Powell River Project Annual Report (2013-2014)

Characterizing microbial community development in reclaimed mine soils

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Summary

A significant amount of the research to date at the Powell River Project (PRP) has been focused on reforestation, with the assumption that tree growth will ultimately lead to the re-establishment of a fully functioning forest ecosystem. Soil microorganisms are a critical component of this system because they mediate many of the ecosystem services for which forests are valued including carbon sequestration, soil formation, nutrient retention, watershed protection, and groundwater purification. We are characterizing the response of soil microbial communities to land reclamation approaches in the PRP to provide critical information about the restoration of the microbial component of the forest ecosystem. The objectives of this project are threefold: 1) characterize the recovery of soil microorganisms over time; 2) determine if alternate reclamation practices affect microbial diversity and community structure; and 3) compare restored microbial communities to un-mined forest soils to identify potential indicators that 'healthy' microbial communities are returning to reclaimed soils. We have identified a variety of reclamation plots within the PRP that represent a range of ages between 5 and 30 years to look at the effects of time. We have also sampled two other sets of to determine effects of reclamation practices: one where soils were amended with biosolids and another that was planted with pines as opposed to the standard hardwood mix. We are using genomic sequencing to fully characterize bacterial and fungal organisms present in soil samples from each plot to determine microbial diversity and community structure. Preliminary results suggest that bacterial communities recover quickly, becoming indistinguishable from communities in undisturbed soils within 10 to 30 years. In addition, certain taxa such as *Bacteroidetes*, *Verrucomicrobia,* and *Gemmatimonadetes* appear to respond to age since reforestation and may contain taxa that can be used to gauge restoration progress.

Introduction

The Powell River Project (PRP) showcases decades of effort to improve reforestation and land reclamation practices of mined lands in the Appalachian coalfields. The major focus of this effort has been the restoration of forest structure using a variety of reclamation approaches, including varying tree and ground cover species, grading and compaction of base material, or soil amendments (Burger *et al.* 2005, Evanylo *et al.* 2005, Fields-Johnson 2011). This work has led to practices that significantly improve the survival and productivity of planted trees following reforestation. One major assumption of this approach is that this restoration of forest *structure* will also lead to restoration of forest *function*. In other words, does the successful establishment of native hardwoods bring about other important changes such as soil formation and development, colonization of other native species, and ultimately the re-establishment of a fully functioning forest ecosystem? Unfortunately, there has not been a sufficient amount of research that focuses on measuring these changes in restored forest systems in order to answer this question. Research on other components of the forest system is required to fully understand the restoration process and gauge which reclamation approaches yield the most successful return of beneficial ecosystem services.

Many of the valuable ecological functions provided by forests, such as carbon sequestration, soil formation, nutrient retention, watershed protection, and groundwater purification, are either completely carried out or at least strongly mediated by soil microorganisms. However, microbiological research has historically depended on methods that require the culture of microorganisms in the laboratory to determine their identities and functions. It is now well established that culture-based approaches only detect about 1-5% of all bacteria existing in the environment, which severely limits our ability to properly characterize the structure and function of the complex assemblages of microorganisms that are known to inhabit natural systems such as forest soil. The development of modern of genomic techniques to identify environmental microorganisms without depending on laboratory culture has finally led to an ability to fully describe natural microbial assemblages and improved understanding of the role of microbes in natural systems. For example, important changes in soil microbial communities during reforestation and ecosystem restoration can include a shift from bacterial to fungal dominance in soil microbial communities (van der Wal *et al.* 2006) and the successful establishment of the mycorhizzae (Teste and Simard 2008). In addition, soil factors such as pH (Kuramae *et al.* 2010, Nacke *et al.* 2011) and nutrient pools (Banning et al., 2011; Kuramae *et al.* 2010) are known to affect the development and successional patterns of soil microorganisms. Over time, microbial communities in restored ecosystems can become more similar to undisturbed reference communities (Banning *et al.* 2011), but this process can take decades (Jangid *et al.* 2010, Lewis *et al.* 2012).

Since the advent of the genomic age in microbiology, further technological advances in DNA extraction, sequencing, and computational analysis have facilitated the characterization of microbial diversity and function, including both culturable and unculturable taxa, at unprecedented levels of detail (Sogin *et al.* 2006; Louzopone and Knight 2007). However, most of the work using genomic techniques has been conducted in ‘pristine’ systems. Meanwhile, ecosystems undergoing restoration provide a valuable opportunity to *experimentally* study succession and restoration of soil microorganisms and how patterns of microbial diversity affect ecosystem function. The PRP serves as a rare research platform in this regard because it contains numerous ecosystems of a wide range of ages that have been restored with alternate reclamation approaches in a relatively controlled manner. From an applied perspective, Harris (2009) posed what is arguably the most critical question related to this research: are

microorganisms simply responding to ecosystem changes or are they facilitating ecosystem succession in a way that can be used to predict or even enhance restoration? By examining the development of the soil microbial community at a range of sites in the PRP we can better understand the speed at which 'normal' soil microorganisms recolonize reclaimed lands and how different reclamation practices can most effectively lead to the restoration of a healthy soil microbial community.

Objectives

The overall goal of this project is to describe the development of soil microorganisms following reforestation by conducting genomic analyses of entire soil microbial communities at numerous sites in the PRP that span a range of ages and reclamation practices. Specifically, we have three objectives:

1. Examine the effect of time since reclamation on microbial diversity and community structure in plots that range in age from 5 to greater than 30 years.

2. Determine if tree diversity or the application of biosolids affects microbial diversity or function in plots of similar ages.

3. Compare restored soil microorganisms to natural assemblages found in un-mined reference soils to identify potential indicators that 'healthy' microbial communities are returning to reclaimed soils.

By measuring diversity, identifying taxonomy, and quantifying indicators of microbial function at each site, we are providing valuable information on the rate at which the microbial component of forest ecosystems is restored as a result of mining reclamation practices.

Methods and Procedures

Sampling Sites. Objective 1 focuses on the effect of time since reclamation on the development of soil microorganisms. Sampling for this objective took place in collaboration with another project concurrently funded by the PRP and awarded to Brian Strahm in the Virginia Tech Department of Forest Resources and Environmental Conservation. Dr. Strahm has established a chronosequence within the PRP, which consists of several sites that differ from each other in the amount of time since reforestation, but are otherwise relatively similar. The chronosequence consists of sites that represent times since reforestation of approximately 6 yr (Fields-Johnson 2011), 11 yr (Burger et al. 2005), 21 yr (Burger and Fannon 2009), and 31 yr (Torbet et al., 2005), as well as un-mined native forest reference sites within the PRP. Full descriptions of these sites can be found in the report by Strahm and Avera (2014) within this same document. The 6, 11, and 21 yr sites, as well as the un-mined reference sites, are located on the Powell River Project. The 31 yr site is along the Roaring Fork River at Kent Junction, VA. The priority for choosing sites was to find locations in each age cohort that had been intentionally reforested with hardwoods. The mine soils are a mix of weathered and unweathered sandstone and siltstone, with shale and coal fragments, and sites have steep slopes. Site characteristics of the mined sites are summarized in Table 1.

The sites for Objective 2 were chosen to highlight two alternative reclamation strategies –

altering tree species and amending soils with biosolids. The tree species comparison was accomplished by comparing the mixed hardwood plots from the chronosequence to similarly aged plots that were reforested exclusively with white pines (*pinus strobus*) at a similar age (Schoenholz and Burger, 1984). Plots for biosolid amendment were sampled from a prior experiment in the PRP where reclaimed soils were amended with biosolids and planted with forage grasses. Controlled plots were amended with a 50:50 mixture of wood chips and biosolids at levels equivalent to ¼, ½, ¾, 1, or 1½ times the base rate of 350 wet tons/acre (780 Mg/ha). A control plot (no biosolid amendment) was also sampled (Haering and Daniels, 1991; Evanylo *et al.*, 2005).

Sampling at the chronosequence sites occurred on a bimonthly basis from May-November 2013. Three sampling plots were established at each chronosequence site. At each sampling time, five replicate soil samples were collected from each plot and pooled to account for small-scale heterogeneity within the soils, and each pooled sample was analyzed independently (see below) so that there were three replicate samples for each site and time. The pine and biosolid sites, along with the reference sites from the chronosequence, were sampled once in May 2014 to obtain a comparison at a single point in time. At each site at least 5 spatially distinct soil samples (with each sample being a composite of three samples from a 1 m^2 area) were collected and analyzed as independent replicates.

Microbial analysis. Upon return to the laboratory, all soils were 4 mm sieved and processed within 24 h. Microbial communities were characterized via respiration, fungal to bacterial biomass ratios, and total bacterial and fungal diversity and community structure. Respiration was estimated using substrate-induced respiration, following the protocol of Strickland et al. (2010). Fungal to bacterial biomass ratios were estimated via quantitative polymerase chain reaction (qPCR). Total DNA was extracted from each pooled soil sample using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) and then quantified prior to qPCR. The 16S rRNA gene was used to estimate bacterial abundance and the ITS region was used for fungal abundance using the primer sequences and reaction conditions outlined by Fierer *et al*. (2005). Amplification was performed on a StepOnePlus real-time thermocycler (Applied Biosystems, Grand Island, NY), with each run including no template controls and a full set of standards ranging from 10^1 to 10^8 copies of the target gene.

For total microbial community analysis, aliquots from the DNA extracts obtained from the triplicate soil samples for qPCR analysis were also sequenced for microbial community structure. Bacterial and fungal diversity and taxonomy was characterized by sequencing amplified taxonomic genes, using the V4 hypervariable region of the 16S rRNA gene of bacteria (Caporaso *et al.* 2012) and the ITS region of fungi (O'Brien *et al*. 2005). Amplification was performed in triplicate to smooth out PCR biases and samples were barcoded and pooled into sequencing runs. Sequencing was performed at the Virginia Bioinformatics Institute using the Illumina MiSeq platform. Sequences were quality filtered, aligned, clustered, and classified using QIIME (Caporaso *et al.* 2010), mothur (Schloss *et al.* 2009), and USEARCH (Edgar, 2013). Sequences were clustered into operational taxonomic units (OTUs) at a similarity cutoff of 97% and representative sequences for each OTU were classified against the Greengenes reference database for bacteria (DeSantis et al., 2006) and the UNITE reference database for fungi (Kõljalg et al., 2013). Alpha and beta-diversity measures were analyzed using the R statistical software platform (www.r-project.org).

Results to Date

All soil sampling has been completed for this project. For the chronosequence sites, all microbial analyses have been completed. Data has been analyzed for the bacterial communities and we are in the process of analyzing data for fungal:bacterial ratios and fungal communities. For the May 2014 samples from the pine, reference, and biosolids sites, all sample prep has been completed and we are awaiting sequencing results to finish analysis.

Bacterial communities. Preliminary analysis of bacterial diversity and taxonomy from the PRP chronosequence sites has shown that soil microorganisms can potentially colonize reclaimed mine soils and develop a community structure that is highly similar to unmined reference soils within 20-30 years. When comparing the presence and abundance of bacterial OTUs present in each age class of soils using principal coordinate analysis (PCoA), the most distinct age class is the 5 yr plots, which are relatively dissimilar from the other ages. The 10 and 20 yr plots have an intermediate structure, and the 30 yr plots cluster tightly and are indistinguishable from the unmined reference soils (Figure 1).

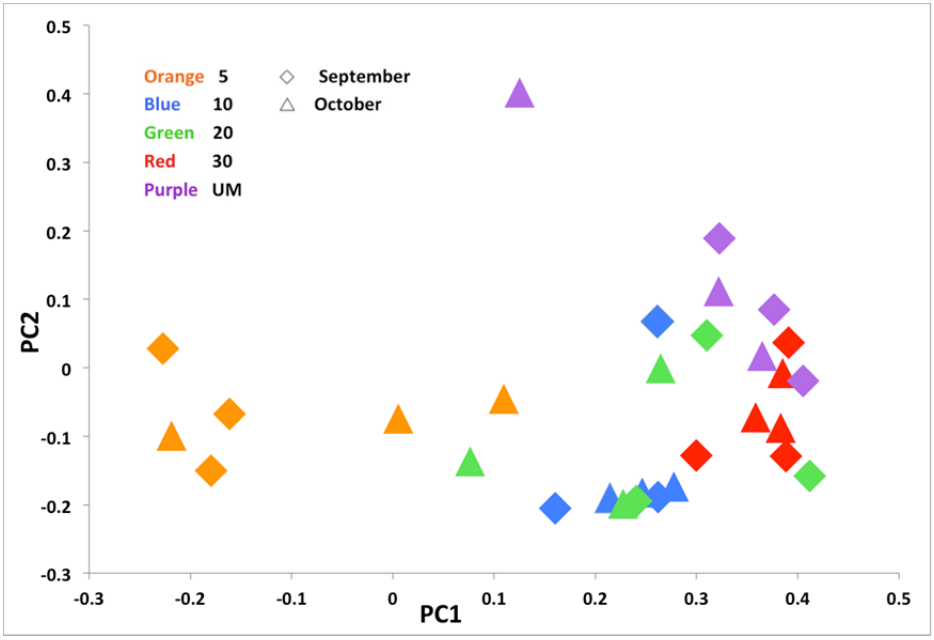

Figure 1. *Principal coordinate analysis of microbial communities present in soil samples from plots within the PRP that represent different ages since reforestation (5, 10, 20, 30 yr) compared to un-mined reference soils. Samples are from September and October 2013.*

This trend is evident looking at the taxonomy as well. At the phylum level, all samples had relatively similar proportions of total individuals represented by each phylum (Figure 2). The dominant phyla in the samples included *Acidobacteria*, *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia, Planctomycetes*, and *Bacteroidetes.* While there was some variability among the relative proportions of each phylum across plot ages, dramatic shifts in dominance did not occur and no major phyla were gained or lost as plots increased in age.

Another important question related to the microbial ecology of reclaimed mine soils is whether particular microbial taxa can potentially be used as indicators of ecosystem restoration. We are currently in the process of exploring this question from a rigorous statistical perspective. However, just looking at the phylum level, a few groups do show trends related to age since reforestation. For example, during the months of May and July, which were very wet, *Bacteroidetes* abundance was proportionally increased in the younger plots (Figure 3). Given

that this phylum consists of anaerobic bacteria, this may be related to the development of soil structure and porosity as plots age, which allows better oxygenation of the soil. *Gemmatimonadetes*, which also appears to decrease with age (Figure 4), has been found to be correlated with low soil moisture (DeBruyn et al., 2011), another characteristic of poorly developed soils in younger plots. And finally, although little is known about *Verrucomicrobia* because this phylum is as yet uncultured, it has been found to be more abundant in less disturbed soils in other systems (Fierer et al., 2013), suggesting that the increase in PRP soils with age may also be an indicator of desirable microbial development (Figure 5).

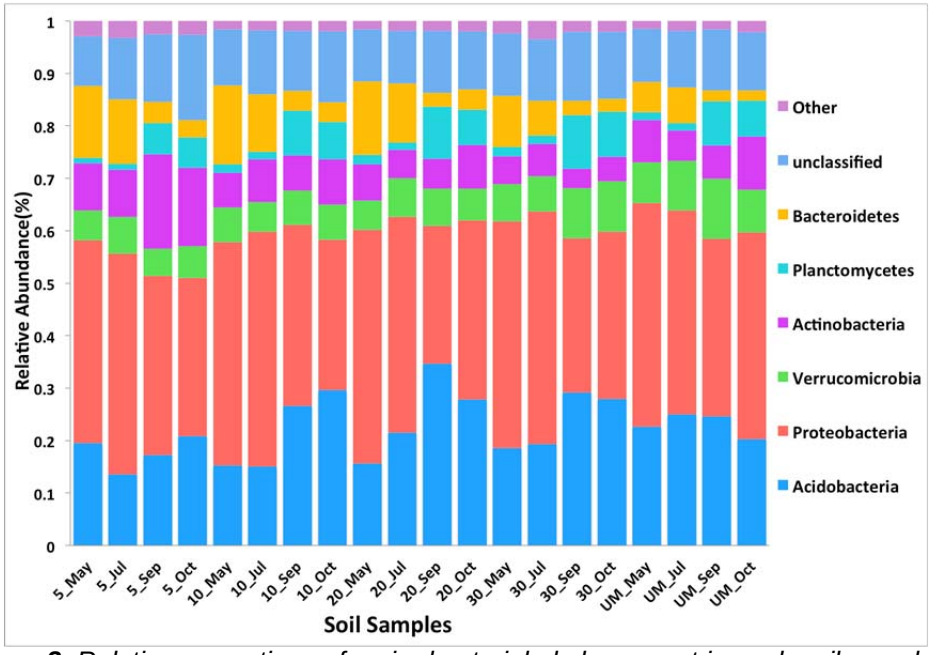

Figure 2. *Relative proportions of major bacterial phyla present in each soil sample collected from the chronosequence sites within the PRP in 2013. Each column represents the average proportion observed at that site for each month (n=3).*

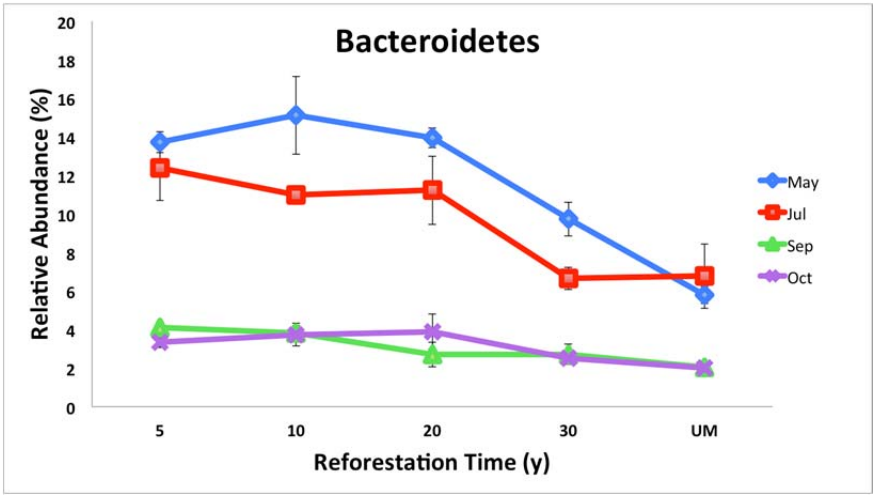

Figure 3. *The relative abundance of the bacterial phylum* Bacteroidetes *expressed as the percent of total bacteria identified from PRP soil samples at the chronosequence sites in 2013. Each point represents the average proportion observed at that site for each month (n=3).*

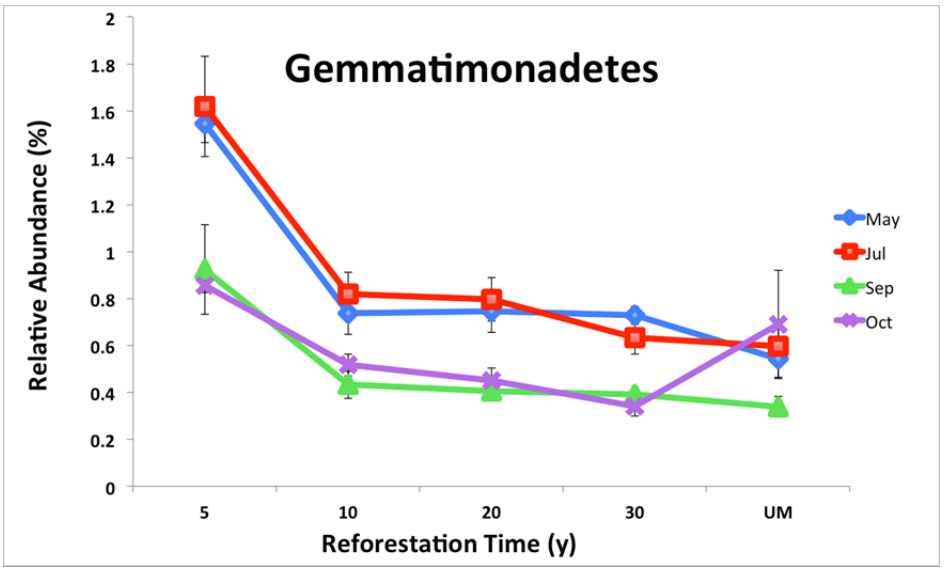

Figure 4. *The relative abundance of the bacterial phylum* Gemmatimonadetes *expressed as the percent of total bacteria identified from PRP soil samples at the chronosequence sites in 2013. Each point represents the average proportion observed at that site for each month (n=3).*

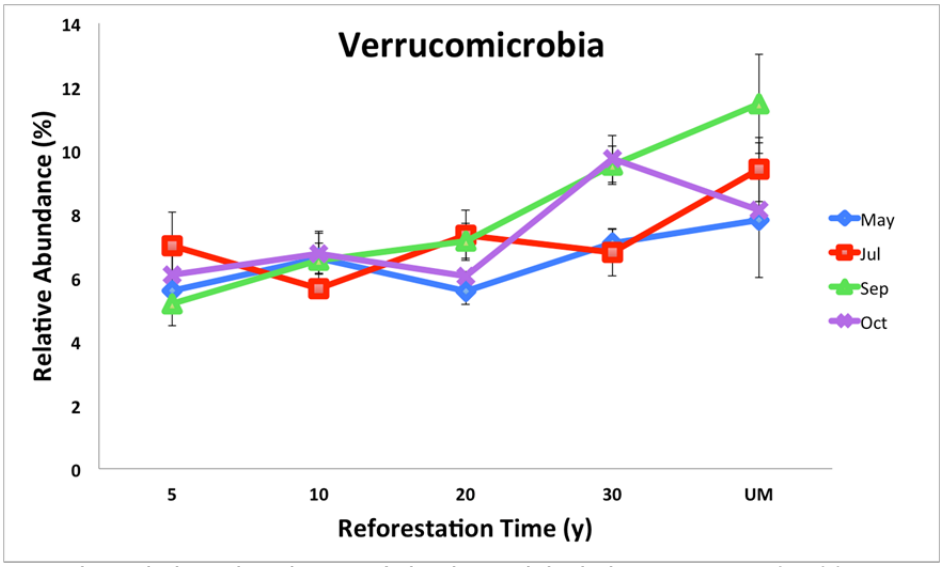

Figure 5. *The relative abundance of the bacterial phylum* Verrucomicrobia *expressed as the percent of total bacteria identified from PRP soil samples at the chronosequence sites in 2013. Each point represents the average proportion observed at that site for each month (n=3).*

Deliverables

The funds received for this project have helped support the research for one CSES Ph.D. student (S. Sun) and research experience for two VT undergraduate students (J. Franklin, Biology; M. Orentas, Environmental Science). These results have already lead to one presentation at a national conference and one federal proposal submission. At least one manuscript, as well as additional presentations and proposals for additional funding are planned.

Sun S, Badgley BD (2014) Microbial community development during reforestation of reclaimed mine soils. *General Meeting of the American Society for Microbiology*, Boston, MA, USA.

Badgley BD, Strahm, BD (2014; declined) *Preliminary Proposal*: Environmental genomics to ecosystems: How do we best link big data on microbial communities to real changes in ecological function? Sent to: Ecosystem Science Cluster, Division of Environmental Biology, National Science Foundation.

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