Metagenomics of Glen Rose Soil Microbial Systems and their Role(s) in Carbon Cycling and Native Plant Restoration at the Black Capped Vireo Research Area (West Austin Macrosite) and Wild Basin Wilderness Preserve

Permit #: Hauser – 2013

Charles Hauser
Bioinformatics Program
St. Edward's University
Austin TX 78704

November 25, 2013

Table of Contents

Abstract	……..	3
Introduction	……..	3
Materials and Methods	……..	4
Results	……..	5
Discussion	……..	11
References	……..	12
Publications	……..	12
Supplemental Information	……..	13

Abstract

The long-term goals of the project are to: first, characterize the microbial populations characteristic of black-capped vireo habitat present at the Black-capped Vireo Research Site and the Wild Basin Wilderness Preserve; second, to evaluate the effectiveness of soil remediation techniques in establishing microbial populations characteristic of black-capped vireo habitat in order to better manage BCP lands. Soil microbiome analyses were conducted at Vireo Preserve/Wild Basin Wilderness Preserve in June 2012, October 2013 and May 2013. In May 2013, Soil samples were obtained from two vireo habitat positive control sites (01WB13 and 02WB13), and three vireo habitat restoration sites (VP06, VP07, VP08). The three habitat restoration sites each consist of 5 mulch sock treatment areas: 1) the control treatment is mulch socks only; 2) onsite mulch containing local juniper and hardwoods recently processed; 3) onsite composted mulch containing local juniper and hardwoods that have been fermenting on the ground for 10-15 years; 4) Mulch and biochar combined; 5) composted mulch and biochar combined. Total microbial DNA was isolated from the 15 treatment areas and both control areas and have been submitted for bacterial and fungal diversity sequencing.

Preliminary analyses based on operational taxonomic unit (OUT) richness estimates indicate each kingdom is responding differentially to each treatment, with per-treatment richness estimates different for each kingdom. OTU abundance estimates also indicate treatment-specific signatures. Lastly, preliminary analyses of the β-diversity for fungi and eubacteria indicate treatment-specific populations. A preliminary analysis of the data obtained to date is summarized.

Introduction

The Black-Capped Vireo Preserve (VP) and Wild Basin Wilderness Preserve (WBWP) are part of the Balcones Canyonlands Preserve (BCP), protecting eight endangered species, as well as 27 species of concern. As part of an adaptive management experiment designed to improve habitat restoration techniques for black-capped vireos, this project aims to characterize the soil microbial communities present in the Glen Rose soils present at the treatment site, and the response of these communities to soil treatments by employing microbiome sequencing approaches. Central Texas has a region of uplifted limestone, the Edwards Plateau, providing an island of karst soils that fostered the speciation of many organisms and forms one of North America's areas of endemism. Within the Canyonlands division of the BCP, and characteristic of the VP treatment sites, exists the stair-stepped Glen Rose limestone, with slopes range from very steep to moderate, and the soils varying from shallow and stony on steep slopes and benches to moderately deep in ravines and small creek corridors.

With an extreme and unpredictably variable climate, the survival of regional flora is increasing recognized to be dependent on tight relationships with soil fauna, none of which have been described. The soils are extremely alkaline, requiring unexplored plant – microbe adaptations and novel biochemistry. The region was deforested in the late 1800's to provide the railroad ties that still underlie the nation's train network. Post-deforestation, the region was heavily grazed, principally with goats. Reforestation efforts have been largely unsuccessful because of insufficient soils and a lack of understanding of plant-soil microbial relationships that underlie biotic yields. The climate of central Texas has historically been extremely unpredictable. However as demonstrated by the historic

drought and heat wave of 2011 and recent climatic trends, we can expect increasing variability in the near future. Soil microorganisms constitute a significant fraction of the Earth's biomass, with surface soils estimated to contain 10^9- 10^{10} microbial cells per gram including bacterial, archaeal, fungal species, plus viruses and protists (Torsvik et al., 2002, Tringe et al., 2005, Gans et al., 2005). Despite this abundance and the importance of soil microorganisms for key ecosystem functions, the diversity and structure of soil microbial communities remain poorly studied. With the development of metagenomic techniques, efforts to characterize the full extent of microbial diversity, their role in a variety of global ecological functions including carbon balance, cycling of nutrients and promoting plant growth have been initiated. Recent large-scale surveys have revealed that different ecosystems support unique microbial populations (Angel et al., 2010, Fierer et al., 2008).

The goals of this project are to: (1) characterize the genomic complexity of the bacterial, archaea and fungal communities characteristic of Glen Rose soils, (2) Determine the impact(s) on soils and plant-microbe communities / interactions following remediation treatment, (3) Assess the capacity of the microbial community to sequester carbon. The long-term goal of the project is to provide a baseline data set that characterizes microbial populations characteristic of black-capped vireo habitat and the ability to approximate these populations using soil remediation techniques to better manage BCP lands for black-capped vireo habitat and ecological health.

Materials and Methods

Soil Samples

Soil samples were collected from the Vireo Preserve/Wild Basin Wilderness Preserve adaptive management treatment areas (VP06, VP07, VP08) and two positive control (WB0113, WB0213) (Supplemental Tables I).

The treatment area (VP06-08) is comprised of 3 rows, with each row consisting of five separate treatments: 1) The control treatment is mulch socks only; 2) Onsite mulch containing local juniper and hardwoods recently processed; 3) Onsite composted mulch containing local juniper and hardwoods that have been fermenting on the ground for 10-15 years; 4) Mulch and biochar combined; 5) Composted mulch and biochar combined. Samples were obtained from each treatment within rows 1 and 2 (Fig. 1). Vireo habitat positive control samples (01WB13, 02WB13) were collected in triplicate, combined and stored -80 prior to DNA isolation.

DNA Isolation

Total DNA was prepared using PowerSoil DNA Isolation Kit (Mo Bio Labs). DNA was prepared in triplicate from each treatment area and pooled to generate a composite sample for sequence analysis.

Microbiome Sequencing

rDNA regions from the total DNA were amplified using PCR primer sets designed to amplify archaea, eubacterial, and fungi. Pyrosequencing was performed using Roche Titanium chemistry (454) at MrDNA.

Primer Sets

16S rDNA genes were amplified using:
515F:GTGCCAGCMGCCGCGGTAA
806R:GGACTACHVGGGTWTCTAAT

18S rDNA genes were amplified using:
SSUfungiF: TGGAGGGCAAGTCTGGTG
SSUFungiR:TCGGCATAGTTTATGGTTAAG

Archeae 16S rDNA genes were amplified using:
archea349F: GYGCASCAGKCGMGAAW
archaea806R: GGACTACVSGGGTATCTAAT

Sequence Analyses

Sequences were analyzed using the QIIME software package (Quantitative Insights into Microbial Ecology) using default parameters for each step (Caporaso, 2010a).

Sequences were removed if their lengths were shorter than 200 nt, their average quality score was <25, and they contained ambiguous bases, primer mismatches, homopolymer runs in excess of six bases or error in barcodes. Filtering of noisy sequences, chimera checking and operational taxonomic unit (OTU) picking was performed using the usearch series of scripts. De novo and reference-based chimera checking was performed and sequences that were characterized as chimeric by both methods were removed. Sequences were chimera-checked and clustered into OTUs with a minimum pair-wise identity of 97%. Each cluster was represented by its most abundant sequence. Representative OTUs sequences were then aligned to the Greengenes database (DeSantis, 2006) using the PyNAST algorithm (minimum percent identity was set at 80%) (Caporaso, 2010b). A phylogenetic tree was built using FastTree (Price 2010). Taxonomy was subsequently assigned to each representative OTUs using the Greengenes database classifier with a minimum support threshold of 80%. Summary plots were generated using the R statistical package, Phyloseq (McMurdie, 2013).

Results

The first phase of the black-capped vireo adaptive management experiment spans three plots across the Black-Capped Vireo Preserve/Wild Basin Wilderness Preserve. Two plots occupy the south-facing slopes and the third is NE of the central knoll. The plots have already been prepared with mulch socks laid perpendicular to the angle of the slope to reduce soil erosion and capture soil runoff during rain events. The south facing plots each contain three tiers. Each treatment is arranged vertically along the slope across the three tiers so there is no contamination of lower tiers by upper tier treatments. The data obtained in this study were obtained from the westernmost south-facing plot (VP06-08, Figure 1).

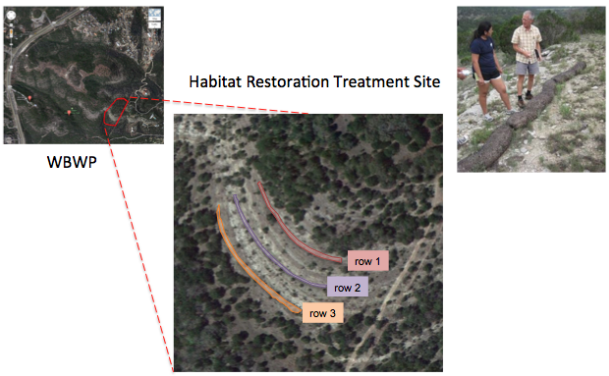

Fig. 1 Soil Metagenomic Research Site. Wild Basin Wilderness Preserve Overview (left), Soil

sample sites (middle), mulch sock (right).

Each row consists of the following treatments: 1) The control treatment is mulch socks only; 2) Onsite mulch containing local juniper and hardwoods recently processed; 3) Onsite composted mulch containing local juniper and hardwoods that have been fermenting on the ground for 10-15 years; 4) Mulch and biochar combined; 5) Composted mulch and biochar combined. Soil samples were obtained from each of the five treatment areas from row 1 in May 2012, and row 3 in May 2013. DNA was isolated in in triplicate from each soil sample obtained from row 1 samples and pooled. The pooled samples from each treatment were amplified using rDNA primer sets for archaea, fungi and eubacteria, and sequenced using 454 pyrosequencing technology.

Soil Analyses

Soils at treatment site 1 (VP06-08) and the 2 positive control sites (WB0113, WB0213) have been characterized by elemental analyses prior to the addition of the treatments. The data are not yet in for the positive control sites, however the data for all treatment sites were completed (Table I, Fig. 1). The carbon/nitrogen ratio is inversely related to elevation: max at VP08 (66), min at VP06 (23). While a similar trend is seen for calcium levels, the opposite trends are found for phosphorous, magnesium and potassium.

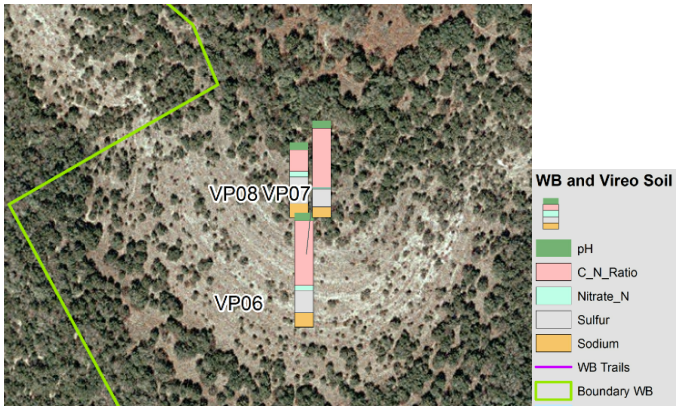

Fig 2. Sample Sites and Soil Characterization. Treatment site 1 (VP06, 07, 08). Histogram representing carbon/nitrogen ratio (pink), nitrate (blue), sulfur (grey) and sodium (orange).

Treatment Effects on α-Diversity

To directly compare the α-diversity (within-sample diversity) of the samples with differing sequencing depth (reads/sample) the fungal and eubacterial data were rarefied using chao1 method within QIIME (Fig. 3). The rarefaction curves generated using the chao1 estimator show the observed number of operational taxonomic units (OTUs) as a function of sequencing depth. Based on the number of curves that asymptote, the archaea and fungal data indicate that sequencing depth was likely inadequate to wholly capture the diversity present, whereas some of the eubacterial samples appear to have been sequenced sufficiently to fully capture the population.

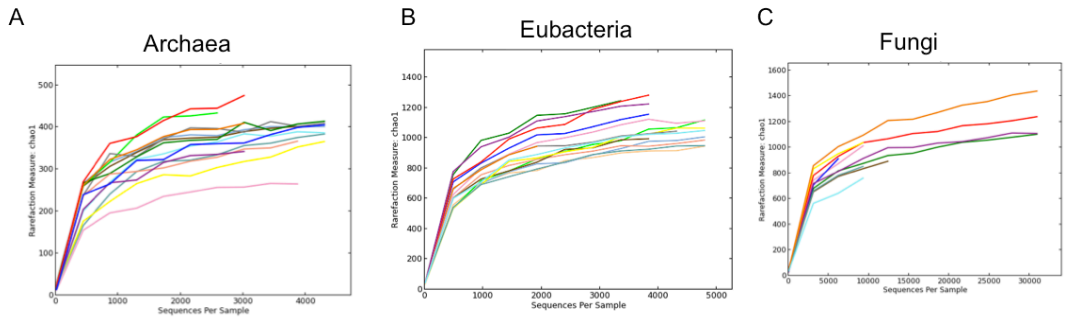

Fig 3. Chao1 rarefaction. Rarefaction curves (chao1) for Archaea (A), Eubacteria (B) and Fungi (C) samples.

Using primer pairs for each kingdom, and considering only the OTUs assigned to the particular phylum, richness (number of species/OTUs represented in a sample were estimated for each of the three kingdoms. Richness was estimated using three measures: Observed, Chao, Ace (Fig. 4). Considering archaea (Fig 4A) the consensus richness measures indicated that the number of species/OTUs present in a sample increases in the following order: [Composted Mulch; Mulch; Control]; Biochar; Biochar+Composted Mulch, with the first three treatments nearly identical. Considering eubacteria, all three richness measures indicated that the number of species/OTUs present in a sample increases in the following order: Composted Mulch; Biochar+Composted Mulch; Biochar; Mulch; Control (Fig. 4B). Considering fungi, all three richness measures indicated that the number of species/OTUs present in a sample increases in the following order: Mulch; Biochar; Biochar+Composted Mulch; Composted Mulch; Control (Fig. 2).

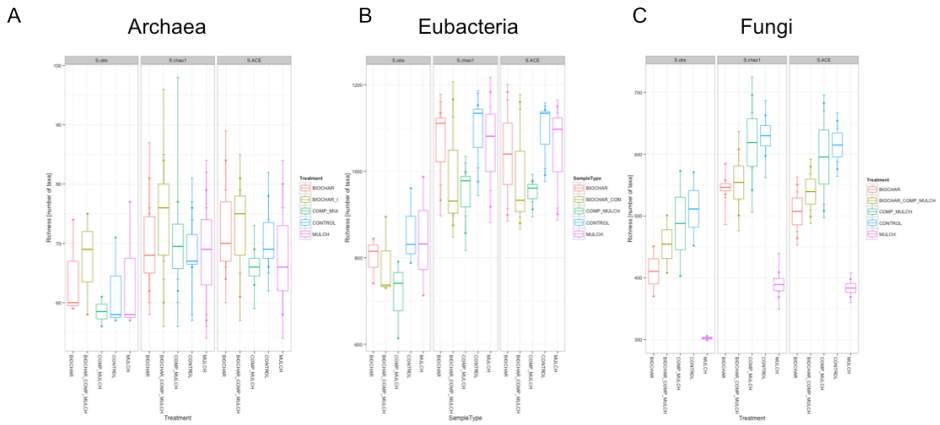

Fig 4. Species richness estimates. Richness estimates using three measures, 0bserved, Chao, Ace for (A) Archaea, (B) Eubacteria and (C) Fungi.

A preliminary analysis of OTU taxonomic classification and taxon abundance is in progress. OTUs were assigned to phylogenetic groups by aligning to either the Greengenes database (archaea, eubacteria) or Silva database (fungi) using the PyNAST algorithm. Trees representing the top 50 OTUs at the order level and their distribution among treatments are shown in Fig. 5.

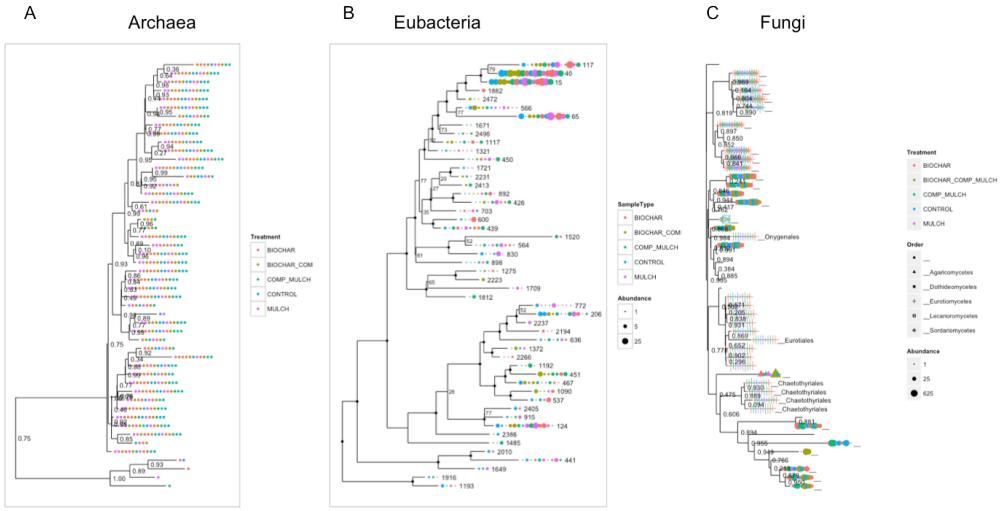

Fig. 5. Phylogenetic tree for the top 50 OTUs. Tree represents order-level phylogeny for the top 50 OTUs. A) Archaea, B), Eubacteria, C) Fungi. Each treatment is designated by colored circles, with diameter reflecting abundance.

Relative OTU abundance estimates are also in progress for each of the three kingdoms. As seen in Fig 6 (relative abundance) and Fig 7 (absolute abundance), each of the five treatments results in slightly different fungal diversity profiles at the class level. Further analyses will focus on identifying correlations between treatment and OTU diversity and abundance.

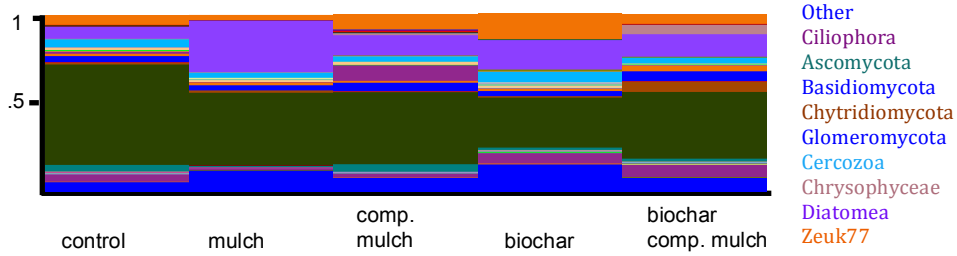

Fig 6. Species abundance estimates. Relative abundance estimates for Fungi.

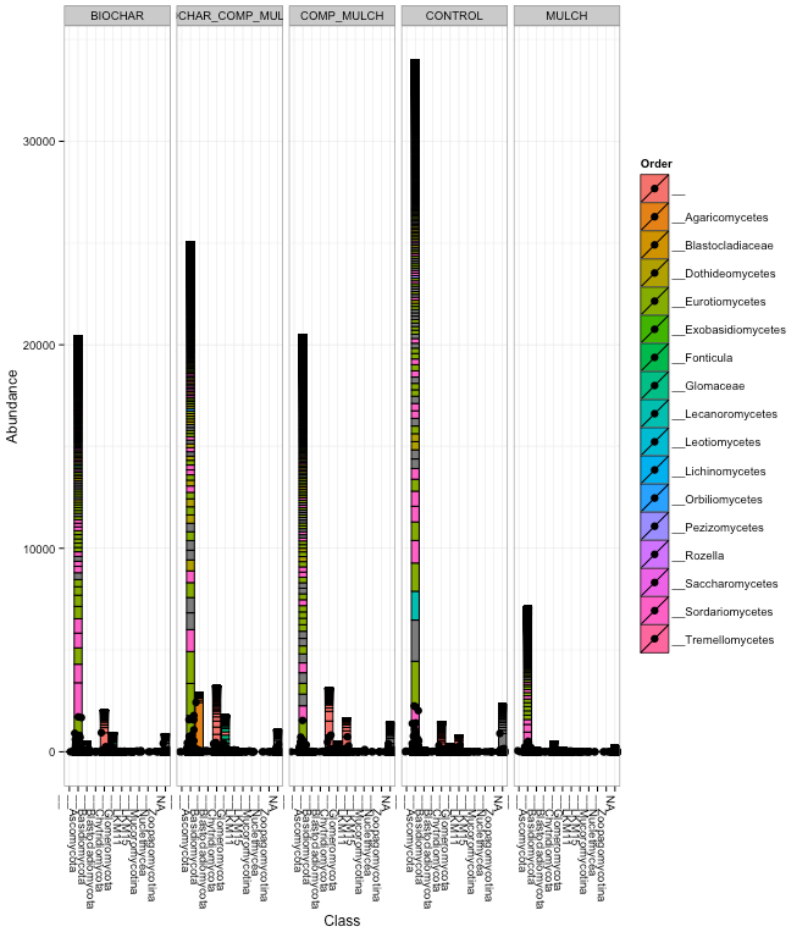

Fig 7. Species abundance estimates. Absolute abundance estimates for Fungi.

Treatment Effects on β-Diversity

We used unweighted (insensitive to OTU abundance, sensitive to rare taxa) and weighted (sensitive to OTU abundance) UniFrac distance metrics to estimate fungal β-diversity. The first principal coordinate (weighted, Fig 8 B) revealed that the treatments are differentiated from the control. The second principal component distinguishes the mulch treatment into 2 classes: (1) composted mulch, biochar, biochar+mulch, and (2) mulch. When allowances are made for rare taxa (unweighted, Fig 8A) treatments are distinguished into two groups: (1) biochar+mulch, composted much; (2) control, biochar, mulch. The unweighted distance metric (Fig 8A), suggests that the 10.91% (2nd component) of the variance in the fungal diversity is attributable to treatment.

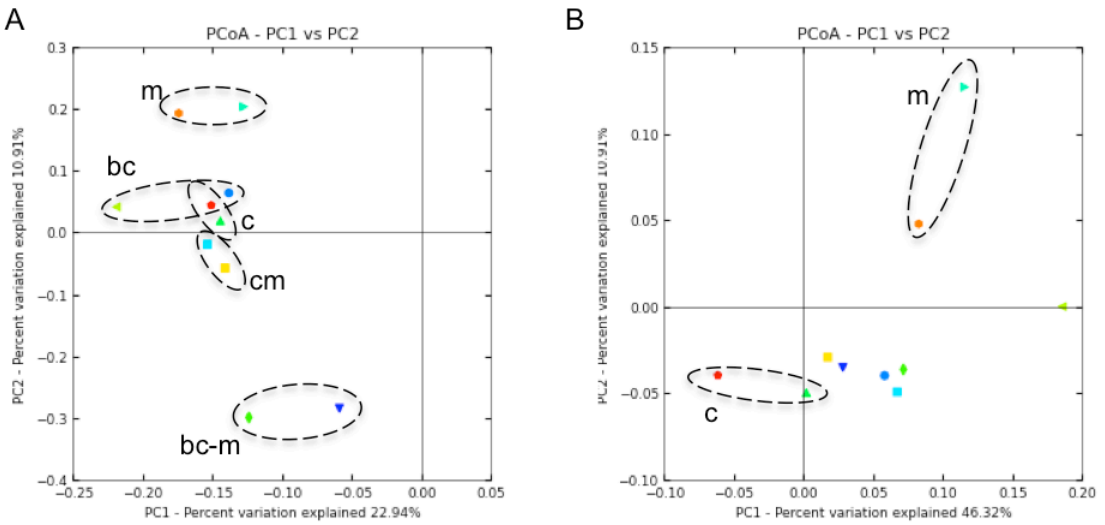

Fig. 8 Factors influencing soil fungal microbiome diversity. Variation in unweighted (A) and weighted (B) UniFrac dispersion.

Specifically, microbial communities that arise due to treatment with composted mulch either separately or together with biochar are distinguishable from those lacking it. The analysis employing the weighted distance metric (Fig 7B) further indicates that the treatments compared to the control contribute 32% of the variance (1st component), and secondarily (10%) resolves mulch from other treatments. Further analyses are needed to confirm and expand upon these findings.

Similar preliminary analyses have been carried out on the eubacterial microbiome data (Fig 9A,B). Using the unweighted distance metric, the first component (11%) resolves mulch and biochar from composted mulch and biochar plus composted mulch, and the second component (9%) groups biochar samples together separate from the others. The results are not as clear when using the weighted distance metric (Fig 9B). The majority of the biochar plus composted mulch samples are grouped distinct from non-biochar samples by the first component (28% variance), however 1 sample groups with the non-biochar samples on the first dimension. These analyses will be rerun to verify the results, and the same techniques will be applied to the archaea data.

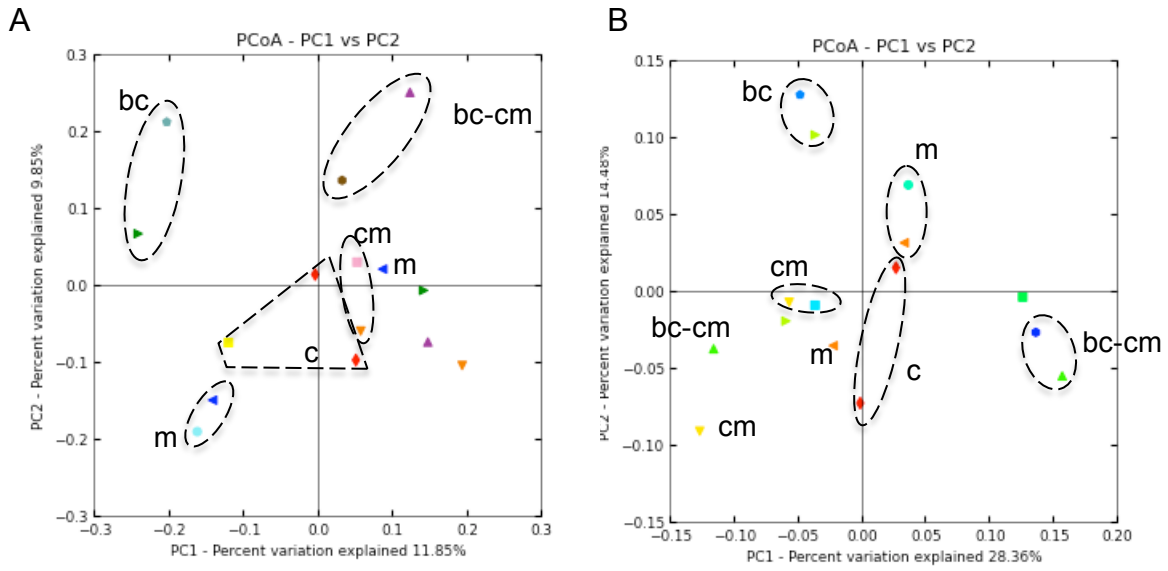

Fig. 9 Factors influencing soil eubacteria microbiome diversity. Variation in unweighted (A) and weighted (B) UniFrac dispersion.

Discussion and Conclusions

Our preliminary phylogenetic assignments of OTUs indicate a rich diversity of all three kingdoms, Archaea, Eubacteria and Fungi at the Vireo Preserve Site 1 treatment areas. Interestingly, OTU richness estimates indicate each kingdom is responding differentially to each treatment, with per-treatment richness estimates different for each kingdom. OUT abundance estimates also indicate treatment-specific signatures. Lastly, preliminary analyses of the β-diversity for fungi and eubacteria indicate treatment-specific populations.

Further analyses are required to identify the microbial composition within each treatment area, and to identify correlations between microbial populations and soil composition characteristics. Additionally, the data obtained from the positive vireo habitat control areas (01WB13, 02WB13) will allow a comparison of microbial populations derived on the basis of soil amendment treatments with populations endemic to established habitat.

One potential complicating factor in these analyses lies with the steep slopes on which VP06-08 treatment sites are arrayed. Mulch socks were placed to retain treatment materials, however much of the original treatment material has been lost due to rains. As each treatment row has the same *order* of treatments, down-slope sites should not be contaminated with differing treatments from above.

To our knowledge this study represents the first metagenomic analyses of the Glen Rose soils microbial biome at Wild Basin, and the first to examine the impact of soil restoration techniques on the microbial populations. The primary benefits of a longitudinal metagenomic analyses will be to provide data regarding: the efficacy of each the five treatments to restore black capped vireo habitat; the effects of soil treatments on microbial populations, and potentially identify unique plant-microbe interactions unique to the Glen Rose soils present at WBWP.

Publications

Cody Ramirez[1,2], Samantha Parsons[2], Lisa O'Donnell[3], Jim O'Donnell[3], Mitchell Robinson[4], Monica Swartz[4], Charles Hauser[2] "Impact on Fungal Microbiomes in Response to Kart Soil Remediation Treatments at Wild Basin Wilderness Preserve" Brother Lucian Blersch Symposium, Oct. 18, 2013, St. Edward's University, [1]Wilems Fellow, Brother Romard Barthel Student Research, St. Edward's University, [2]Bioinformatics Program, St. Edwards University Austin TX, [3]City of Austin, Sr. Biologist, Balcones Canyonlands Preserve, [4]Wild Basin Wilderness Preserve, St. Edward's University Austin TX

References

Angel, R., Soares M., Ungar, E., Gillor, O. (2010). Biogeography of soil archaea and bacteria along a steep precipitation gradient. ISME Journal 4, 553-563.

Capone, K. A., S. E. Dowd, et al. (2011). "Diversity of the human skin microbiome early in life." J Invest Dermatol **131**(10): 2026-2032.

J Gregory Caporaso, Justin Kuczynski, Jesse Stombaugh, Kyle Bittinger, Frederic D Bushman, Elizabeth K Costello, Noah Fierer, Antonio Gonzalez Pena, Julia K Goodrich, Jeffrey I Gordon, Gavin A Huttley, Scott T Kelley, Dan Knights, Jeremy E Koenig, Ruth E Ley, Catherine A Lozupone, Daniel McDonald, Brian D Muegge, Meg Pirrung, Jens Reeder, Joel R Sevinsky, Peter J Turnbaugh, William A Walters, Jeremy Widmann, Tanya Yatsunenko, Jesse Zaneveld and Rob Knight. QIIME allows analysis of high-throughput community sequencing data Nature Methods, 7, 335–336, 2010

J. Gregory Caporaso, Kyle Bittinger, Frederic D. Bushman, Todd Z. DeSantis, Gary L. Andersen, and Rob Knight. (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26: 266-267

DeSantis, T. Z., P. Hugenholtz, et al. (2006). "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB." Appl Environ Microbiol **72**(7): 5069-5072.

Dowd, S. E., T. R. Callaway, et al. (2008). "Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP)." BMC Microbiol **8**: 125.

Dowd, S. E., J. Delton Hanson, et al. (2011). "Survey of fungi and yeast in polymicrobial infections in chronic wounds." J Wound Care **20**(1): 40-47.

Dowd, S. E., Y. Sun, et al. (2008). "Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned Salmonella-infected pigs." Foodborne Pathog Dis **5**(4): 459-472.

Edgar, R. C. (2010). "Search and clustering orders of magnitude faster than BLAST." Bioinformatics **26**(19): 2460-2461.

Eren, A. M., M. Zozaya, et al. (2011). "Exploring the diversity of Gardnerella vaginalis in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation." PLoS One **6**(10): e26732.

Fierer, N. (2008) Microbial biogeography: patterns in the microbial diversity across space and time. In Zengler K. (ed) Accessing Uncultivated Microorganisms: from the Environment to Organisms and Genomes ans Back. ASM Press: Washington DC pp 95-115.

Gans, J., Wolinski, M., Dunbar, J. (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309:1387-1390.

McMurdie and Holmes (2013) phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE. 8(4):e61217

Price, M.N., Dehal, P.S., and Arkin, A.P. (2010) FastTree 2 -- Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE, 5(3):e9490.

Swanson, K. S., S. E. Dowd, et al. (2011). "Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice." ISME J **5**(4): 639-649.

Torsvik, V., Ovreas, L., Thingstad, F. Prokaryote diversity: magnitude, dynamics, and controlling factors. Science 296: 1064-1066. (2002)

Tringe, C., Mering, S., Kobayashi, A., Salamov, A., Chen, K., Chang,H., Podar, M.,Short, J., Mathur, E., deter, J., Bork, P., Hugenholtz, P., Rubin, E. Comparatievmetagenomics of microbial communities. Science308:554-557 (2005)

Supplemental Table I Vireo Habitat Sites

Site	Date Collected	X	Y
VP06	8-Jan-12	613772	3353623
VP07	8-Jan-12	613772	3353660
VP08	8-Jan-12	613770	3353681
02WB13	3-Oct-2013	613220	3353955
01WB13	3-Oct-2013	613336	3353910

Supplemental Table II Soils Elemental Analysis

Site	Total N	Total C	N/C Ratio	C/N Ratio	pH	conductivity	Nitrate-N	Phosphorus	Potassium	Calcium	Magnesium	Sulfur	Sodium
VP06	1719	118144	0.01	68.73	8.1	161	5	5	82	25824	344	24	15
VP07	1891	118134	0.02	62.47	8.2	126	2	6	83	16698	327	19	11
VP08	8539	195618	0.04	22.91	7.9	328	6	19	148	10352	940	28	15
02WB13	Samples Submitted for Analysis (10/2013)												
01WB13	Samples Submitted for Analysis (10/2013)												