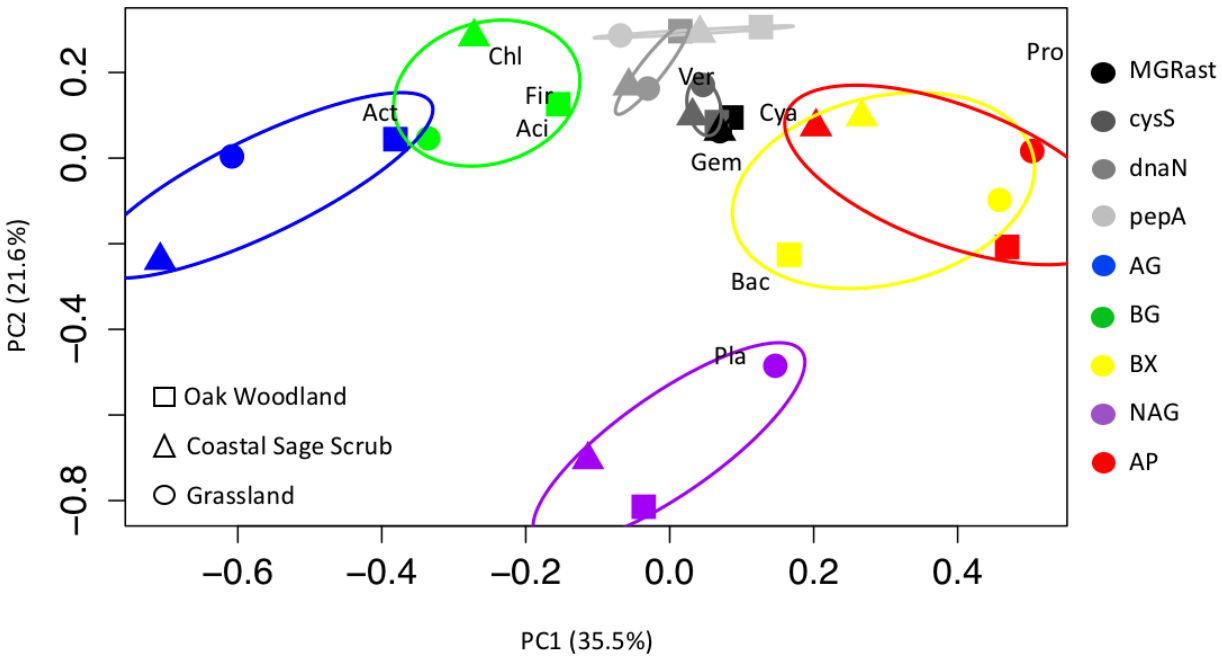


**Figure 5.** Principal Components Analysis of extracellular enzyme genes by phylum based on relative abundance of reads in each metagenome (N = 3). Standard error is indicated by the ellipses.

**Table 1. Enzymes assayed for potential extracellular enzyme activity**

| Target for Degredation | Functional Enzyme | | Fluorescently Labeled Substrate | Target Genes |
|---|---|---|---|---|
| Carbohydrates | α 1,4 glucosidase | (AG) | 4-MUB- α-D-glucoside | EC: 3.2.1.20 |
| Cellulose | Cellobiohydrolase | (CBH) | 4-MUB- ß-D-cellobioside | EC: 3.2.1.91* |
| Cellobiose | ß glucosidase | (BG) | 4-MUB- ß-D-glucoside | EC: 3.2.1.21 |
| Hemicellulose | ß xylosidase | (BX) | 4-MUB- ß-D-xyloside | EC: 3.2.1.37 |
| Chitin | N acetylglucosaminidase | (NAG) | 4-MUB-N-acetyl- ß-D-glucosaminide | EC: 3.2.1.52 |
| Organic phosphate | Alkaline Phosphatase | (AP) | 4-MUB-phosphate | EC: 3.1.3.1 |

EC, enzyme commission classification, * no reads mapped to these genes

**Table 2. Correlations between extracellular enzymes and environmental variables, Pearson's r**

| Extracellular Enzyme | Soil Moisture | pH | TDS | Soil % Nitrogen | Soil % Carbon | Litter % Nitrogen | Litter % Carbon | Litter C:N |
|---|---|---|---|---|---|---|---|---|
| **AG** | 0.07 | 0.03 | 0.56*** | 0.32** | 0.45*** | -0.42*** | 0 | 0.47*** |
| **CBH** | -0.04 | -0.11 | 0.48*** | 0.45*** | 0.52*** | -0.26* | 0.09 | 0.34** |
| **BG** | -0.08 | -0.14 | 0.44*** | 0.40*** | 0.50*** | -0.18 | 0.09 | 0.26* |
| **BX** | 0.03 | -0.05 | 0.51*** | 0.31** | 0.45*** | -0.30** | 0.12 | 0.40*** |
| **NAG** | 0.2 | -0.17 | 0.46*** | 0.38*** | 0.47*** | -0.14 | 0.07 | 0.24* |
| **AP** | 0.28** | -0.1 | 0.51*** | 0.12 | 0.35*** | -0.18 | 0.1 | 0.29** |

Significant values are indicated as follows: $p < 0.05$ *, $p < 0.01$ **, and $p < 0.001$***, TDS stands for Total Dissolved Solids,

**Table 3. Metagenomic shotgun seqeuncing summary**

| Habitat type | # Seqeuncing Reads | | Average Read length | | # Seqeuncing Reads | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | After MG-RAST QC | Total | after MG-RAST QC | cysS | pepA | dnaE | AG | BG | BX | NAG | AP |
| **Grassland** | 474,812 | 461,118 | 657 ± 236 bp | 482 ± 236 bp | 162 | 116 | 246 | 57 | 171 | 66 | 52 | 63 |
| **Oak woodland** | 367,560 | 358,627 | 672 ± 231 bp | 497 ± 236 bp | 109 | 63 | 156 | 45 | 112 | 23 | 22 | 23 |
| **Coastal Sage Scrub** | 331,979 | 322,637 | 673 ± 236 bp | 490 ± 238 bp | 117 | 101 | 188 | 31 | 87 | 28 | 28 | 38 |
| **Total** | 1,174,351 | 1,142,382 | 667 ± 234 bp | 490 ± 237 bp | 388 | 280 | 590 | 133 | 370 | 117 | 102 | 124 |

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CHAPTER 3

# Microbial community succession during animal decomposition in a Southern California tar seep

## INTRODUCTION

Over the millennia, many animals have become entrapped and died in natural tar seeps. Millions of fossils have been recovered from the still-active tar seeps of Rancho La Brea California, but most of the skeletons recovered are incomplete, disarticulated, and mixed together with little stratigraphic order (Friscia et al. 2008). The rate of decomposition of a carcass contributes to this jumble of bones. Initially, animal carcasses entrapped in tar are only partially submerged (Spencer et al. 2003), and take an estimated 17-20 weeks for total submergence in tar (Holden et al. 2013). The anaerobic decomposition of submerged portions can occur in as few as two months. These rapidly skeletonized portions of the carcass are less likely to be transported by scavengers or the surface flow of tar and water than the exposed portions of the carcass (Brown in prep 2016). Microbial communities are responsible for the majority of decomposition in anaerobic environments, but knowledge of anaerobic organic matter decomposition in tar seeps is limited. Diverse communities of microorganisms persist in these habitats (Kim and Crowley 2007, Schulze-Makuch et al. 2011, Meckenstock et al. 2014), and these microbial communities likely play a role in the rapid time to skeletonization of animal components submerged in tar.

The microbial community involved in the decomposition of animal tissue submerged in tar is not known (but see Brown in prep 2016). Organic matter added to hydrocarbon saturated habitats can increase metabolism of petroleum products and is often used to stimulate microbial

metabolism for remediation of hydrocarbon polluted sites (Bell et al. 2013, Zhang and Lo 2015) and in experimental microcosms (Aburto-Medina et al. 2012). The succession of microbial communities during decomposition can indicate changes in microbial community metabolism (Scherr et al. 2012) and selection of different taxa due to changes in resources (Jurelevicius et al. 2013). In aerobic habitat, the rate of animal tissue decomposition is related to succession in microbial communities and the associated changes in function over the course of decomposition (Cobaugh et al. 2015). Microbial community succession has also been observed in environments fouled by hydrocarbons (Aburto-Medina et al. 2012, Scherr et al. 2012, Koo et al. 2015), and individual microbes and microbial consortia are implicated in the metabolism of hydrocarbons (Fowler et al. 2012, Gutierrez et al. 2013, Fowler et al. 2014) and decaying animal matter (Carter et al. 2015, Cobaugh et al. 2015, Finley et al. 2015, Metcalf et al. 2016). Although microbial succession and associated stages of decomposition are better understood for mammal decomposition in soils (Carter et al. 2015, Cobaugh et al. 2015, Finley et al. 2015), they are less known in anaerobic environments, particularly in hydrocarbon saturated environments.

Understanding the composition of native microbial communities in tar seeps and their relationship with animal tissue decomposition is a first step towards understanding the microbial ecology of decomposition in tar and how microbial communities may impact the rate of decomposition in tar environments. The first goal of this study was to investigate differences in microbial diversity between different locations in tar environments and characterize those involved in animal tissue decay. I hypothesize that the diversity of the microbial communities differs between groups, and is the highest in resource poor environments and the lowest in resource rich environments (Margalef 1963). Because resources are limited in environments saturated with hydrocarbons (e.g. recalcitrant carbon sources and toxic compounds (Kim and

Crowley 2007)), but are high in animal decomposition environments (e.g. high concentrations of energy rich lipids and proteins (Kovacs et al. 2013)), I hypothesize that the community diversity is the greatest in tar environments.

The second goal of this study is to determine whether community composition differs between locations in the tar environment and animal decomposition, and, if the shifts of the major taxa associated with these habitats follow general successional patterns of microbial decomposition found in other hydrocarbon or animal decomposition environments. I hypothesized that shifts in communities differed by tar environment and that tar environmental communities are dominated by hydrocarbon degraders (e.g. Proteobacteria (Kim and Crowley 2007, Schulze-Makuch et al. 2011) ) and methanogens (Kim and Crowley 2007, Schulze-Makuch et al. 2011, Meckenstock et al. 2014), whereas that animal decomposition communities are dominated by taxa commonly found in the anaerobic stages of animal decomposition (e.g. higher abundances of proteolytic members of the Firmicutes (Kovacs et al. 2013, Kovacs et al. 2015)). I sampled a tar seep and bobcat limbs that were submerged in the seep and characterized microbial communities using 16S rDNA sequencing of the V4 region.

## Materials and Methods

*Animal tissue specimens, experimental design, and microbial community sampling*

For a detailed description of the experimental design see Brown and colleagues (in prep 2016). In brief, limbs were dissected from the carcasses of 12 bobcat (*Lynx rufus*) specimens that were stored frozen for over two years and thawed prior to use. The limbs were placed on the surface of an asphalt seep (located in Simi Valley California) and allowed to naturally sink in to tar. The limbs were placed in small cages. These cages allowed the movement of tar, while

protecting the limb from macro-scavengers (i.e., birds and mammals). The area was further protected by a chain-link enclosure to deter human and animal disturbance. I sampled and characterized the microbial communities from tar environments and the leg scrapes. The sampled tar environments include the head waters of the tar seep ~ 4 m from the bobcat limbs (H); and the surface tar (S), tar water (W), and tar sediments (B), all sampled ~ 2 meters from the bobcat limbs. The leg scrape samples include the first sample take three days after the limbs were deposited into the tar on 3/24/2014 (L1), the second sample taken on 4/1/2014 (L2), and leg decay samples from deep tissue collected on 4/1/2014 (D1) and 5/16/2014 (D2). Animal decay microbial communities were sampled from limbs that were sequentially removed and not replaced in the seep. Samples were frozen at -20C until DNA extraction.

*DNA Extraction and Sequencing*

To characterize the tar microbial community, I first extracted DNA in triplicate using a PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA) with a minor modification. During cell lysis, samples were heated to 80 C for 5 minutes prior to bead beating. Microbial communities were characterized based on the V4 region (corresponding primers 515F/806R) (Caporaso et al. 2012) of the 16S rDNA sequence. The sequencing libraries were prepared and sequenced as follows. Sequencing primers were synthesized with a recognition sequence compatible with Nextera Indexing Primers (Nextera® Index Kit, Illumina, San Diego, CA). Amplification was carried out in triplicate using Platinum® Taq DNA Polymerase (Life Technologies, Grand Island, NY, USA) and 1 uM of each primer. The reaction conditions were as follows: Hold at 95$^{o}$C for 3 min; 30 cycles of 95$^{o}$C for 45 sec, 50$^{o}$C for 60 sec, and 72$^{o}$C for 90 sec; and a hold at 72$^{o}$C for 10 min and a final hold at 12$^{o}$C. Amplification was verified and PCR products were

purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Amplicon libraries were amplified using Nextera Indexing Primers (1.25 uL of each primer) using the KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Woburn, MA) under the following conditions: Hold at 95$^{o}$C for 5 min; 5 cycles of 98$^{o}$C for 20 sec, 56$^{o}$C for 30 sec, and 72$^{o}$C for 3 min; and a hold at 72$^{o}$C for 5 min and a final hold at 8$^{o}$C. Amplicons were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). Amplicons were quantified by qPCR sing the Library Quantification Kit – Illumina for the LightCycler® 480 (Roche, Indianapolis, Indiana, USA). Amplicons were adjusted to an equimolar concentration, pooled and loaded onto a MiSeq Personal Sequencer using a MiSeq Reagent Kit v3.

*Microbial Diversity and Statistical Analyses*

After high throughput sequencing, MiSeq reads were subjected to quality control using Galaxy (Blankenberg et al. 2001, Giardine et al. 2005, Goecks et al. 2010). Sequencing reads were retained if they had a minimum quality score of 30 and length of greater than 75 bp. The remaining reads were assigned taxonomic classification using open reference methods at 97% identity (Edgar 2010, McDonald et al. 2012) using Qiime (Caporaso et al. 2010). If an operational taxonomic unit (OTU) had five or fewer occurrences across the data set, it was removed from subsequent analyses to minimize the influence of sequencing artifacts. I used within community diversity (alpha diversity) and community turnover (beta diversity) to investigate differences in microbial communities. For each of the following diversity measurements, I took the average of 10 rarefactions to 6,000 sequences. Alpha diversity was measured as richness by the number of observed OTUs, Faith's phylogenetic diversity, and evenness by the Simpson index (Caporaso et al. 2010). Differences in alpha diversity and the

presence of abundant OTUs between groups were determined using Wilcoxon rank sum tests. Beta diversity was determined as the Hellinger distance between communities based on the relative abundances of genus level OTUs (Legendre and Gallagher 2001, Dixon 2003). Beta diversity was visualized using hierarchical clustering (Wickham 2009), and Non Metric Multidimensional Scaling (NMDS) (Venables and Ripley 2002 ). Differences in community composition between tar environments and animal decay were determined by PERMANOVA, using vegan function ADONIS (Dixon 2003).

**Results**

There were taxonomic differences in the microbial communities between tar environments and animal decay. I identified 413 microbial genera (Bacteria and Archaea), of which 94 were found exclusively in tar environmental samples and 42 microbial genera were found only in the animal decay communities. The top 40 abundant microbial genera across all samples comprised 76.5% of the sequencing reads and were found in each community regardless of sample origin (Figure 1). There was considerable variation in the relative abundances of these genera within communities, and more than half of the genera are significantly more prevalent either in the tar environmental communities or in the animal decay communities (Figures 2). Genera from the Oceanospirillales, NRA6 of the class Methanomicrobia, BA021 of the phylum OP9, the unclassified order of the phylum WWE1, and the Thermotogales orders were enriched in tar environmental sample communities. Genera from the Enterobacteriales, Clostridiales, Desulfovibrionales, Rhodocyclales, and Burkholderiales orders were enriched in animal decay sample communities.

Tar environment communities had a higher alpha diversity (Wilcoxon rank sum test p = 0.036) than decay communities (Figure 3). The diversity of these communities measured by OTU richness (97%) was greater in communities from tar environments than from those in animal decay samples. There was 2.1x more OTU richness in the extremes in these groups; W versus D1. Evenness did not vary between community types (Wilcoxon rank sum test of Simpson index, p = 0.48), but D1, D2, and H were less even than the other communities.

Community composition also differed between tar environment and animal decay samples. Approximately one quarter of the variation in community composition across samples is explained by community type (ADONIS r2 = 0.234, p = 0.057). Hierarchical heat map and NMDS show that microbial communities clustered by tar environment and animal decay (Figures 4 and 5). The environmental community W is more similar to the other environmental communities than they are to each other. The environmental community S is most similar to environmental community W followed by the animal decay communities, specifically D2. Within animal decay communities, D1 and D2 are most similar to each other as are L1 and L2. NMDS also shows the centroids of the top 40 microbial genera associated with microbial community sample origin (Figure 5), genera cluster with sample type where they are most abundant (Figures 1 and 2).

**Discussion**

In tar seeps, I found distinct microbial communities associated with tar environments and animal decomposition. Energy rich environments are predicted to contain lower community diversity relative to energy poor environments (Margalef 1963, Valentin 1971). Environments with high concentrations of nutrient rich resources should favor copiotrophic species (Fierer et

al. 2007) that can rapidly grow and outcompete other taxa (Bell et al. 2013). Animal tissue contains high levels of proteins, lipids, and other energy rich compounds, and in this study communities sampled from animal decay had lower species richness. Although species evenness did not differ between tar environments and animal decay communities, D1 and D2 communities were less even than the other communities and dominated by Clostrium, which can be copiotropic (Janaway et al. 2009). Tar environments, and in particular tar water, should have fewer nutrients than decay samples. Such low energy environments should contain more oligotrophs that are energy efficient and slow growing (Fierer et al. 2007).

The most abundant genera identified in this study are all found in tar environments and animal decay communities, but the relative abundances of the genera vary considerably between tar environments and animal decay communities and even within communities of each group. For example, Clostridium is found in very low abundance in the tar environment samples, increased in abundance in the leg scrape samples (the interface between the surface tar and animal decay), and was the dominant Genus in decay samples. I do not know the initial community of the bobcat leg prior to placement in the tar, but the overlap of genera between tar environments and animal decay communities and the enrichment of specific genera in animal decay suggest that decay communities may have originated in tar environments. Similarly in soils, the majority of mammal cadaver decomposition communities likely originate from soil communities rather than from the preexisting flora of the mammal cadaver (Carter et al. 2015, Metcalf et al. 2016). The tar surface community (S) shows similarity to animal decay communities D1 and D2. The similarity between D2 and S, is likely due to the advanced stage of decomposition in this sample. D2 sample had little remaining tissue and the community could be shifting in composition towards that of surface tar. This indicates high temporal variability and

likely rapid microbial succession between communities able to decompose animal tissue and/or tar. Despite the low number of communities sampled in this study, rapid microbial succession in this system is consistent with other studies that find rapid changes (on the order of weeks, depending on sampling strategies) in microbial communities in petroleum contaminated sites supplemented with organic material (Bell et al. 2013, Zhang and Lo 2015), or in petroleum free habitats (Coulon et al. 2012, Jurelevicius et al. 2013, Koo et al. 2015) and organic anaerobic digesters (Aburto-Medina et al. 2012, Scherr et al. 2012) that are supplemented with petroleum products.

Community compositional differences between tar environments and animal decay suggest that there may be groups of organisms that are specialized on utilizing available resources. The animal decomposition communities contained higher levels of Enterobacteriales than the environmental samples. Some members of this order have been observed to increase in abundance during the active stage of decomposition in human cadavers (Cobaugh et al. 2015). It is common to find Enterobacteriales in earlier stages of decomposition of cadavers followed by a shift to groups such as the Clostridiales toward the end bloat stage (Hyde et al. 2013). This is consistent with our observations of higher relative abundances of Enterobacteriales in L1 and L2 than D1 and D2, albeit the reason cited for this shift is a change in conditions from aerobic to anaerobic metabolism. The shift from aerobic to anaerobic conditions for animal decay samples presumably occurred rapidly after the leg was submerged in tar, yet the Enterobacteriales persisted on the exterior of the leg for more than a week. The majority of these OTUs are unclassified at 97% identity, and likely originated from the tar environment. The Enterobacteriales found (in low abundance) in a tar lake in Trinidad and Tobago were also unclassified (Meckenstock et al. 2014), and many Enterobacteriales OTUs have been identified

at low abundance in petroleum environments (Khorasani et al. 2013, Meckenstock et al. 2014, Pavlova-Kostryukova et al. 2014, Zhang et al. 2015).

Clostridiales, in particular the genus Clostridium, was found in all samples in this study but was enriched in the animal tissue samples. Species within this group are implicated in the breakdown of human tissues, lipids and complex carbohydrates (Janaway et al. 2009). The abundance of Clostridiales increases in the end stages of cadaver decomposition (Hyde et al. 2013), however, members of this group have also been identified as potential members of methogenenic n-alkene (Wang et al. 2011) and toluene (Fowler et al. 2012, Fowler et al. 2014) degrading community. The enrichment of Clostridiales in animal decay communities is consistent with decomposition studies, and the presence of Clostridiales in tar environments is consistent with other studies in hydrocarbon environments. Clostridiales have been found in tar microbial communities (Kim and Crowley 2007, Schulze-Makuch et al. 2011, Meckenstock et al. 2014), and they are also abundant in many petroleum (Koo et al. 2015, Zhang and Lo 2015) and animal decomposition (Hyde et al. 2013, Kovacs et al. 2013) environments.

Desulfovibrionales are also associated with animal decomposition samples, but less so with the last decay sample (5/16/14). Members of the Desulfovibrionales include sulfate reducing bacteria that produce hydrogen sulfate as the end product of anaerobic digestion rather than methane (Magot et al. 1992). The prevalence of this order near and within animal tissue decay samples (Cobaugh et al. 2015) releasing sulfur compounds (Rosier et al. 2015) is consistent with observed increases in sulfate reducing bacteria across sulfur gradients (Bae et al. 2015). The reduction in Desulfovibrionales in the late decay sample could indicate that most of the sulfur compounds from the animal tissue had been reduced.

The bacteria orders Rhodocyclales and Burkholderiales showed similar patterns of relative abundance across samples, and they were most prevalent on the leg scrape samples. Taxa from both orders have been identified as major bacterial degraders of polycyclic aromatic hydrocarbons (PAH) in soils (Chemerys et al. 2014). It is not known if these groups become more abundant in hydrocarbon environments enriched in organic compounds. Rhodocyclales were most abundant in the intermediate stages of petroleum decomposition in saltmarsh sediment (Koo et al. 2015) and were the dominant bacterial species degrading PAHs in a polluted marine systems experiencing an algal bloom (Gutierrez et al. 2011), presumably organic matter was abundant in both of these studies. Both groups were found in bioreactor sludge, where the carbon source changed from yeast extract to phenol (Silva et al. 2013). Rhodocyclales were also prevalent in oil well injection waters (Ren et al. 2011) and some tar environments (Schulze-Makuch et al. 2011). Burkholderiales were also found in tar environments (Kim and Crowley 2007, Schulze-Makuch et al. 2011, Meckenstock et al. 2014) that presumably did not have a high organic matter content. Burkholderiales and Rhodocyclales species can be incredibly metabolically diverse (Perez-Pantoja et al. 2012) and degrade a wide variety of compounds found in petroleum (Bacosa et al. 2011, van der Zaan et al. 2012). It is not clear why these groups are more prevalent in animal samples than in tar environment samples, but it is possible that they are better competitors in tar environments enriched with organic matters.

The bacterial groups associated with the tar environmental samples are similar to those found in other petroleum saturated habitats. The Oceanospirillales was most abundant at the headwaters of the tar seep. Oceanospirillales have been found in early microbial succession communities associated with crude oil decomposition (Hazen et al. 2010, Gutierrez et al. 2013, Koo et al. 2015) and are most likely involved in the degradation of aliphatic hydrocarbons

fraction of petroleum environments (Mason et al. 2012, Gutierrez et al. 2013). These aliphatic hydrocarbons are more easily biodegraded than PAHs. The tar in the seep sample would have been the least biodegraded of the tar environment samples and would have the largest concentration of aliphatic hydrocarbons which would explain the prevalence of Oceanospirillales.

The archaea order NRA6 of the Methanomicrobia was most abundant in the tar seep sample, but also prevalent in most of the environmental samples with the exception of the final decay sample (5/16/2014). In experimental anaerobic digesters, the prevalence of Methanomicrobia decreased with increasing levels of protein (Kovacs et al. 2013). This group was also the dominant archaea in oil waters (Zhao et al., 2012, Ren et al., 2011) and has been found in tar environments (Anderson et al., 2009, Meckenstock et al., 2014). The order BA021 of the phylum OP9 and an unclassified order of the phylum WWE1 were more prevalent in environmental samples and were most prevalent in tar sediment and tar water. Members of the OP9 have been found in tar (Schulze-Makuch et al., 2011) and associated with methanogenic consortia found in marine and freshwater sediment (Webster et al. 2004) and deep sediments near methane seeps (Siegert et al. 2011). Members of the WWE1 were found in wastewater treatment and likely anaerobically degrade cellulose and/ or uptake fermentation products (Limam et al. 2014). However, to the best of our knowledge, they have not been found in any petroleum associated habitats.

The Thermotogales were most prevalent in surface tar, tar sediment, and tar water. Although it is not clear what their role is in petroleum degradation, members of this group have been found in tar (Nesbo et al. 2010, Meckenstock et al. 2014), oil formation water (Pavlova-Kostryukova et al. 2014), and a variety of hydrocarbon saturated habitats (see (Nesbo et al.

2010). The prevalence of orders of microbes found in tar environments was consistent with what has been found in other hydrocarbon associated environments.

**Conclusions**

The addition of animal tissue, in this case a bobcat limb, into a tar seep appeared to lead to rapid microbial community succession. The major groups of microbes associated with tar environments and animal decay are consistent with what is known in analogous systems. In animal decomposition in soil, soil microbes are known to accelerate the rate of decomposition (Lauber et al. 2014). The tar microbial community studied here may provide insight on an analogous function in anaerobic digestion of animal tissues in tar. Future experiments of decomposition and time to skeletonization in tar and other anaerobic conditions are required to verify our findings and to better understand the role of microbial communities and microbial succession in determining the rate of decomposition in tar environments.

## Figures

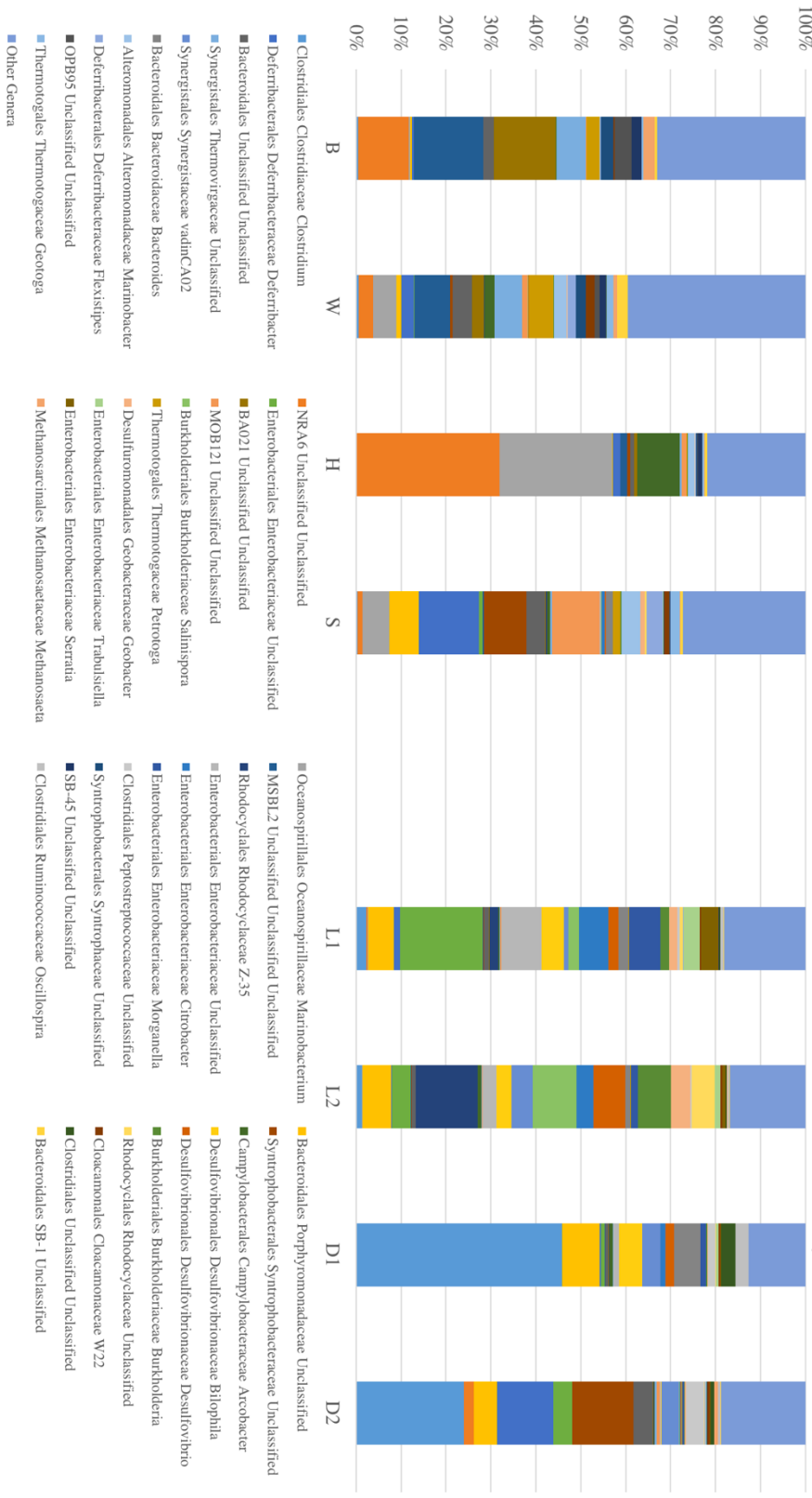
Tar Environment
Animal Decay
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
B
W
H
S
L1
L2
D1
D2
Clostridiales Clostridiaceae Clostridium
Deferribacterales Deferribacteraceae Deferribacter
Bacteroidales Unclassified Unclassified
Synergistales Thermovirgaceae Unclassified
Synergistales Synergistaceae vadinCA02
Bacteroidales Bacteroidaceae Bacteroides
Alteromonadales Alteromonadaceae Marinobacter
Deferribacterales Deferribacteraceae Flexistipes
OPB95 Unclassified Unclassified
Thermotogales Thermotogaceae Geotoga
Other Genera
NRA6 Unclassified Unclassified
Enterobacteriales Enterobacteriaceae Unclassified
BA021 Unclassified Unclassified
MOB121 Unclassified Unclassified
Burkholderiales Burkholderiaceae Salinispora
Thermotogales Thermotogaceae Petrotoga
Desulfuromonadales Geobacteraceae Geobacter
Enterobacteriales Enterobacteriaceae Trabulsiella
Enterobacteriales Enterobacteriaceae Serratia
Methanosarcinales Methanosaetaceae Methanosaeta
Oceanospirillales Oceanospirillaceae Marinobacterium
MSBL2 Unclassified Unclassified Unclassified
Rhodocyclales Rhodocyclaceae Z-35
Enterobacteriales Enterobacteriaceae Unclassified
Enterobacteriales Enterobacteriaceae Citrobacter
Enterobacteriales Enterobacteriaceae Morganella
Clostridiales Peptostreptococcaceae Unclassified
Syntrophobacterales Syntrophaceae Unclassified
SB-45 Unclassified Unclassified
Clostridiales Ruminococcaceae Oscillospira
Bacteroidales Porphyromonadaceae Unclassified
Syntrophobacterales Syntrophobacteraceae Unclassified
Campylobacterales Campylobacteraceae Arcobacter
Desulfovibrionales Desulfovibrionaceae Bilophila
Desulfovibrionales Desulfovibrionaceae Desulfovibrio
Burkholderiales Burkholderiaceae Burkholderia
Rhodocyclales Rhodocyclaceae Unclassified
Cloacamonales Cloacamonaceae W22
Clostridiales Unclassified Unclassified
Bacteroidales SB-1 Unclassified

**Figure 1. Relative abundances of the 40 most abundant microbial genera by sample.** I found a total of 413 genera; 277 were shared between sample groups, 94 were found only in tar environmental communities, and 42 were found only in animal decay communities. B = sediment at the bottom of the tar seep, W = water between the sediment and surface tar, H = head waters of the tar seep, S = surface tar, L1 = first leg scrape, L2 = second leg scrape, D1 = first tissue decay, D2 = second tissue decay.

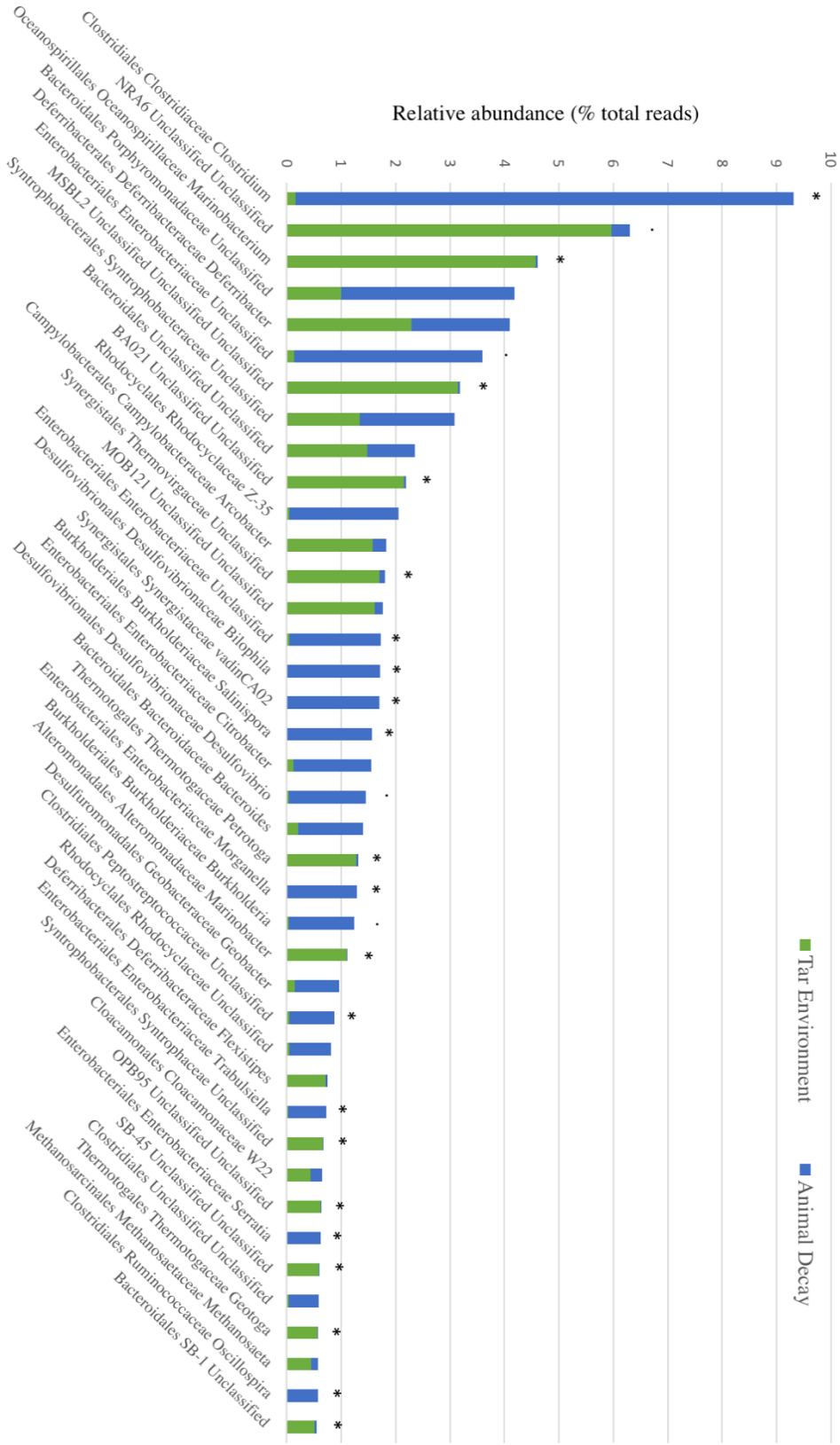
Relative abundance (% total reads)
0
1
2
3
4
5
6
7
8
9
10
Tar Environment
Animal Decay
Clostridiales Clostridiaceae Clostridium
Oceanospirillales Oceanospirillaceae Marinobacterium
NRA6 Unclassified Unclassified
Bacteroidales Porphyromonadaceae Unclassified
Deferribacterales Deferribacteraceae Deferribacter
Enterobacteriales Enterobacteriaceae Unclassified
MSBL2 Unclassified Unclassified
Syntrophobacterales Syntrophobacteraceae Unclassified
Bacteroidales Unclassified Unclassified
BA021 Unclassified Unclassified
Campylobacterales Campylobacteraceae Arcobacter
Rhodocyclales Rhodocyclaceae Z-35
Synergistales Thermovirgaceae Unclassified
MOB121 Unclassified Unclassified
Enterobacteriales Enterobacteriaceae Unclassified
Desulfovibrionales Desulfovibrionaceae Bilophila
Synergistales Synergistaceae vadinCA02
Burkholderiales Burkholderiaceae Salinispora
Enterobacteriales Enterobacteriaceae Citrobacter
Desulfovibrionales Desulfovibrionaceae Desulfovibrio
Bacteroidales Bacteroidaceae Bacteroides
Thermotogales Thermotogaceae Petrotoga
Enterobacteriales Enterobacteriaceae Morganella
Burkholderiales Burkholderiaceae Burkholderia
Alteromonadales Alteromonadaceae Marinobacter
Desulfuromonadales Geobacteraceae Geobacter
Clostridiales Peptostreptococcaceae Unclassified
Rhodocyclales Rhodocyclaceae Unclassified
Deferribacterales Deferribacteraceae Flexistipes
Enterobacteriales Enterobacteriaceae Trabulsiella
Syntrophobacterales Syntrophaceae Unclassified
Cloacamonales Cloacamonaceae W22
OPB95 Unclassified Unclassified
Enterobacteriales Enterobacteriaceae Serratia
SB-45 Unclassified Unclassified
Clostridiales Unclassified Unclassified
Thermotogales Thermotogaceae Geotoga
Methanosarcinales Methanosaetaceae Methanosaeta
Clostridiales Ruminococcaceae Oscillospira
Bacteroidales SB-1 Unclassified

**Figure 2.** Relative abundances of the 40 most prevalent genera found in the tar environment and decay **samples. Differences in relative abundance** between groups were determined using Wilcoxon rank sum tests. P values between .1 and 0.05 are indicated with "." and P values equal to 0.05 or between 0.05 and 0.01 are indicated with "*".

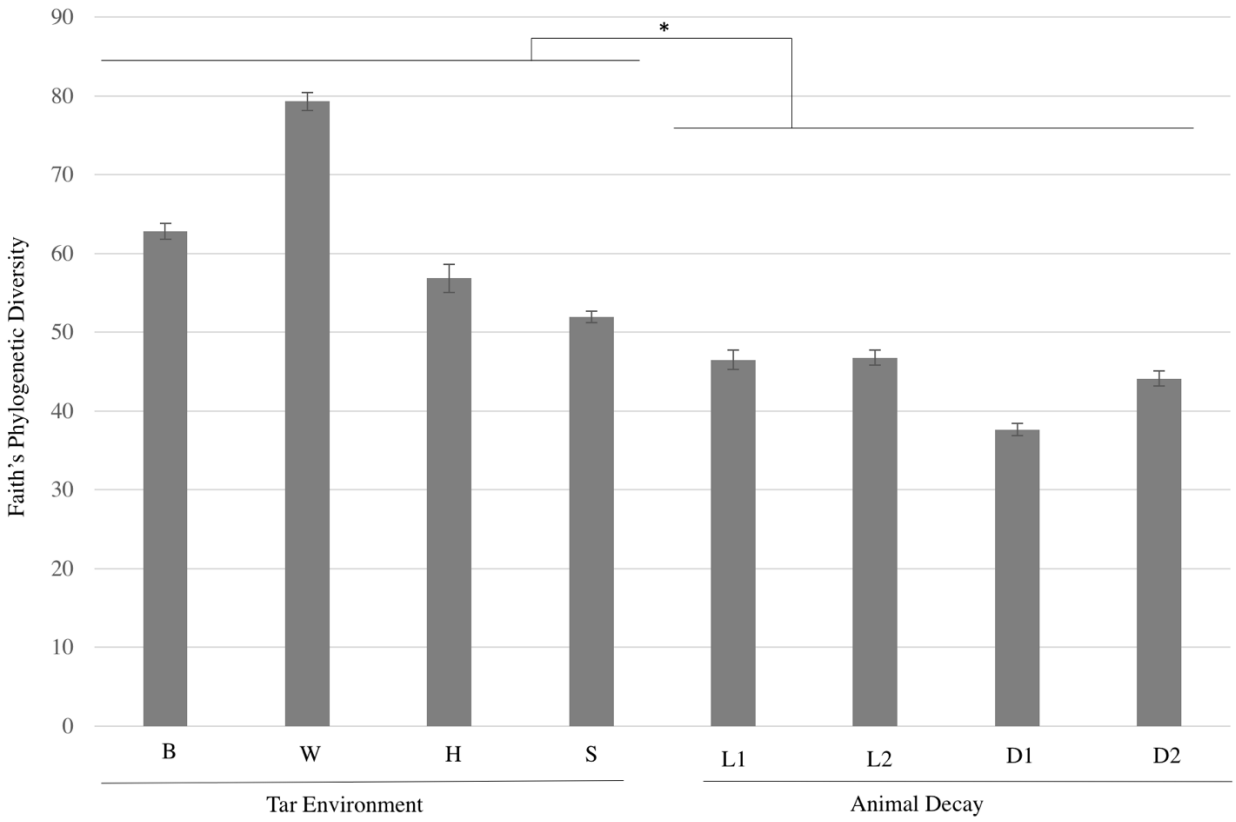


**Figure 3.** Average diversity within microbial communities. Alpha diversity, measured by Faith's Phylogenetic Diversity, is given as the average of 10 rarefactions of communities to 6,000 sequences. Error bars indicate the standard deviation. Communities from tar environments are more diverse than animal decay for Faith's Phylogenetic diversity (Wilcoxon rank sum test p = 0.036) and for the number of observed genera (Wilcoxon rank sum test p = 0.036), which are not shown. B = sediment at the bottom of the tar seep, W = water between the sediment and surface tar, H = head waters of the tar seep, S = surface tar, L1 = first leg scrape, L2 = second leg scrape, D1 = first tissue decay, D2 = second tissue decay.

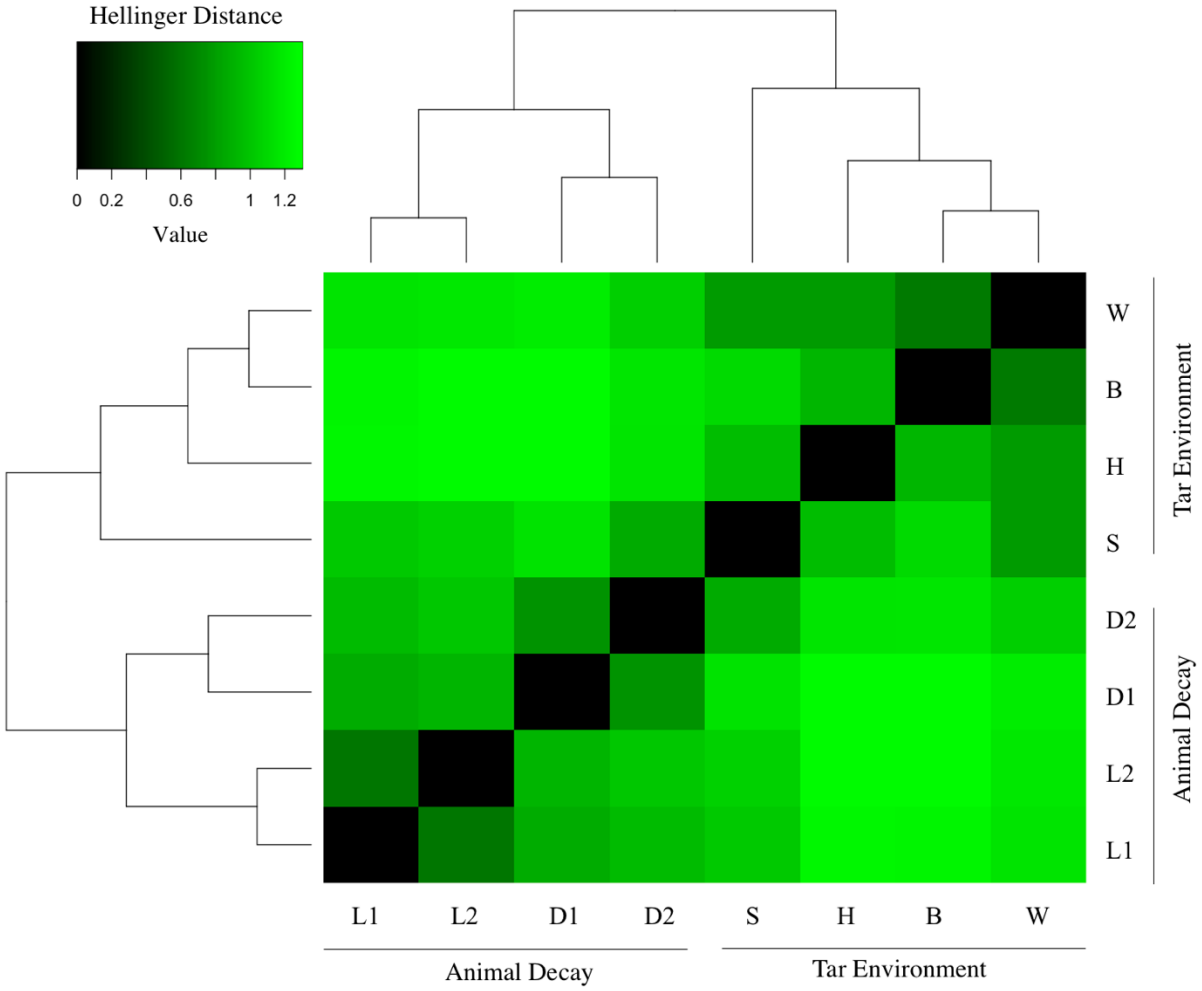


**Figure 4.** Heat map of Hellinger distance between microbial communities. Hellinger distance is calculated from the relative abundances of genera between microbial communities. B = sediment at the bottom of the tar seep, W = water between the sediment and surface tar, H = head waters of the tar seep, S = surface tar, L1 = first leg scrape, L2 = second leg scrape, D1 = first tissue decay, D2 = second tissue decay.

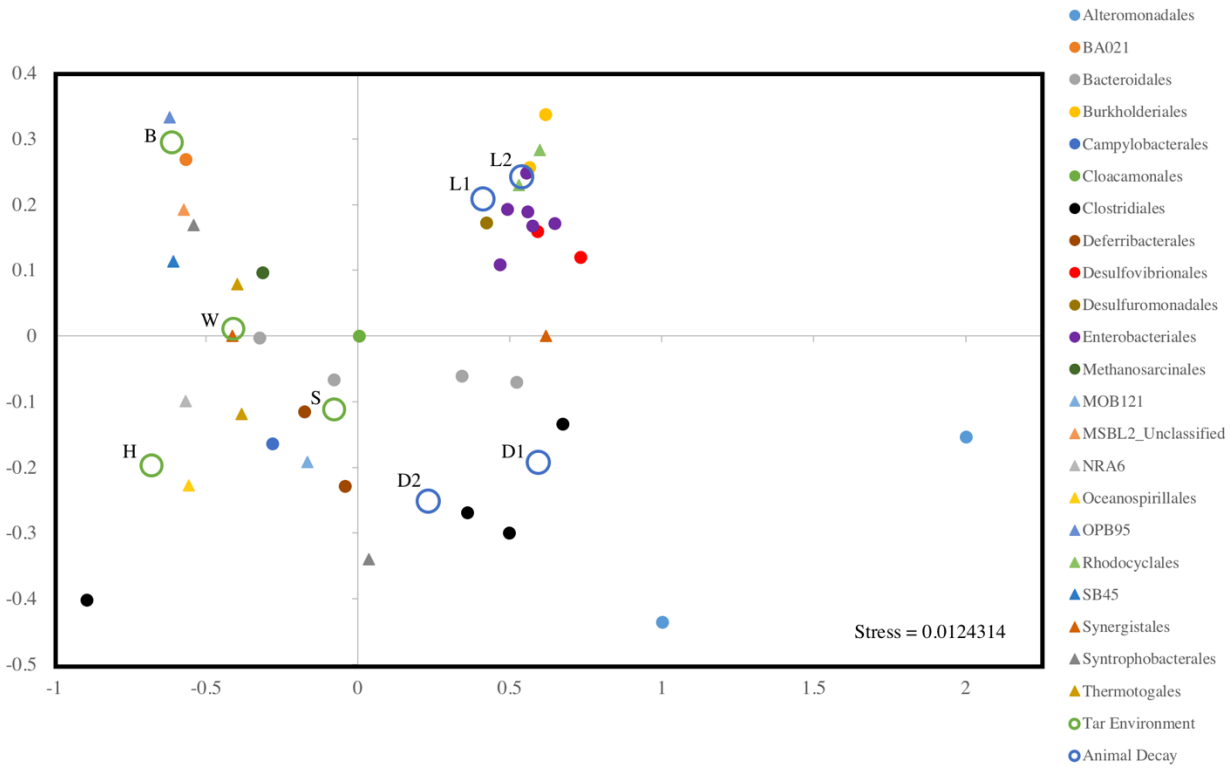


**Figure 5. Non-metric multidimensional scaling plot of between community diversity.** Beta Diversity was measured as the Hellinger Distance of the relative abundances of genera between microbial communities. Communities from tar environments are indicated in blue, and animal decay communities are indicated in green; B = sediment at the bottom of the tar seep, W = water between the sediment and surface tar, H = head waters of the tar seep, S = surface tar, L1 = first leg scrape, L2 = second leg scrape, D1 = first tissue decay, D2 = second tissue decay. The centroids for the 40 most abundant genera are given and colored by order.

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APPENDIX I

# Grassland invasions and their effects on native woody habitat in California's Mediterranean Ecosystems

"Invasions that alter ecosystems represent a particularly significant threat to native populations and communities: they don't merely compete with or consume native species, they change the rules of the game by altering environmental conditions or resource availably." (D'Antonio and Vitousek 1992)

## Threats to Mediterranean Ecosystems

There are five global Mediterranean ecosystems; California / Baja California, Chile, South Africa, Australia and the Mediterranean Basin. These ecosystems are characterized by summer drought and cool wet winters with highly variable precipitation events (Burcham 1956) between November and April (Kirkpatrick and Hutchinson 1980). Although these regions comprise less than 5% of the Earth's land mass they contain nearly 20% of the worlds' vascular plant species (Cowling et al. 1996; Greuter 1994). These ecosystems contain a large number of endemic species and comprise more biodiversity than that which is found in tropical Africa or Asia (Cowling et al. 1996; Underwood et al. 2009). Mediterranean ecosystems are under threat from many stressors and by 2100 they are expected to have the largest change in biodiversity relative to other biomes (Sala et al. 2000). As of 1994, of the 4300 native plants in Mediterranean California, 718 were considered threatened (Greuter 1994). These ecosystems are highly sensitive to land use change, and change in climate (Sala et al. 2000). Human activity is directly and indirectly responsible for much of the land use change. This is primarily through

habitat destruction, land conversion for agriculture, but pollution and changes in fire regimes may also benefit exotic invasive species and facilitate habitat conversion (Kimball et al. 2014; Pardo et al. 2011; Wood et al. 2006). To exacerbate the problem, human population growth is occurring rapidly in these climate regions. For example, in California, population increased by 13% between 1990 and 2000 (Underwood et al. 2009). This leads to increases land use change both directly and indirectly. Maintaining native Mediterranean habitats and protecting them from conversion to invasive exotic vegetation is of critical concern (Cox and Allen 2011; Kirkpatrick and Hutchinson 1980; Myers et al. 2000; Sharma et al. 2010; Vilà et al. 2001).

**Invasive Annual Grasslands**

Species invasions and conversion to non-native habitat, occur when the natural barriers to dispersal break down. Exotic annual grasses are successful invaders in part because they are actively moved by humans (Heady 1977; Parsons 1972; Minnich 1988). Grasses are successful in many ecosystems and change the composition of species and ecosystem functions. This promotes the establishment of invasive exotic grassland habitats (Keeley and Swift 1995; Wood et al. 2006; Cox and Allen 2011; D'Antonio and Vitousek 1992; Dickens et al. 2013; Wolkovich et al. 2010). They also change fire regimes in these ecosystems which can also facilitate further habitat conversion to non-native grasslands (O'Leary and Westman 1988; D'Antonio and Vitousek 1992; Talluto and Suding 2008; Vilà et al. 2001). Invasions by non-native species decrease the likelihood that native species will persist (Gilbert and Levine 2013). It is estimated that species invasions are responsible for more extinctions than human induced climate change (D'Antonio and Vitousek 1992). Non-native grassland invasions are a global phenomenon

(D'Antonio and Vitousek 1992), and their effects are long-term and frequently irreversible (Coblentz 1990).

In California, non-native grasses have been present since at least 1769 and were likely transported on the ships of early explorers in the form of feed stocks (Bartolome, Klukkert, and Barry 1986; Heady 1977; Minnich and Dezzani 1998; Minnich 1988). Early invaders (*Hordeum leporinum*, *Lolium multiflorum* and *Poa annua*) can be found in the bricks used to build early Franciscan missions (Heady 1977; Burcham 1956). Mediterranean annuals (e.g. *Avena fatua, Brassica nigra, Erodium cicutarium*) naturalized in valleys (Minnich 1988; Minnich and Dezzani 1998) and replaced native herbaceous cover (Bartolome, Klukkert, and Barry 1986; Minnich 1998; Timbrook, Johnson, and Earle 1982). By 1850, most of the perennial bunch grasses were replaced by *Avena fatua* and *Brassica nigra* (Burcham 1956). A later wave of Mediterranean annuals (*Bromus madritensis, Bromus diandrus, and Brassica geniculate*) naturalized on hillsides (Cione, Padgett, and Allen 2002). Today, most Californian soils support non-native annual grasslands which frequently co-occur with shrubland and woodland habitat.

Grasses are good competitors for resources. Dense grass canopies absorb a lot of light and reduces photosynthesis for competitor species (Kjelgren and Rupp 1997; Tang et al. 1990). The dense shallow root system of grasses (Davis and Mooney 1985; Goldstein and Suding 2014; Ward, Wiegand, and Getzin 2013) can reduce water availability for other species and suppress seeding establishment (Davis and Mooney 1985; Eliason and Allen 1997; Schultz, Launchbaugh, and Biswell 1955; Gordon and Rice 2000). Grassland soil has higher surface temperature and lower canopy humidity than woody species, which favors the persistence of grasses and reduces the establishment of woody species, particularly through outcompeting woody seedlings (D'Antonio and Vitousek 1992). The changes in microclimate associated with grasses and the

high flammability of grasses can lead to increased fire frequency and intensity. Grasses recover quickly from fires relative to woody species and can lead to further declines in woody establishment and grassland conversion (Keeley, Baer-Keeley, and Fotheringham 2005; Talluto and Suding 2008). Californian Mediterranean habitats have different sensitivities to annual grass invasion.

**Annual Grassland Invasion in Coastal Sage Scrub Habitat**

Coastal sage scrub is one of the most endangered habitats in the United States (Noss, LaRoe, and Scott 1995), and is only found in California's Mediterranean climatic zone (Kirkpatrick and Hutchinson 1980) between central and Baja California (Mooney 1977). Human activities are responsible for large losses of coastal sage scrub habitat. These include land development due to urbanization and agriculture (Kirkpatrick and Hutchinson 1980; Minnich and Dezzani 1998; Davis, Stine, and Stoms 1994; Talluto and Suding 2008; Taylor 2005) and also the introduction of exotic species (Minnich and Dezzani 1998; Allen 2005; Cione, Padgett, and Allen 2002; Eliason and Allen 1997). Between 1931 and 1995 37% of coastal sage scrub habitat was lost in Orange (22%), Riverside (26%) and San Diego (46%) counties (Taylor 2005). Less than 4% of coastal sage scrub habitat occurs on protected land (Davis, Stine, and Stoms 1994), however, there are still major declines in coastal sage scrub on undeveloped lands due to invasions of non-native grasses including *Bromus sp.* and *Avena sp.* (Freudenberger, Fish, and Keeley 1987; Minnich and Dezzani 1998). For example, of the undeveloped land in Riverside and San Diego Counties that consisted of coastal sage scrub in 1930, 49% was converted to nonnative grasses by 2008 (Talluto and Suding 2008). Physical characteristics of coastal sage

scrub plants contribute to the success of annual grass invasions and habitat conversion to exotic grasslands.

Many of the adaptations used by coastal sage scrub plants to persist in California's Mediterranean climate can be exploited by invasive exotic grasses. For example, coastal sage scrub is dominated by shrubs (e.g. *Salvia leucophylla* and *Artemisia californica*) that are widely spaced with characteristic bare ground patches and / or soil crusts between plants (Desimone and Burk 1992). Bare ground between shrubs is thought to promote shrub seedling establishment. However, this open space can provide open soil for exotic grass invasion and establishment (Minnich and Dezzani 1998). In addition, coastal sage scrub is drought deciduous, which means that the majority of growth occurs during wet periods and leaf loss occurs during hot dry periods (Rundel 2007; Jacobsen et al. 2007). This strategy helps minimize the effects of drought on coastal sage scrub which is vulnerable to dry conditions that lead to xylem cavitation (Jacobsen et al. 2007; Lambrecht et al. 2011). In fact, drought is a contributing factor for coastal sage scrub decline (Kimball et al. 2014; Heady 1977) (Keeley, Baer-Keeley, and Fotheringham 2005). Annual grasses, in contrast, are active earlier in the growing season and senesce in the late spring and early summer. They use a lot of soil water early in the growing season, and reduce amount of soil moisture available to shrubs. This reduction in moisture is detrimental to shrub establishment and persistence (D'Antonio and Vitousek 1992; O'Leary and Westman 1988; Westman 1981). Grasses also differ from shrubs in root structure and rooting depth and can deplete surface moisture, which also is detrimental to shrub seedling establishment and survival (Eliason and Allen 1997; Wood et al. 2006). Grassland species are good competitors for space and water to the detriment of shrub seedling establishment.

Other ecosystem characteristics benefit annual grasses over coastal sage scrub plants. These characteristics include fire and nitrogen deposition from pollution. Coastal sage scrub shrubs are subligneus, which means that they are partially or somewhat woody, and have mesophytic leaves that can be aromatic and flammable (Kirkpatrick and Hutchinson 1980). Annual grasses lack woody structure and generate large amounts of flammable biomass at the end of the growing season. Grass litter promotes more frequent fires (D'Antonio and Vitousek 1992; Ziska, Reeves, and Blank 2005) and grasses grow much faster than coastal sage scrub after fires (Minnich and Dezzani 1998; O'Leary and Westman 1988). Coastal sage scrub can regenerate after fire (Keeley and Keeley 1984), but the level of disturbance (including the presence of annual grasses (Eliason and Allen 1997)) influences the rate of recovery (O'Leary and Westman 1988). Fire can be very detrimental to shrubland habitat and in the past it was used as a strategy to clear this habitat for grazing (Keeley 2002; Tyler 2007). Pollution is also detrimental to coastal sage plants. Pollutants can cause leaf damage and increase leaf turnover. High leaf turnover diverts energy from away from root biomass (Allen 2005; Westman 1985) which affecting the heath and subsequent growth of the plant. Pollution also changes soil nitrogen concentrations (Padgett et al. 1999) through nitrogen deposition. High levels of nitrogen deposition favors the growth of annual grasses over coastal sage scrub (Cione, Padgett, and Allen 2002; Kimball et al. 2014; Padgett et al. 1999; Wood et al. 2006). Coastal sage scrub is very sensitive to annual grass invasion, however, not all Californian habitats have the same relationships with annual grasses.

**Annual Grassland Invasion in Oak Woodland Habitat**

Oak woodland habitat is also negativity impacted by annual grassland invasions. Oak

trees make up roughly a quarter of the California's forests and woodlands and provide numerous ecosystem services (Pavlik 1991). Oak woodland and savanna used to cover 10 -11 million acres but have been reduced by nearly 50% (Bolsinger 1988). Between 1945 and 1975, 1 million acres of oak habitat were removed for cattle grazing through a state sponsored range improvement programs (Bolsinger 1988), and were replaced by annual grasslands. These habitats are also negatively impacted by urban development (Gaman 2006), wine production (Merenlender 2000), and diseases (Lynch et al. 2013; Lynch et al. 2014). To compound these losses of habitats and trees, oak seedling recruitment is declining within habitats (Tyler, Kuhn, and Davis 2006; Zavaleta, Hulvey, and Fulfrost 2007; Griffin 1976). Across the state, oak woodlands interface with grasslands. Oak woodlands are frequently adjacent to patches of annual grass or have grassy understories. There are many ways in which grasslands are impairing the maintenance and regeneration of oak woodlands.

The current oak woodland habitats are facing regeneration problems. The age structures of several species of oak across different habitat patches suggest that trees are not being replaced in oak habitats adjacent to grassland (Callaway and Davis 1998). This is supported by observations of few seedlings and low sapling recruitment within these habitats. Low recruitment is particularly apparent in oak savannah (Callaway and Davis 1998) and in open grasslands adjacent to oak woodlands (Griffin 1976; Callaway 1992). Oak recruitment is negatively affected by competition from non-native grasses (Gordon and Rice 2000; Griffin 1971; Gordon et al. 1989), herbivory (Borchert et al. 1989; Davis, Tyler, and Mahall 2011; Gordon et al. 1989; Griffin 1971), low soil moisture (Borchert et al. 1989; Mahall et al. 2009), and cattle grazing (Davis, Tyler, and Mahall 2011; Pearse et al. 2014; Lopez-Sanchez et al. 2014). The causes of low recruitment may differ by oak species (Tyler, Kuhn, and Davis 2006; Pearse et al. 2014) and

the impact of annual grasses may be species dependent. For example, most of the detrimental effects of annual grasses on coastal live oak (*Quercus agrifolia*) are associated with cattle grazing and herbivory in annual grasslands (Pearse et al. 2014). Coastal live oak regeneration is more common in shrubland (Callaway and Davis 1998), where *Artemisia californica* and other shrub species enhance seedling survival. This is accomplished by reducing herbivory near seedlings, and also through increased acorn survival under shrubs (Callaway and D'Antonio 1991; Callaway and Davis 1998). Oak woodlands are directly and indirectly impacted by annual grasslands and as a consequence many oak species are declining due to reduced recruitment.

**Summary**

Human activities facilitate annual grassland invasion in California's Mediterranean habitats. Coastal sage scrub is particularly sensitive to invasion because grasses compete directly with sage scrub plants for water, space, and other soil resources. Indirectly, invasive annual grasslands promote higher frequencies of fire and recover more rapidly than coastal sage scrub plants. Increased levels of anthropogenic pollution favor annual grass species over coastal sage scrub. In contrast, oak woodlands are indirectly impacted by annual grasslands through the clearing of habitat for range land and maintenance of annual grasslands by grazing. This impacts seedling recruitment by reducing favorable habitat (e.g. sage scrub) for seedling recruitment, and increased herbivory and disturbance of seedlings through cattle grazing. Invasive annual grasslands are changing habitats across Mediterranean California, but the impacts of these grasses are specific to habitats types.

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