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Changes in microbial communities of a passive coal mine drainage bioremediation system

Hannah Roth[1], Samantha Gallo[1], Paul Badger[1], and *Melissa Hillwig[1]

[1] Department of Science, Robert Morris University, Moon Township, PA 15108, USA.

* Corresponding Author email: hillwig@rmu.edu

Abstract

Drainage from abandoned mines is one factor greatly affecting the streams and vegetation in and around Pittsburgh and the Appalachian Mountains where coal mining occurred. This drainage may be more acidic, alkaline, or metal based. Different methods for remediation exist. Passive remediation is one method used to naturally allow the metals to precipitate out and aid in cleaning up the water. The goal of this study is to sample different holding ponds in a sequential passive remediation system and determine microbial communities present at each site of an abandoned coal mine drainage (CMD) site. 16s rRNA gene sequencing of the sediment indicated the most abundant phyla at each of the 5 ponds and wetlands area included Proteobacteria (36-43%), Bacteroidetes (12-37%), Firmicutes (3-11%), and Verrucomicrobia (6-11%). Analysis of genera between the first, and most polluted pond includes S*olitalea, Pedosphaera,* and *Rhodocyclus*; while the microbial community from the wetlands site at the end of the remediation system included *Ignavibacterium, Pelotomaculum,* and *Petrimonas*. The results of our microbial community composition study of sediment from a passive treatment system are in line with organisms commonly found in sediment regardless of iron oxide precipitation, while others are preferentially found in the less polluted wetlands site.

Keywords: mine drainage, microbial communities, sediment, meta-genomics, passive MD water treatment

Introduction

Excavation of coal from the earth has been taking place for many years. As demands change and resources are consumed many of these mines are shut down and the entrances sealed. Often times the network of tunnels begin to fill with water and eventually ends up flowing into an established water way. Coal mines often contain ferrous iron and as the water comes in contact with it, the iron is dissolved into the water. When oxygenated, the ferrous iron is converted to ferric iron and is easily recognized by the reddish-orange color. This precipitate settles to the bottom of the river bed and begins to coat the surroundings. Additionally, flora and fauna are affected by changes to pH, heavy metals, and a reduction in the amount of dissolved oxygen present (Senko et al. 2008; Branter and Senko 2014). Metagenomics of this type of mine drainage (MD) sediment is of great interest (Klein et al. 2013).

In western Pennsylvania, CMD contributes to a multitude of changes that other organisms must face. Abandoned mines may also leach out other dissolved metal ions such as $Mn^{2+,}$ Cu^{2+}, Ni^{2+}, and Zn^{2+}, along with low valency iron cations (Rakotonimaro et al. 2018; Nancucheo et al. 2017; Pinto et al. 2018). Metagenomics of microbial communities found in soil from abandoned coal mines the Appalachian mountains includes organisms from the phyla of Proteobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Acidobacteria, and Verrucomicrobia (Brantner and Senko 2014; Sanchez-Andrea et al. 2014).

Bioremediation is often used in areas of MD. Many scientists in the Appalachians are studying ways in which MD and CMD contaminated soil and water can be cleaned and treated. One method is the fabrication of soil. This often involves the mixing of organic material with soil to introduce new microbes and nutrients to the affected earth (Kalevitch and Kefeli 2013). Another possible treatment plan includes phytoremediation where select vegetation is grown in the contaminated soil, such as the

growth of Phragmites to chelate the heavy metals (Guo and Cutright 2015). The vegetation used must be matched with the unique combinations of metals found in the environment (Kim and Lee 2010). In addition, studies have shown that combining microorganisms along with suitable plants can influence metal uptake by the plants (Kuffner et al 2008; Becerra-Castro et al 2012). Nancucheo, et al. 2017 states that the use of sulfidogenic microorganisms for bioremediation, particularly in areas affected by acid MD may help with the redevelopment of these metal-rich and acidic environments. Treatment strategies often use microbes that metabolize these metals and sulfur compounds (Klein et al. 2013). These are a few types of the methods currently being researched for bioremediation of MD soil.

Passive water treatment systems are one way naturalists are trying to limit the amount of heavy metal water entering into streams and rivers. In southwestern Pennsylvania, a passive water treatment system was completed in 2009 to help clean-up the CMD and iron flowing into Chartiers Creek (Allegheny Land Trust 2018). At this site approximately 5600-7500 liters per minute of MD water are treated in a passive system that utilizes gravity to treat the iron discharge. The system designed by Bob Hedin, allows the water to pass through 5 sequential ponds followed by a wet-land area before finally flowing into Chartiers Creek. Precipitation of iron (III) oxide can be seen in many of the early ponds. The flow pattern of the ponds has been modeled and areas of low flow are noted (Buxton 2018). Environmental data was not reported by the authors at this time as part of this study, however water was collected with the intent of determining metal ion concentration from it at a later date. Information regarding pH, temperature, dissolved oxygen, turbidity, and conductivity has been collected from other groups surveying the system (Kester 2019). Much of the environmental chemistry data presented on the website is a result of Ed Schroth and other educational groups.

The purpose of this study is to sample and describe the microbial communities found in the various ponds and wetland areas of the passive MD site in Allegheny County, Pennsylvania.

Material and Methods

Location and collection of sediment

The sampling of MD contaminated sediment was from Wingfield Pines Passive remediation treatment Figure 1 (coordinates: 40.341362 - 80.109656; Google Maps). Each pond is numbered following the flow of water through the system. Sediment was collected by using a long reach pole with a 100ml beaker attached to the end. The beaker was dipped into the pond and a single, undisturbed scoop of sediment was collected and stored in labeled clean sterile bags. Excess air was carefully removed from the bag and sealed on-site. The beaker was cleaned between each sample. Upon arrival to the lab, 2 grams of sediment from each bag was measured out for DNA extraction.

Isolation of genomic DNA

Genomic DNA was isolated from 0.5g of each sample immediately upon returning to the lab. The Zymo soil microbe Quick DNA kit (D6010) was used and the directions were followed as indicated. DNA from each site was labeled and stored in the -20°C for further processing. Concentrations of nucleic acids were determined using a UV-VIS Spectrometer, measuring absorption (@ 260 nm) and sent for genomic sequence analysis. Extractions were performed in duplicate.

Sequencing reactions

DNA was aliquoted and sent to Molecular Research LP (MR DNA) Lab in Clearwater Texas, by overnight shipping. For each sample, the V4 variable region of the 16srRNA gene was amplified using 515(F-GTGCCAGCMGCCGCGGTAA) and 806 R (5' GGACTACHVGGGTWTCTAAT)(Caporaso et al. 2012) . PCR conditions are as follows: 94°C for 3 minutes one time, then 94°C for 30 seconds, 53°C for 40 seconds, 72°C for 1 minutes and cycled 28 times. A 5 minute final elongation step at 72°C was performed following the final cycle. An Ion Torrent PGM was used for sequencing at according to the

manufacturer's guidelines. Proprietary analysis pipeline software was used for processing the sequence data. Briefly, all barcodes and primers were removed from the sequences and any remaining sequence less than 150bp was removed from the sample. Additionally, any sequence with homopolymer runs more than 6pb were also removed prior to denoising the sample, generating OTU's and removing chimeras. Operational taxonomically units (OTU's) were determined by clustering at 97% similarity (3% divergence). BLASTn was used to taxonomically classify each OTU. Sequences were grouped taxonomically. Analysis of phylum, family, and genus were used for comparison of genomic diversity at each site. Data files were arranged by percent presence in each sample.

Results

Sample collection

Sediment from areas of low flow were collected from each holding pond (Figure 1) 1-5 along with a sample from the wetlands area where the water courses before flowing into Chartiers Creek. Six samples were collected for this study.

rRNA Gene Sequencing

16s rRNA gene sequences were found through PGM sequencing. Sequences are deposited into NCBI with the SRA accession of PRJNA518724. Over 370,400 sequences were found and compared using Blastn at the National Center for Biotechnology Information (NCBI). Data was organized by phylum, class, family, genus and species. Table 1 indicates the number of genra and species found at each site along with the total number of reads from that site. The top 4 phyla at each site included Proteobacteria, Bacteroidetes, Verrucomicrobia, and Firmicutes. The next 6 phyla varied, but each site had Acidobacteria, Spirochaetes and Chloroflexi represented. The remaining 3 phyla at each site was from one of the following groups: Tenericutes, Planctomycetes, Ignavicacteriae, Nitrospinae, or

Cyanobacteria. Figure 2 illustrates the percent of organisms from these 12 groups and other. As noted in Figure 1, the majority of organisms belong to the phyla of Proteobacteria and Bacteroidetes (52-75%).

Comparison of sequences at the genus taxonomic level included more than 850 members. Each location had between 528-655 genera represented. Among the samples, two were found in the top 3 groups of all five sites; *Geobacter* and *Cytophaga*. The top 20 organisms at each site were compared between sites (Table 2). Further analysis of the data examining organisms having more representation in either Pond 1 or the Wetlands sample was noted in Table 3. These organisms were present at least 3 fold higher in one of the samples relative to the other.

Discussion

Many of the organisms identified through this study are common between samples. The most diverse sample was from pond 4 with 1022 species identified. The least diverse was pond 2 with 796 unique species. To better compare these samples, percent of each group was calculated and used for data analysis. Relative abundance of each varied and some of those detected in one site were not identified in other samples. Those found in one or a few sites are often among the lowest percent of organisms represented in their group. The two most abundant phyla represented in the sediment samples are Proteobaceria and Bacteroidetes (Figure 2). These two are among some of the most commonly represented phyla in previously reported mine drainage samples (Bruneel et.al. 2017, Chaput et al. 2015). Five additional abundant phyla are among the remaining six in each sample: Acidobacteria, Verrucomicrobia, Firmicutes, Spirochaetes, and Chloroflexi. Beyond these phyla, the other three vary between the sites with some phyla appearing in up to four or five of the sites.

From the Proteobacteria, the genera *Anaeromyxobacter*, *Desulfobacterium*, *Desulfobulbus*, and *Pelobacter* are represented in the top 20 of 5 of the 6 sites (Table 2). *Anaeromyxobacter* is a facultative organohalide respiring bacteria and grow anaerobically (Sanford et al. 2002). Sanford et al reports that

this genus of organisms may use nitrate, fumarate, and chlorophenolic molecules as terminal electron acceptors. Additional reports also identify that members of this genus are capable of reducing iron and uranium (He and Stanford 2003; Treude et al 2003; Wu et al. 2006; Sanford et al. 2012; Onley et al. 2018) The sediment collected from the passive remediation ponds (1-5) was under 60-90cm of water in low flow areas (Buxton, 2018). As such, the microorganisms found here would have less oxygen present in the sediment. The *Deltaproteobacteria* family includes the genera *Desulfobacterium*, *Desulfobulbus*, and *Pelobacter*. Species from the *Desulfobacterium* and *Desulfobulbus* are part of the sulfur cycle studied in aquatic sedimentation using various fermentation pathways (Pagani et al. 2011). The genus *Pelobacter* consists of microorganisms which are strictly anaerobic (Schink, 2006).

In the phylum Bacteroidetes, the genera *Anaerophaga*, *Bacteroides*, *Cytophaga*, and *Solitalea* were present in the top 20 of at least 5 of the 6 samples. *Anaerophaga* was first described in 2002 by Denger et al. as a new genus of strictly anaerobic microorganisms which are non-photosynthetic and chemo-organotrophic. These organisms are rod shaped and in aging cultures produce an orange-red pigment similar to flexirubins. *Bacteroides* in general are nonendospore forming bacteria. These organisms have been identified in many MD samples (Delmont et al 2011; Llyod et al. 2004). *Cytophaga* are a group of rod-shaped Gram-negative bacteria commonly found in soil. These organisms are known for their ability to rapidly digest cellulose (Stanier 1942; McBride et al. 2014). The genera listed here are only a small subset of those identified through this study.

Comparison of the relative microbe representation between Pond 1 and the Wetlands (Table 3). Those genera more abundant in the first pond include *Solitalea, Pedosphaera, Rhodocyclus, Alkaliflexus, Chintinophaga,* and *Rhodoferax* (Table 3A). Of these, *Solitalea* is described as a nitrate reducing microorganism found in the soil, suitable in aerobic environments or facultative anaerobic (Weon et al 2009). *Pedosphaera* and *Chitinophaga* have been identified in multiple soil samples (Brewer et al 2016). While many of these genera have little published data. The wetlands area of the passive water

treatment center has an expanded amount of flora and fauna. In this area, the fourth most abundant genus found is *Ignavibacterium* (Table 3B) had more than a 5 fold change in relative community percent between the less polluted wetlands and the first sedimentation pond (wetlands 2.51; pond 1 0.44-Table 3B). This genus was identified in all samples, with ponds 1 and 2 having 34 and 30 other groups more abundant (data not shown). This organism was first identified from microbial mats from a hot spring, but has been successfully cultured in the lab (Iino et al. 2010). The genomic sequence of *Ignavibacterium* suggests it may utilize a variety of electron donors and can live both aerobically and anaerobically permitting it to grow in both low flow and wetland areas (Liu et al. 2012). *Pelotomaculum* had the largest fold change of 27 between the wetlands and pond 1. This genus has only a few reported species (Qui et al 2006; Imachi et al. 2007; Imachi et al. 2002). Reports state these organisms utilize organic compounds and cooperate with methanogens and oxidize propionate (Lueders et al. 2004; Gan et al 2012; Ishii et al 2005; Kato et al. 2009). Additionally, some of the organisms we identified with our study correlate to those found by others examining CMD.

Environmental data was not collected as part of this study, but data was found online from prior studies conducted at this passive remediation site. The information presented on the webpage was interpreted by the authors in this discussion. In 2014, the pH of the different sites was found to increase from 6.5 at the source of the water flow to 8.3 in the wetlands (Kester 2019). The amount of dissolved oxygen has an upward trend as the water flows from the source to the wetlands during the years of 2011-2014. While there is no consistent increase from year to year, each year reported has the same pattern. This irregular pattern from one year to the next may, in part, be due to sampling at different temperatures as noted in 2013 and 2014 the temperature was less than -6^{o}C (less than 20^{O}F) and in years 2010, 2011, and 2012 the temperature was between 10-18^{o}C.

Summary

Many of the genera identified in this study are anaerobic or facultative and commonly have alternate electron acceptors and fermentation pathways. This is beneficial for organisms subjected to harsh conditions of heavy metal sedimentation (iron oxide) precipitating out of CMD contaminated water. Microbial diversity varies at the genus level for organisms utilizing the available nutrients and lack of oxygen in the contaminated and remediated sediment. Future analysis of this site includes continued sampling and investigation into changes of the microbial community along with environmental and elemental analysis of the water and sediment.

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326 Figure 1. Map of AMD Passive Remediation site. Image from Google maps. Coordinates 40. 341362 -
327 80.109656.

328 Figure 2. Major phyla represented in each site.

329

	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Wetlands
Number of Genra	585	527	654	629	599	591
Number of Species	892	796	1016	1022	913	921
Number of Sequences	60124	58781	73543	53022	66827	43615

330

331 Table 1. Comparison of total number of OTU‘s and reads from each pond.

332

Genus	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Wetlands
Acholeplasma	0.38	1.81	2.67	1.68	1.06	0.39
Acidobacterium	1.82	0.86	1.61	4.95	1.35	2.03
Alkaliflexus	1.95	1.12	0.60	0.40	1.12	0.55
Anaeromyxobacter	1.76	1.45	1.12	2.75	2.06	2.02
Anaerophaga	10.12	13.85	3.83	1.88	8.48	7.82
Bacteroides	1.59	1.81	1.83	0.99	2.96	2.33
Chitinophaga	1.74	1.66	0.06	0.19	0.38	0.19
Clostridium	0.88	1.85	0.83	0.88	0.76	0.61
Cytophaga	8.17	7.12	6.10	3.96	5.18	5.60
Desulfobacterium	1.21	0.91	3.21	1.88	2.03	2.40
Desulfobulbus	1.42	2.04	1.58	0.58	2.58	0.98
Desulfococcus	1.37	0.97	0.88	0.41	1.98	1.06
Gemmatimonas	0.16	0.14	0.29	1.69	0.06	0.16
Geobacter	11.24	10.78	7.00	5.77	7.55	5.31
Holophaga	0.30	0.41	0.67	1.27	0.54	0.60
Ignavibacterium	0.44	0.49	3.90	1.47	1.41	2.51
Longilinea	0.12	0.08	1.39	1.21	0.34	0.87
Luteolibacter	3.24	3.71	1.09	1.71	1.30	2.05
Moorella	0.15	0.07	1.52	2.56	0.68	1.08
Nitrosovibrio	3.19	4.52	1.34	0.78	3.33	1.83
Nitrospina	1.75	0.21	0.56	0.26	2.29	2.45
Nitrospira	0.54	0.12	0.22	1.49	1.17	0.80

Opitutus	1.66	3.02	2.06	1.88	1.70	2.04
Pedosphaera	3.57	2.74	1.43	1.22	3.00	1.19
Pelobacter	2.51	1.33	1.35	3.03	3.48	1.93
Pelotomaculum	0.09	0.11	0.35	1.78	0.63	2.39
Petrimonas	0.47	0.08	1.84	0.84	1.00	2.21
Planctomyces	0.31	0.10	2.13	2.96	0.59	1.03
Prolixibacter	0.97	1.74	0.71	0.98	0.83	0.69
Rhodocyclus	2.05	4.35	0.80	0.40	0.83	0.17
Solitalea	6.99	4.21	1.57	0.26	4.00	2.10
Sphingobacterium	1.85	2.23	0.86	0.37	1.61	1.79
Spirochaeta	1.14	0.95	2.51	2.30	1.25	1.43
Sulfuricurvum	0.68	0.38	3.43	0.87	1.24	0.70
Syntrophus	0.50	0.36	0.88	2.43	0.66	0.83
Verrucomicrobium	1.40	0.97	1.10	1.53	1.08	0.84

333

334 Table 2. Accumulation of the top 20 genera from each site, ordered alphabetically. Values represent the

335 percent of each genera found in the sample.

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337

Genus	Pond 1	Wetlands
Solitalea	6.99	2.10
Pedosphaera	3.57	1.19
Rhodocyclus	2.05	0.17
Alkaliflexus	1.95	0.55
Chitinophaga	1.75	0.19
Rhodoferax	1.04	0.10

Genus	Pond 1	Wetlands
Ignavibacterium	0.44	2.51
Pelotomaculum	0.088	2.39
Petrimonas	0.47	2.21
Moorella	0.15	1.08
Dechloromonas	0.14	1.04
Bellilinea	0.12	1.04
Planctomyces	0.31	1.03

338

339 Tables 3A & 3B. Genera found in Pond 1 or Wetlands with at least a 3 fold difference than the other site.

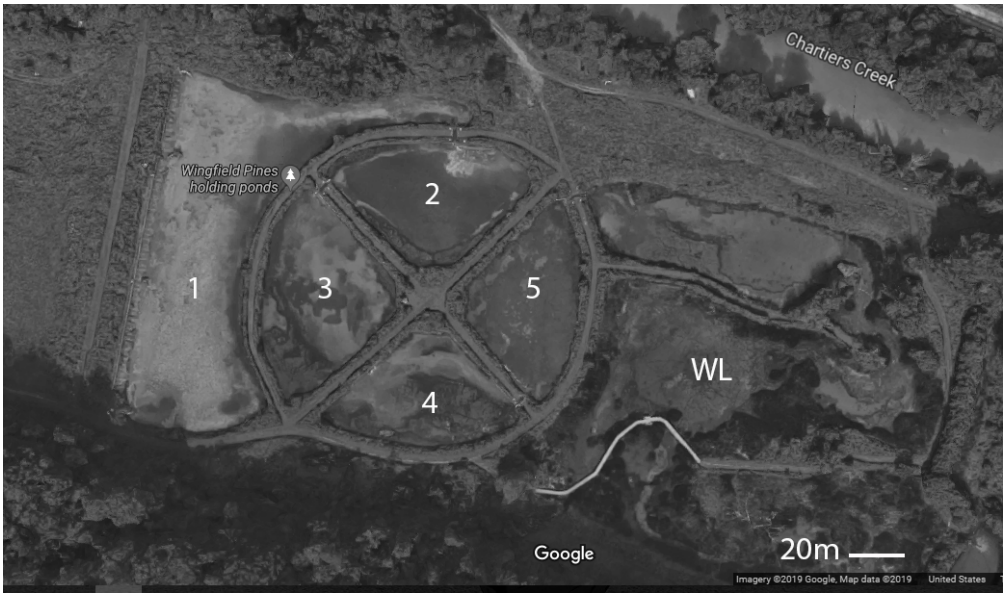
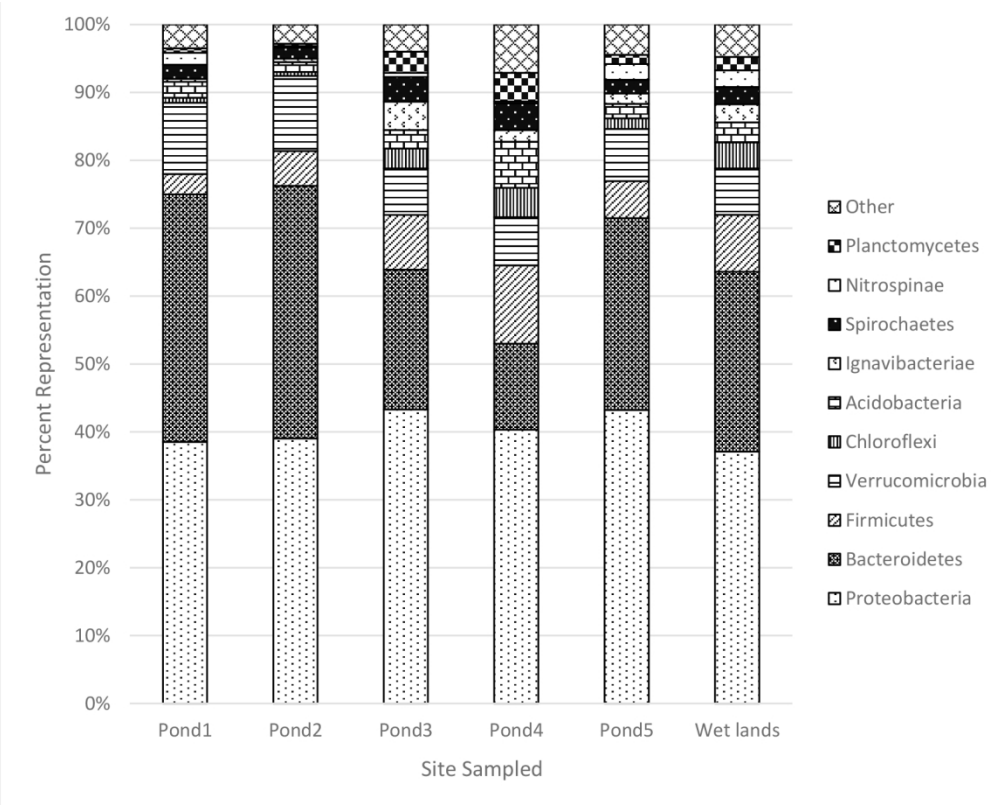


Figure 1. Map of AMD Passive Remediation site. Image from Google maps. Coordinates 40. 341362 - -80.109656.



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3a

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3b