UNIVERSITY OF CALIFORNIA

Santa Barbara

Environmental Controls on Extracellular Polysaccharide Production in a Mediterranean Grassland Soil

A Thesis submitted in partial satisfaction of the

requirements for the degree Master of Arts

in Ecology, Evolution, and Marine Biology

by

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June 2016

The thesis of Kenneth Allen Marchus is approved.

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ACKNOWLEDGEMENTS

I couldn't have done this without the financial support and awards provided by NSF, the Earth Research Institute, the Dept. of Ecology, Evolution and Marine Biology and the Worster family for their generous support. Thanks to the Schimel Lab, both past and present, and the Holden Lab for guidance and support through this process. Last but not least, I'd like to acknowledge my amazing family and friends for their continuous love and support.

ABSTRACT

Environmental Controls on Extracellular Polysaccharide Production in a Mediterranean Grassland Soil

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The Mediterranean climate has two clear seasons—the cool wet winter growing season, and the hot dry summer, which routinely experiences 6 months or more without rain and is routine in southern California. Microbes survive and biochemical processes continue even during the driest parts of the long summer. Biofilms, or extracellular polymeric substances (EPS), are thought to be an important means for microbes to survive through physically stressful times, (i.e. drought). Do EPS concentrations increase with the length of the dry season? Do EPS concentrations vary with different levels of carbon (C) inputs? We hypothesize that drier soils will have greater microbial EPS due to the amplified need for survival; additionally, soils with higher C inputs will have more C to allocate to EPS production, but may be dominated by plant produced EPS.

To answer these questions, we manipulated plant cover and dry season length and measured EPS in seasonally dry grassland soils and evaluated pools of total EPS in the soils as well as the mix of sugars making up EPS. Soil cores were collected monthly from our research plots to capture the transition from the dry dormant summer to the wet winter growing season, from July 2014 to February 2015. Because EPS are largely made up of sugars, we used extractable sugar residues as a proxy for EPS and we analyzed them using Gas Chromatography coupled with Mass Spectroscopy (GC-MS).

The GC-MS data shows a significant decrease in sugar concentrations with increased moisture across all sample dates. Drier soils show greater accumulation or production of EPS, which supports our hypothesis. Plant removal does lessen EPS accumulation or increase consumption and drive overall concentrations down slightly.

We conclude that after subjecting the soils to a range of dry season length treatments, there were reductions in EPS accumulation with moist conditions. However, these changes were not as drastic as we expected thus suggesting that other microbial survival mechanisms may be involved.

LIST OF FIGURES

1. Introduction

The Mediterranean climate has two clear seasons—the cool wet winter growing season, and the hot dry summer, which routinely experiences 6 months or more without rain (Bolle, 2003). Although many plants senesce or go dormant, microbes survive and biochemical processes continue even during the driest parts of the long summer (Parker and Schimel 2011). While it was previously assumed that microbial activity in the dry summer months ceases, in California grasslands, microbial biomass may actually increase during these harsh times (Waldrop and Firestone, 2006; Parker and Schimel 2011).

The mechanisms remain unclear that microbes use to survive in Mediterranean environments, as well as others that experience dry or seasonally dry conditions. Yet, moisture can be limited for prolonged periods of time and drought stress severe. Extended dry periods are often followed by strong rewetting events, which have been argued to cause severe osmotic stresses, potentially causing microbes to burst (Kieft et al. 1987; Schimel et al. 2007), but recent work suggests this may be rare (Boot et al. 2013). During dry conditions, microbes may simply dehydrate or become disconnected from substrate, eventually leading to starvation (Manzoni et al. 2012; Parker and Schimel, 2011). Yet these stressors are routine in seasonally dry ecosystems and microbes continue to function.

Microbial physiology is in part regulated by substrate and water availability in soils (Skopp, 1990; Stark and Firestone, 1995). When soils wet up, micro and macro-pores fill and soils become hydrologically connected (Manzoni et al. 2012; Parker and Schimel, 2011), which allows for diffusion of substrates through the soil (Parker and Schimel, 2011). When soils dry, diffusion becomes limited and soil pore spaces become physically disconnected, separating microbes from their resources leading to reduced activity and may drive microbes into dormancy (Fierer et al. 2005; Manzoni et al. 2012). However, they don't

shut down completely (Waldrop and Firestone, 2006; Parker and Schimel 2011). Therefore, to survive dry conditions, microbes have adapted the ability to shift resources from acquiring substrate and growth to survival mechanisms (Schimel et al. 2007). This begs the question, what are microbes doing to survive?

One proposed mechanism for overcoming environmental stressors is the production of extracellular polymeric substances; this is predominantly polysaccharide, but also contains DNA, protein, and other constituents. Extracellular polysaccharides (EPS) are produced by both plants and microorganisms (Oades, 1972) and acts as glue-like binding agents that adhere to soil particles, which promote soil aggregate formation and stability (Blankenship et al. 2016; Martin 1946; Whistler and Kirby 1956). It is thought that microbes encapsulate themselves in EPS as a survival mechanism under stressful conditions, particularly starvation (Chen et al. 2014; Colica et al. 2014; Rossi et al. 2012; Steinberger and Holden 2004; Wolfaardt et al. 1999) and desiccation (Chenu, 1993; Chenu, 1995; Harris, 1981; Roberson and Firestone 1992). During dry periods, when resource pools shrink and concentrate in soil pore microsites, EPS creates a matrix that physically connects microbes to substrates (Chenu and Roberson, 1996; Or et al. 2007). Further, as soils dry, EPS films may help maintain a beneficial microhabitat within soil aggregates to retain moisture by dramatically increasing soil water-holding capacity and delaying drying (Oades, 1984; Pointing and Belnap 2012; Roberson and Firestone 1992). However, producing EPS may be taxing and energy intensive for microorganisms (Harder and Dijkhuizen 1983; Wolfaardt et al. 1999). Thus, attributing microbial EPS production to an environmental condition or specific stress response remains uncertain (Schimel et al. 2007).

In this study, we ask: what environmental drivers in a natural grassland ecosystem control the production or accumulation of extracellular polysaccharides? Do EPS

concentrations increase with the extended length of the dry season and greater plant inputs? We hypothesize that with longer dry periods (i.e. extended drought), soils will have greater levels of EPS due to the amplified need for survival from desiccation and starvation. We also hypothesize that soils with more plants (i.e. higher C inputs) will have more C to allocate to EPS production, but will be dominated by plant produced EPS whereas soils without plants (i.e. lower C inputs) will have less EPS, due to a lack of available C substrate and will be dominated by microbial EPS. To answer these questions, we manipulated plant cover and dry season length and measured EPS in seasonally dry grassland soils and evaluated pools of total EPS in the soils as well as the mix of sugars making up EPS.

2. Methods

2.1 Field Methods

We designed and implemented a plant cover and soil moisture field manipulation experiment in a seasonally dry grassland in Santa Barbara County. The site is located at the University of California, Sedgwick Reserve in Los Olivos, California (34.712036°, -120.038797°). The reserve is approximately 28km from the coast, and 370m above sea level in a North-South oriented valley in the Santa Ynez Valley. The sampling area is dominated by exotic annual grasses -primarily *Bromus diandrus*, *Bromus hordaceous*, and *Avena fatua*. The area has a Mediterranean climate regime, with long dry summers and cool wet winters. Average annual precipitation is 380mm, however, during our 2 year study we were in the midst of a drought, where annual rainfall was approximately 50% below normal (175mm in 2013 and 201mm in 2014). Daily average air temperature is 16.8^{o} C, with highs in the summer months reaching into the 30's and winter lows below freezing are not uncommon. Micro meteorological data was obtained (IDEAS, UCSB Geography Dept.

http://www.geog.ucsb.edu/ideas/) from a site on the Sedgwick reserve, approximately 2km southwest of the field site. Air temperature and relative humidity readings were taken from 75cm above the ground, rain and fog events were collected and soil temp was measured from 15cm depth.

Soils are described as Pachic Haploxerolls with silty clay loam texture and granular structure on nearly flat slopes (< 2%). The soil pH is 6.0, with 2.2% C, 0.21% N, and a bulk density of 1.2 g cm^{-3} in the upper 10 cm (Blankinship et al. 2016; Homyak et al. 2016).

Plant thinning treatment

We created a gradient of fresh plant inputs into the surface soils by thinning live plants. The treatments consist of 0%, 33%, 66% and 100% removal and was done by hand throughout the growing season. All plants were removed with the 100% removal, but we can't assume all plants inputs were excluded. For this study, we sampled from plots with plants (0% removal) and without plants (100% removal) to focus on the extreme manipulations.

Dry season manipulation

Dry season length was manipulated to alter the amount of time during which dry-season processes would continue (**Fig. 1**). Water manipulations included: dry, control, short dry, and wet (i.e. no dry season). Water was added to the short dry plots (May 23rd – July 8th) and wet plots (May 23rd – Nov 28th) biweekly using backpack sprayers. Each scheduled watering consisted of two applications of 15L each, roughly an hour apart, which equated to 1.5cm of water. This maintained >10% volumetric water content (VWC). Based on Fierer et al., 2005, we deemed 10% VWC to be an important moist/dry threshold, which was due to a noticeable decline in soil respiration, suggesting a loss of access to C substrate. In dry treatment plots, we excluded precipitation from the from October 28th – February 2nd using

rain-out shelters made of clear corrugated polycarbonate roof panels (Suntuf, Palram Americas, Kutztown, Pennsylvania).

After 2 years of field manipulation treatments we collected 0-10cm soil cores monthly from our research plots to capture transition from the dry dormant season to the wet growing season, from July 2014 to February 2015. Samples were collected from near center of each plot to prevent any edge effect from neighboring plants and plant roots from outside the plots.

2.2 Laboratory Methods

To analyze the EPS material we extracted polysaccharides from soils sampled from each of the treatment plots using a modified hot-water extraction method (Ball et al. 1996). Hot water was used to hydrolyze microbial EPS and extract the stabilized materials that are bound to minerals and tied up in soil aggregates. Hot water is an intermediate between the dilute sulfuric acid method, which hydrolyzes plant tissues and other organic matter (OM) and so overestimates microbial polysaccharide pools (Redmile-Gordon et al. 2014), and the cold-water extraction method, which only accesses free detritus and OM (Lutzow et al. 2007).

Soils were brought back to lab and prepared that same day by passing intact cores through a 4mm sieve to remove rocks and homogenize each sample. 1g (wet weight) subsamples of the fresh soils were mixed with deionized water (10mL) and then heated and shaken in an 80^{o}C water bath at 150 rpm for 16 hours. At the end of the 16 hours the extracts were cooled for 20 minutes and vacuum filtered to 2.7µm using Whatman #542, hardened ashless filter paper to obtain liquid extracts of each soil sample. Sample extracts were stored at -20^{o}C while standard solutions (1 mg sugar/mL) of each sugar of interest were prepared (arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, mannose,

galactose, glucose (dextrose), N-acetyl-galactosamine, N-acetyl-glucosamine, N-acetyl-mannosamine) and an internal standard (myo-inositol). The internal standard was added (20μL) to all samples and standards to track the derivatization process and provide quality control throughout the process. Samples were refrozen and then freeze-dried until ready for the derivatization process.

To prepare freeze-dried samples for analysis, we hydrolyzed polysaccharides to their constituent monomers and then derivitized them to convert sugars into methyl glycosides and glycosyluronic acids into methyl ester methyl glycosides (i.e. glycosyl residues). This was done by adding 500μL of 1M methanolic HLC and heating the sample at 80^{o}C for 16 hours; after cooling for 20 minutes, the methanolic HLC was removed by adding 200μL of methanol and evaporated using filtered N_2 gas two times. N_2 gas was filtered to remove any moisture present using an inline Hydro-Purge II filter from Alltech Associates Inc. (Deerfield, Illinois). Then to re-acetylate any N-acetyl compounds present, 100 μL methanol, 50 μL pyridine, and 50 μL acetic anhydride was added to each tube, mixed and then set at room temperature for 30 min. The samples were again evaporated with N_2 gas and 100μL of Tri-Sil HTP Reagent (2:1:10 HMDS:TMCS:pyridine) was added. The reaction mix was heated at 80^{o}C for 20 minutes, cooled for 10 minutes then evaporated again with N_2. Samples were filtered to 8μm through mini-columns packed with glass wool and evaporated again. Derivatives were re-dissolved with 0.5mL hexane, evaporated, rinsed with 100μL hexane and transferred to 2ml vials (with 200μL inserts). Samples were stored at -20^{o}C until analysis.

The derivatives were analyzed using an Agilent Technologies gas chromatograph, with mass spectrometer (GC-MS, HP 6890/5973 GC-MS system) to analyze the glycosyl composition of polysaccharides by forming trimethylsilyl ethers of methyl glycosides (York

et al. 1986). The GC-MS provides accurate concentrations of 12 specific sugars of interest: Arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, mannose, galactose, glucose (dextrose), galactosamine, glucosamine and mannosamine. This quantitative analysis is performed on a fused-silica DB1 column (J&W Scientific), 30-meter by 0.25 mm by 0.25 μm. Auto-injections of 1μL were made with a split ratio of 10:1, and a column flow rate of 1mL/min helium. The initial oven temperature was 160°C with an immediate ramp to 200°C at 2°/min. The column was then conditioned for the next sample by an increase to 260°C at 10°/min and holding at 260° for 5 min. Since each sugar results in several derivatives, the major peak areas for each sugar are summed before calculating response factors and determining glycosyl compositions.

2.3 Statistical Analysis

Treatment effects (harvest date, plant thinning and moisture (i.e. dry season length)) on total EPS, microbe:plant EPS and individual sugar concentrations, were tested by Three-way, full-factorial ANOVA. No multi factor effects were significant with the full-factorial ANOVA. Therefore a One-way test was done on individual treatments. Tukey HSD was also run to separate samples and treatments from one another. All statistical analyses were performed in JMP Pro 12.0.1 (SAS Institute, Cary, North Carolina). Mean and standard errors were also calculated for each sample date, treatment and EPS concentration.

4. Results

4.1 Seasonal variation in EPS concentrations

Total sugars

Total EPS (i.e. glycosyl residue) concentrations across all treatments started relatively low and generally rose, oscillated then declined across the sampling dates

(**Fig. 2**). Plots with plants in dry and ambient conditions (i.e. control), concentrations notably increased to the end of the summer dry season, reaching 363±30(μg C g^{-1} soil) (**Fig. 2**). After the first rain of winter (October 31st, 2014) concentrations began to decline and dropped to below early summer levels 188±27(μg C g^{-1} soil). EPS concentrations in short dry and wet (i.e. moist, >10% volumetric water content) as well as dry and control plots without plants, did not show a notable summer/fall increase and oscillated more mildly thru November and December before concentrations dropped.

Individual sugars

Individual EPS sugars generally track the overall pattern of total sugars (**Fig. 3**). The exceptions were galacturonic acid and the N-acetyl compounds (N-acetyl-galactosamine, N-acetyl-glucosamine and N-acetyl-mannosamine). The N-acetyl compounds were bordering detection limits, whereas N-acetyl-mannosamine was consistently below the limits of detection. Galacturonic acid concentrations increased in the September and October samplings, opposite to the pattern of the other sugars; concentrations went from 1.08(μg C g^{-1} soil), up to 9.8(μg C g^{-1} soil), and then returned back to 3.76(μg C g^{-1} soil) between the October-November sampling dates. All the other sugars concentrations decreased during September and October.

4.2 Ratio of Microbe:Plant EPS

Galactose (gal) and mannose (man) are thought to be predominantly microbial products while arabinose (ara) and xylose (xyl) are thought to be more plant based (Oades, 1972; Oades, 1984). In these soils, EPS was always dominated by microbial products, as indicated by the ratio of microbial to plant sugars (gal + man : ara + xyl). The microbe to plant EPS ratio reached as high as 1.97±0.10 in

ambient conditions with plants, whereas at the lowest, it was only down to 1.72 (**Fig. 4**).

The ratio, however, shifted significantly across the sampling period ($p=0.02$). These low ratios were found in winter (Dec, Feb) sampling dates, while high ratios were found typically in the fall (Aug, Sept, Oct). Yet, none of the individual sampling dates showed significant differences between treatments.

Plant thinning increased the ratio of microbial to plant sugars noticeably across all treatments ($p < 0.0001$). Effects were significant on plant sugars; ara ($p=0.07$) and xyl ($p=0.02$), but no significance was shown on microbial sugars; gal ($p=0.75$) and man ($p=0.99$).

Dry season manipulations lowered the ratio of microbe to plant sugars with drier conditions ($p < 0.0001$). Effects were also significant for both plant sugars; ara ($p=0.001$) and xyl ($p=0.0005$) and microbial sugars; gal ($p=0.07$) and man ($p=0.003$).

4.3 Treatments

Dry season manipulations

In plots that had been watered, notably in the no dry season treatment, EPS concentrations were lower than in drier plots; this held true throughout the sampling period (**Fig. 5**). The EPS levels in constantly moist plots were significantly lower than the dry ($p=0.006$), control ($p=0.001$) and short dry ($p=0.06$) plots, roughly 50μgC/g $soil^{-1}$ lower on average across the sampling period.

Plant thinning

Plant thinning tended to reduce EPS concentrations slightly. Effects were most apparent in both dry and control plots during the fall (Oct, Nov), when total EPS concentrations were highest (**Fig. 2; Fig. 5**). With short-dry and wet plots,

differences were more apparent in the winter sampling dates (Dec, Feb) and both remained lower without plants. The overall plant thinning treatment effect was significant at $p = 0.07$. Again, plant thinning did significantly reduce individual sugars that are thought to be more associated with plant production; ara ($p= 0.07$) and xyl ($p=0.02$).

5. Discussion

In this study we examined which environmental conditions (i.e. moisture and plant C) alter EPS (i.e. glycosyl residue) accumulation in a California grassland soil. We hypothesized that dry season length would alter microbial EPS production and that drier conditions would promote greater EPS production. We also hypothesized fresh plant C would increase total EPS levels and be more dominated by plant products, while removing plants would greatly reduce EPS levels.

We found that, to our surprise, EPS concentrations were not influenced by a lack of fresh plant C—rather EPS levels were only slightly lower and in some cases higher (but not significantly so) in plots without plants (**Fig. 5**). Fresh C inputs may not be as essential to available substrate pools and microbial EPS production as previously thought.

Contrary to what we hypothesized, the response of EPS accumulation indicates that microbes are not C limited in the absence of plants. After 2 years of plant removal one would think the fresh C pools would be consumed and the difference between plant and no plant treatments would be more evident. However, that was not the case in this study. Microbes may in fact be more limited by physical constraints, given the heterogeneous nature of soil, rather than deficient organic C

pools (Manzoni et al. 2012; Parker et al. 2011). This could also be due to a legacy of plant C in plant removal plots.

The plant removal treatment did, however, decrease plant EPS products (arabinose and xylose) and raise the microbe:plant ratio signature of the EPS composition. Plant EPS was much lower, yet still a significant fraction of total EPS pool. Microbial production of EPS dominates across all plots and treatments and is an important process in this system. Plant EPS increases with the wet season when plants start growing and reallocating resources into root and soil storage, which was what we initially hypothesized.

With our dry season length manipulations, EPS patterns indicate a strong positive response to dry conditions and negative response to moist conditions (**Fig. 2; Fig. 5**). We found that EPS concentrations declined with increased moisture (both irrigation treatments and rain events) and increased with a longer dry season. We interpret this to mean that microbes produce EPS as an adaptation and response to drought, which supports our original hypothesis. If a key role of EPS is to maintain connection between microbes and resources as soils dry and hydrological connectivity fails (Manzoni et al. 2012; Parker and Schimel, 2011), then in moister soils, there may be less need to produce EPS. Alternately, in moister soils, there may be more EPS consumed. If EPS can be used as a microbial resource, then microbes may not break it down as readily in dry soils and pools may therefore accumulate. (Chenu and Roberson, 1996; Cheshire, 1977; Foster, 1981; Holden, 2011; Manzoni et al. 2012; Or et al. 2007; Parker et al. 2011)

The pattern of EPS in dry and control plots follow one another closely, even after soils in the control plots received rain. Moisture created hydrologic connections

and so substrate may be accessible with the moist soil treatments, but this doesn't explain why the dry plots follow the same pattern. One possible explanation for the decline in EPS, even in the rain exclusion treatment, might simply be relative humidity and dew. When the winter rains begin, fog, humidity and leaf wetness all increase regardless of direct exposure to rain (as measured by micrometeorological stations near our study site; IDEAS, UC Santa Barbara, Geography Department). Moisture in the air may have a strong influence on dry surface soils (McHugh et al. 2014), and so could shift soils out of the "dry season" pattern even without actual rainfall.

6. Conclusion

Our measurements suggest that soil moisture is controlling factor of microbial EPS dynamics, while plants have limited effects. We conclude that after 2 years of plant removal, plants did not play a major role in microbial EPS dynamics—EPS concentrations were the same in the presence and absence of plants. Although, plant associated sugars did decrease with plant removal. After subjecting the soils to a range of dry season length treatments, there were reductions in EPS accumulation with moist conditions. However, these changes were not as drastic as we expected thus suggesting that other microbial survival mechanisms may be involved. It is also possible that drought stress in seasonally dry climates has been overestimated and is simply business as usual for microorganisms.

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Figures

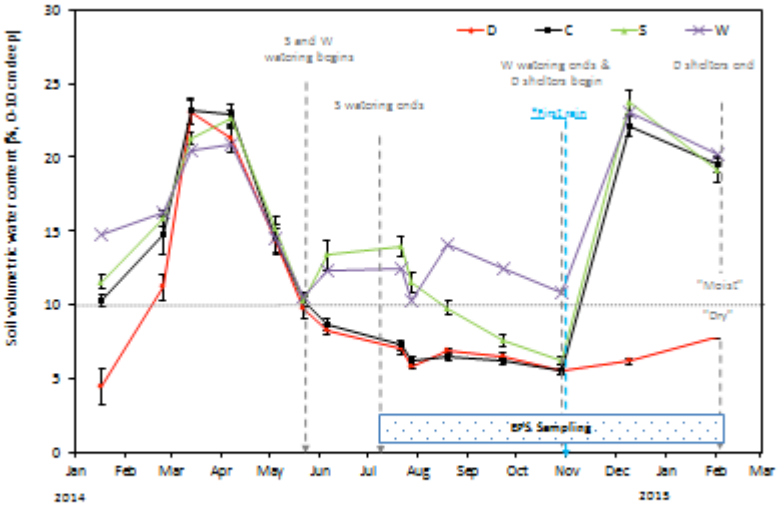

Figure 1. Seasonal soil moisture patterns with ambient and manipulated conditions before and during EPS sampling. Treatments (Dry (D) red diamonds & line; Control (C) black squares & line; Short Dry (S) green triangles & line; Wet (W) purple X & line). Timing of treatments (grey dashed line) and rain event (blue dashed line) (±SE).

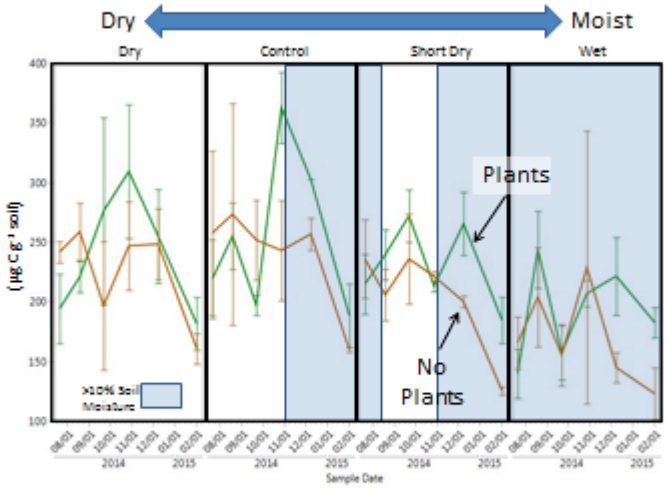

Figure 2. Total EPS patterns across sampling dates with manipulated dry season conditions and plant thinning treatments. Plant treatments; with plants (green line) and without plants (brown line) (±SE).

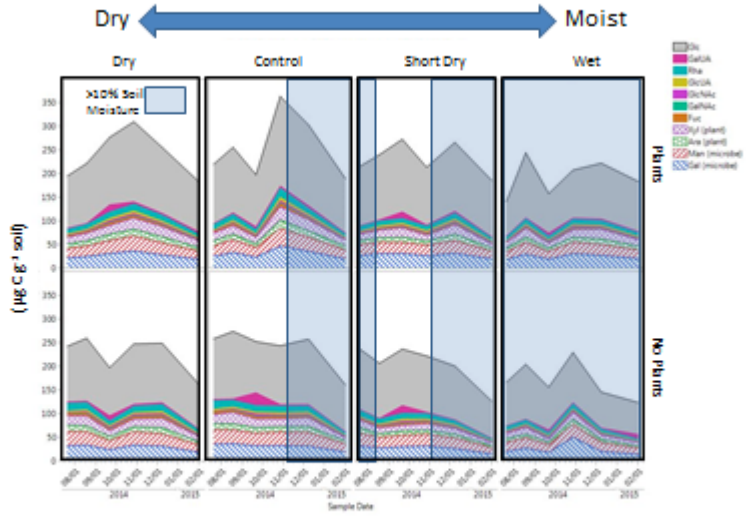

Figure 3. Individual EPS patterns across sampling dates with manipulated dry season conditions and plant thinning treatments.

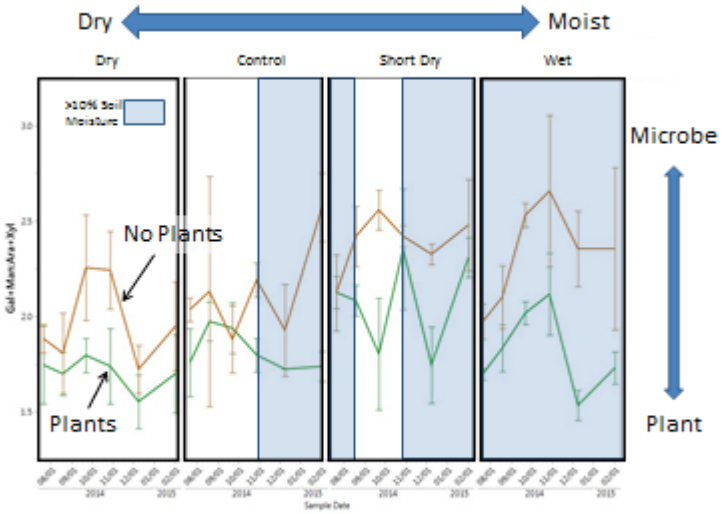

Figure 4. Ratio of microbial:plant EPS patterns across sampling dates with manipulated dry season conditions and plant thinning treatments. Galactose (gal) and mannose (man) are thought to be predominantly microbial products while arabinose (ara) and xylose (xyl) are thought to be more plant based. Plant treatments; with plants (green line) and without plants (brown line) (±SE).

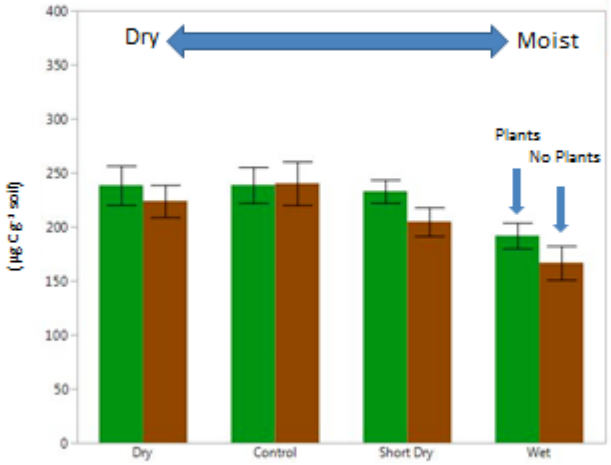

Figure 5. Total EPS averaged across sampling dates with manipulated dry season conditions and plant thinning treatments (±SE).