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COMPARISON OF BACTERIAL DNA FROM SOIL CONTAMINATED BY ACID MINE DRAINAGE AND AMENDED SITES OF BUTLER COUNTY IN WESTERN PENNSYLVANIA, USA

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ABSTRACT

Integrity of soil is extremely important from an ecological standpoint. When mining for natural resources occurs, the soil is disrupted and often times removed leaving behind soil of poor nutritional quality for both flora and fauna. Soil fabrication and/or planting are often employed for remediation purposes to amend the soil quality. The major goal of our study was to compare bacteria using 16s rDNA of soil collected from an acid mine drainage site to nearby soil plots where remediation or fabrication of the soil took place. The diversity of represented phyla from the fabricated sites was 2-3 times greater than the diversity found in the mining soil. The most abundant phyla from the mining soil site were Proteobacteria (66%) and from the amended soil either Proteobacteria (45%) or Firmicutes (45.5-57.5%). The distribution of species varied between samples. The most diverse sample was the compost sample (7.8x increase) compared to the mining soil; while the least diverse sample was the ray grass (4.2 fold increase). Our results indicate that specific methods for environmental improvement of the soil increased microorganism diversity beyond the number of species present in unamended mining soil alone.

Keywords: Pennsylvania, acid mine drainage (AMD), fabrication of soil, amending soil, bacterial communities, high-throughput sequencing.

INTRODUCTION

Acid mine drainage (AMD) can have an ecological impact on areas where mining, mainly of coal, has left behind exposed sulfur containing rock and soil which come in contact with surface water to create sulfuric acid. Often, the areas where the mines are found are very rural and impact the natural habitats of the plants and animals now living near contaminated water sources. The harmful effects from water contamination can affect the soil biota including both flora and fauna at locations along streams and rivers where this water flows. Soil biota is a term often used to collectively group all the organisms present in a soil sample. This includes bacteria, fungi, nematodes, earthworms and various arthropods to name a few.

Decomposition of organic material is important for the recycling of nutrients in the soil ecosystem, and is commonly done by microorganisms present in the soil and environment (Chaudhry *et al.*, 2012). Acid mine drainage is known to have had a deleterious effect on the bacterial communities in the Upper Tioga River Watershed in Pennsylvania (Honey *et al.*, 2013).

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Anthropogenic changes to the soil, such as fabrication, amendment, or disturbances, will also affect the diversity of the microbial community (Chaudhry *et al.*, 2012). Fabrication of soil is routinely done in areas where soil quality is low or where contaminants have been identified including areas impacted with surface mining and acid mine drainage. The fabricated soil (FS) often contains nutrients such as phosphorus, nitrogen, and potassium from decaying substrates (Kefeli *et al.*, 2008). Incorporation of different substrates can affect availability of favorable nutrients and thus increase the microbial community at each site.

The objective of this study was to survey bacteria found in mining soil and compare it to bacteria in soil from fabricated or amended acid mine drainage plots in Butler County of Western Pennsylvania, USA.

MATERIALS AND METHODS

Acquisition of soil

Plots in Butler County, PA were identified as an area which had been strip mined in the 1950's and were further subjected to acid mine drainage afterwards. Sites were amended with organic material such as composted leaves, ray grass, or ray grass and clover. The soil collected from

each site was annotated, dried, and stored at room temperature.

Isolation of DNA

Extraction of DNA began by rehydrating 5.0g of soil with 5mls of deionized water in a sterile 15ml conical tube. The samples were left shaking for 3 days at room temperature. DNA was extracted from the soil using the Zymo ZR Soil Microbe DNA microprep (Cat No. D6003) and resuspended with sterile deionized water. DNA concentration was measured using a spectrophometer. Confirmation of DNA presence in the sample was verified through PCR of 16s rDNA (Nadkarni *et al.*, 2002) and run on a 1.5% agarose gel. Approximately 10ug of sample DNA was sent for sequencing to MR DNA Lab in shallow water Texas.

Sequencing/Data mining

Sequencing and matching was done by Scott Doyd at MR DNA (www.mrdnalab.com). PGM sequencing (Ion Torrent - Personal Genome Machine) was done with 16s rDNA gene primers (Caporaso *et al.*, 2011). Briefly, the sequencing cycle used a 3 minute hot start at 94^{o}C; followed by 30 seconds at 94^{o}C, 40 seconds at 53^{o}C and 60 seconds at 72^{o}C for 28 cycles. The elongation step was 5 minutes at 72^{o}C. All sequencing done at the MR DNA Lab in Texas was performed on an Ion Torrent PGM. Pipeline analysis of the data included removing primer sequences and denoising the samples. The operational taxonomic units (OTUs) were classified using BLASTn (DeSantis *et al.*, 2006). The data was then compiled into analysis files and organized based on taxonomic level and prepared as total hits/annotation or as a percent of total hits in each sample.

RESULTS AND DISCUSSION

Results from sequencing data (Table 1) indicated each sample had at least 10,000 reads and had a unique combination of bacterial DNA from various phyla present. The least diverse sample was the mining soil while the most diverse soil was from the compost plot.

Table 1. Depicts the number of reads from each sample sent for sequencing.

Sample	Number of OTU's
Mining Soil	11775
Compost	48168
Ray Grass	10657
Clover - Ray Grass	53674

Sequences identified in each sample were tabulated and recorded by phyla and are listed in table 2 and represented by percent of reads for each group. There were 5 phyla of bacteria identified in the DNA from the mining site (Table 2). The two abundant phyla were Proteobacteria (66.2%) and Actinobacteria (19.9%) of total OTU's from this DNA set. From the compost sample, our most diverse sample, 13 different phyla were represented (Table 2). The most abundant was Firmicutes (45.5%) and second largest phylum was Proteobacteria (33%) (Table 2). The fabricated soil with ray grass had 10 different phyla represented (Table 2). The top two phyla were Firmicutes (57.5%) and Proteobacteria (28.3%) (Table 2). In the sample of soil fabricated with clover and ray grass 11 different phyla were represented (Table 2) of which 45% were from the phylum Proteobacteria and 40.3% Firmicutes (Table 2). Figure 1 illustrates the percent representation of the 4 most highly represented phyla.

Each sample has a unique combination of phyla identified (Table 2). The mining soil sample had the least diversity with only five phyla represented in the extracted DNA (Table 2). The most abundant phylum was Proteobacteria with 66% of the total OTU's in this DNA sample. This is not surprising as Proteobacteria consists of many different groups of bacteria capable of surviving in harsh conditions by forming endospores, metabolizing nutrients from various sources and bacteria capable of nitrogen fixation. The most abundant class was the Betaproteobacteria comprising over 37% of the identified species (Table 3). In our sample, the most abundant species was *Paucibacter toxinivorans* of which 21.5% of sequences from the mining soil DNA matched (Table 4). It is a gram-negative rod shaped bacterium with single flagella (Rapala *et al.*, 2005). This genus belongs to the Betaproteobactera class and has been found in Canada (Verastegui *et al.*, 2014) and Finland (Rapala *et al.*, 2005). Betaproteobacteria is the most abundant class of DNA found in our mining sample (Table 3).

The second largest phyla represented in the mining soil DNA sample were the Actinobacteria (Table2). From the mining sample, 13% of OTU's were from *Kocuria rosea* (Table 4). A strain of *Kocuria rosea* was even isolated and found to secrete a keratinase capable of degrading feathers as a carbon source (Bernal, *et al.*, 2006). These gram-positive cocci are in the Micrococcaceae family and are widely distributed in various environments including soil, freshwater, saltwater, and even human skin.

Fabrication of soil is one way to incorporate essential nutrients needed by microorganisms. In all of the fabricated soil samples, the diversity of bacterial DNA was greatly increased relative to the phyla identified in the mining soil sample. The minimum number of phyla present in any one of the three fabricated samples was 10 in the ray grass sample and the most diverse sample was the compost sample with 13 different phyla represented (Table 5).

Changes to abundance and species diversity were also identified in our samples (Table 5). The fabricated soil

Table 2. Percent listed by phyla represented in each sample.

Phylum Percent	Mining Soil	Compost	Ray Grass	Clover-Ray Grass
Acidobacteria	0	0.85	0.01	0.31
Actinobacteria	19.90	9.94	4.50	4.89
Bacteroidetes	1.46	4.63	3.48	5.17
Chloroflexi	0	0.91	0.02	0.46
Cyanobacteria	0	0.61	0	0.14
Elusimicrobia	0	0.10	0	0
Firmicutes	11.65	45.50	57.50	40.34
Gemmatimonadetes	0	0.74	0.26	0.97
Nitrospirae	0.76	0.04	0	0.01
Planctomycetes	0	2.91	0.13	2.01
Proteobacteria	66.23	32.97	28.33	44.95
Tenericutes	0	0.01	5.71	0
Verrucomicrobia	0	0.78	0.05	0.75

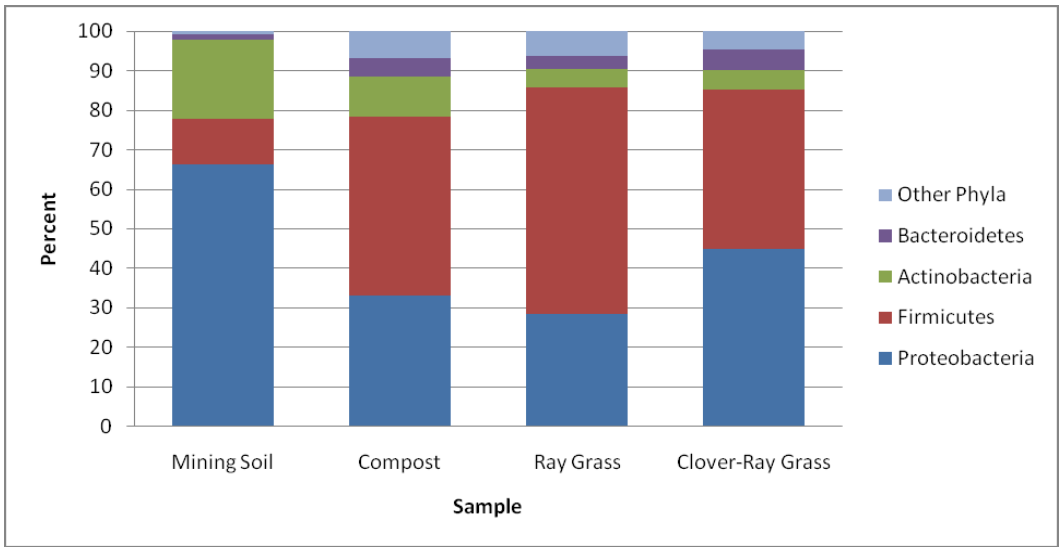

Fig. 1. Percent of sequences grouped by phyla present in each sample.

samples had 4-7x the diversity as the mining sample. With only 47 species spread between 34 families, the mining soil was the least diverse. The compost sample however had the most diversity with 336 different species divided between 127 families (Table 5). All amended samples had an increase in diversity and overall changes to the community structure as demonstrated by the increase in represented phyla and species.

It has been reported by other authors (Gros *et al.*, 2006; Lin *et al.*, 2011) that disturbances to soil in general can impact microbial communities. Soil from a natural *Chamaecyparis* forest in Taiwan was compared to nearby communities where disturbances had occurred due to harvesting of older trees, development of a new tree plantation, and harvesting of downed logs and results suggested that there were changes in microbial communities (Lin *et al.*, 2011). In the present study Proteobacteria was the most abundant phylum in the undisturbed sample at 55% and was reduced in the other three. Actinobacteria was the third most abundant phylum identified in the undisturbed site with 15.5% of clones and fewer in the three disturbed samples as well. Compared to our data, these results follow along with the trends we detected in the DNA samples whereby the mining soil had more sequences in the Proteobacteria and Actinobacteria phyla (Table 2).

Comparison of our data to published literature identifying bacteria that were found in the soil and water of the Great Smoky Mountains contained many of the same represented phyla (O'Connell *et al.*, 2007). They were able to document 69 different genera between the water and soil samples collected from the plots. Firmicutes was the most abundant phyla they found in the soil samples; similar to our fabricated plots. Firmicutes are known to mainly have a gram-positive cell wall and be either in cocci or bacilli form. They are sometimes referred to as having low G/C content, and many of them are able to produce endospores. This phylum contains the Clostridia,

Table 3. Percent listed by major class represented in each sample.

Major Classes	Mining Soil	Compost	Ray Grass	Clover-Ray Grass
Actinobacteria	19.9	6.3	4.05	3.45
Alphaproteobacteria	14.49	16.39	8.11	13.29
Bacilli	6.01	20.27	25.67	10.16
Betaproteobacteria	37.53	7.90	5.40	21.26
Clostridia	5.64	24.73	31.62	30.06
Deltaproteobacteria	7.03	5.16	1.02	8.22
Gammaproteobacteria	7.18	3.52	13.80	2.18
Other	2.22	15.73	10.33	11.37

Table 4. The top 3 species identified in each sample. Data are shown as a percent of total reads for each group from the total number of identified sequences at the specific site.

Sample	Top 3 Species/Site	Percent of Identified Sequences
Mining Soil	*Paucibacter toxinivorans*	21.50
	Kocuria rosea	14.30
	Rhizobium spp.	6.44
Compost	*Bacillus vireti*	11.50
	Anaerosporobacter spp.	4.82
	Clostridium spp.	3.72
Ray Grass	*Anaerosporobacter spp.*	12.10
	Pseudoxanthomonas taiwanensis	11.10
	Clostridium spp.	8.31
Clover – Ray Grass	*Massilia timonae*	13.86
	Bacillus vireti	8.14
	Clostridium spp.	7.80

Table 5. Distribution of taxonomic groups represented in each sample.

Sample	Number of Group Members					
	Phylum	Class	Order	Family	Genus	Species
Mining soil	5	10	19	34	45	47
Compost	13	37	65	127	256	336
Ray grass	10	22	42	92	153	191
Clover - Ray grass	11	31	57	115	233	307

Bacilli, and Mollicute classes. These microbes are able to survive in harsh conditions including desiccation. Representatives of other phyla were also found in either water or soil samples from the Smoky Mountains, including the Proteobacteria, Bacteroidetes, Actinobacteria, Planctomyces, Acidobacteria, and Verrucomicrobia. These phyla were also found to be represented in our DNA sequences (Table 2) with some only in the remediated samples thus increasing diversity in those samples where fabrication of the soil took place.

In work reported in the Journal of Student Research (Lutton *et al*., 2013), the researchers collected soil and water sediment samples from the temperate forest area of Washington, Pennsylvania. Their method for increasing cultarability of bacteria from the soil sample included using 3 different agar plates to maximize diversity. Briefly, they isolated bacteria from 13 different orders, in 24 different families, for a total of 40 different genera. These values correspond to the following phyla distributions Proteobacteria (44%), Firmicutes (12%), Bacteroidetes (30%), Actinobacteria (15%), and Cyanobacteria (0.6%) of total isolates. The representation of Proteobacteria is similar to our results (30-66%) as was the Actinobacteria (4-20%) see table 2. Overall, the sequencing results have similar patterns for these two phyla, however the diversity we were able to detect was greater which would be expected, in part, because our method was to look at genomic DNA in our samples rather than culturing and identifying the microbes present. Previous work involving fabrication of soil and characterization of microbes at similar sites in Butler County, Pennsylvania used traditional microbial analysis where the soil samples were placed in water, incubated, diluted and streaked onto TSA plates (Kalevitch and Kefeli, 2013). This method was used to identify microbes

capable of being cultured and subsequently identified. Our method involved isolating genomic DNA and utilizing high throughput sequencing and BLASTn comparison to identify bacterial communities at our sites. Similarly, both data sets have representation of multiple species of *Bacillus* and *Coryneform bacillus.* However, our results suggest a greater microbial diversity in the soil of this area may exist.

CONCLUSION

The findings of this study indicate that the mining soil had less microbial diversity compared to the plots of amended soil. Fabrication of the soil with organic material provided additional resources and disturbed the soil; thereby providing bacteria with a new source of potential nutrients. Our results provide a unique component to previous work using culture based identification and provoke more questions about community structure between the groups and available nutrients between the sites. Future studies of AMD sites to those where fabrication or remediation occurred will allow for comparison of treatments over time and the effect it has on microbial diversity.

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