

Modeling the effects of acid rain on soil microbial communities: How pH alters the composition, structure, and metabolic capabilities of soil communities

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## **Abstract**

Acid rain is a growing problem in industrialized nations. This highly acidic precipitation is due in large part to human activities, and has been shown to have disastrous effects on numerous ecosystems, including aquatic environments, soils, as well as man-made structures. In this study, in order to understand how altered pH affects the composition of the soil microbial communities, soil samples were treated with buffers at pH 3, 5, and 7 and the resulting soil communities were characterized. Phylogenetic analyses revealed that the treated communities were not significantly different from one another, although taking into account just community membership, UniFrac analyses is suggestive of a difference in community composition between pH 3 treated soil and untreated soil. Despite not being significantly different as indicated by our statistical analyses, one novel group emerged in the pH 3 treated soil, the Gemmatimonadetes. These data suggest that in the time allotted, the soil microbial communities were not given enough time to significantly change. We speculate that if the soil were treated for longer periods of time, we would actually see more pronounced alterations of the vaginal flora.

## **Introduction**

Acid rain refers to the highly acidic precipitation from the atmosphere containing higher than normal amounts of nitric acid and sulfuric acid. This is a huge problem in industrialized nations such as China, Russia, and the eastern third of the United States.

Various human activities/products such as the burning of fossil fuels, and car emissions can result in the release of sulfur dioxides, nitrogen oxides, and volatile organic compounds into the atmosphere. It is here where these compounds interact with water to generate sulfuric acid and nitric acid, and this acid containing precipitation can then fall to the earth.

The soil is a complex environment composed of inorganic and organic matter, as well as air and water. This complex mixture of components can create several different microenvironments supporting the growth of various groups of microorganisms. It has been shown previously that simulated acid rain can have alterations of the microbial community structure. However, this was previously done in a non-specific way, via phospholipid fatty acid (PLFA) analysis(1) of the microbial community. Acid rain can have several detrimental effects leading to altered microbial communities, including the mobilization of several toxic metals (mercury, lead, cadmium, arsenic, selenium), a reduction in the soil pH which could have effects on the viability of various groups of organisms, and leaching of essential nutrients from the soil(2).

Nitrogen fixation is a crucial function carried out by certain soil bacteria, and accounts for the majority of useable nitrogen that can be assimilated into important building blocks such as amino acids and nucleotides. This is accomplished by the function of a nitrogenase enzyme, which has been highly conserved. This multiprotein enzyme complex is encoded by the genes *nifH*, *nifD*, *nifK*. The diversity of nitrogenase genes varies across different habitats(3, 4) and can be grouped into five clusters: the traditional Fe-Mo nitrogenases, the alternative nitrogenases (Fe-V and Fe-Fe nitrogenases), a third cluster with *nifH* sequences from anaerobes, and two more clusters made up of *nifH* genes not

involved in nitrogen fixation(3). We speculate that the alteration of the pH would select for a particular group/class of nitrogenases and potentially result in the discovery of novel nitrogenase genes.

## **Materials and Methods**

### *Sample Collection*

60g soil samples were collected in syringes from the same 2 foot x 2 foot square outside of swope center. Briefly, syringes were plunged into the group, and soil was lifted up to preserve the spatial orientation of the soil. Plants and associated roots were maintained in the syringes.

### *Treatments*

Each syringe of soil was sawed off at its end and capped with wire screen. Each syringe was treated with 25mM HomoPIPES buffer +1% mannitol adjusted to a pH of 3, 5, or 7 with H<sub>2</sub>SO<sub>4</sub>. As a control one syringe was treated with a 1% mannitol solution. Treatments were carried out for 5 days, and the syringes were placed in a rack to allow for the fluid to drain out as it flowed through. Soil was then removed from each syringe, mixed, and stored in sterile bottles. The pH of each soil sample was measured using pH strips (Hydrion)

### *Community Enrichments*

Soil samples were subsequently used for enrichment of aerobic and anaerobic nitrogen fixers. For enrichments of aerobic nitrogen fixers, the respective soil samples were placed into NFMH medium (1X freshwater base, 25mM HomoPIPES, 1X Trace elements solution, 1mM Na<sub>2</sub>SO<sub>4</sub>, 100nM Potassium phosphate, 1X Vitamin solution, 0.2% sodium molybdate,

2% FeSO<sub>4</sub>-7H<sub>2</sub>O, 1% mannitol) adjusted to the appropriate pH with H<sub>2</sub>SO<sub>4</sub> and incubated at 30°C. For enrichment of anaerobic nitrogen fixers, the respective soil samples were placed into 5mL of NFSMH (1x Freshwater base, 1X sulfate, 1X phosphate, 1X trace metals, 1X vitamins, .342% sucrose, 25mM HomoPIPES) adjusted to the appropriate pH with. The bottles were purged with nitrogen, and incubated anaerobically at 30°C. In order to generate solid media, agar was added to the liquid medium, and sterilized. However, in order to generate pH3 NFMH or NFSMH, we had to make both agar and medium at 2x concentration, and mix them after sterilization.

#### *Community 16SrDNA analysis*

DNA was extracted from 0.25g of soil per treatment using the MoBio Power Soil DNA isolation kit. DNA was quantified using NanoDrop (ThermoScientific). Extracted DNA was amplified using universal bacterial primers 8F and 1492R. **Amplification conditions were as follows:** Amplification products were cloned via the TOPO TA cloning kit (Invitrogen) to create clone libraries. The RDP classifier as implemented in the Qiime pipeline for community microbial analysis tool was used to assign taxa to all sequences(5). Sequences were filtered using the filter\_alignment.py protocol in Qiime with lane masking enabled. Trees were visualized using FigTree. In addition to the creation of clone libraries, 454 16S data on soil samples were collected.

#### *Analysis of nitrogenase diversity*

In order to analyze the diversity of nitrogenase genes present in our samples, extracted DNA was amplified using primers for the *nifH* gene: UEDA 19F (5'

GCIWTYTAYGGIAARGGIGG3') and UEDA 407R (5' AAICCRCCRCAIACIACRTC3').

Amplification conditions were as follows: 95°C for 3 min; 95°C 30s, 61°C for 1 min, 72°C for 1 min repeat 35 times. Amplification products were cloned via the TOPO TA cloning kit (Invitrogen) to create clone libraries. The RDP classifier as implemented in the Qiime pipeline for community microbial analysis tool was used to assign taxa to all sequences(5). Sequences were filtered using the filter\_alignment.py protocol in Qiime with lane masking enabled. Trees were visualized using iTOL.

### *FISH*

Alterations in the abundances of different classes of proteobacteria amongst different treatments were monitored by FISH. Soil samples were fixed and probes for the appropriate organisms were hybridized as per Ishii et al. (2004). Probes against Alphaproteobacteria, Deltaproteobacteria, Betaproteobacteria, and Gammaproteobacteria were used for the FISH analysis.

<b>Alphaproteobacteria</b>	<b>Alf 968</b>	GGTAAGGTTCTGCGCGTT
<b>Betaproteobacteria</b>	<b>Bet42a</b>	GCCTTCCCACATCGTTT
<b>Deltaproteobacteria</b>	<b>Delta495-a</b>	AGTTAGCCGGTGCTTCCT
	<b>Delat495-b</b>	AGTTAGCCGGCGCTTCCT
	<b>Delta495-c</b>	AATTAGCCGGTGCTTCCT
<b>Gammaproteobacteria</b>	<b>Gam42a</b>	GCCTTCCCACATCGTTT

## Results/Discussion

### *Clone library and 454 sequence analysis*

Clone libraries yielded 73 sequences for pH 3, 29 sequences for pH 5, 84 sequences for pH 7, 46 sequences for untreated samples. Analysis of taxon abundance demonstrated that *Proteobacteria* represented the dominant species, with *Actinobacteria* representing another major species present (Fig 1A). A further analysis of the *Proteobacteria* composition demonstrated no real change in the abundance of any of the groups of the *Proteobacteria* between samples (Fig1B). Interestingly, we see that those soil samples treated with HOMOPipes buffer had an increase in the *Deltaproteobacteria*, suggesting that HOMOPipes buffer may select/enrich for deltaproteobacteria. Any changes most likely represents normal variation, rather than changes due to treatment. This is not surprising as the major nitrogen fixing species present in the soil belong to these classes. Comparison of the relative taxon abundance across conditions did not reveal any significant differences. Small changes most likely represent just normal variation in the community composition. We carried out both a weighted and unweighted UniFrac analysis to determine if there was a statistically significant difference between the communities present after treatment. Weighted UniFrac analysis (Fig 1D), taking into account the relative abundance of OTUs indicated that the communities were not significantly different, and contained many of the same members. In the unweighted UniFrac analysis (Fig 1C), most of the communities are not significantly different from each other. However, the pH 3 treated soil may be somewhat different from the untreated soil, indicated by a p-value of 0.06. This suggests

that perhaps the communities are beginning to change and transition, and probably required more time and treatment before we see a significant change in the community structure and composition. The dendrogram of the unweighted UniFrac demonstrated that the pH 3 treated soil community was most closely related to pH 7 and untreated soil, and more distantly related to pH 5 treated soil community. However, they are all extremely similar to one another. The dendrogram of the weighted UniFrac analysis, taking into account the relative abundances of the organisms, indicated that none of these communities were significantly different from each other. All of the clone library data is suggestive of a community that may have begun to change during our time period, but requires more time to result in a significant difference in community composition.

The 454 analysis revealed many of the same things that the clone library data also demonstrated. However, it should be mentioned that the number of sequence reads across samples varied widely. The pH 3 treated soil sample had a total of 3,297 reads, whereas pH 5 treated soil samples had a total of 86 reads. Thus, it is difficult to accurately interpret the results of the 454 sequence data. An analysis of the taxa present normalized to the total number of reads demonstrated that in all samples, unclassified (Other) bacteria dominated the reads (Fig 2A). This is in contrast to the clone library data that demonstrated a smaller proportion of unclassified bacteria. This possibly is due to deeper sequencing, picking up more unclassified bacteria, or possibly represents cloning bias (where fragments from the unclassified bacteria are more resistant to cloning). Interestingly, there was one class of organisms that was detected that was unique to the pH 3 treated soil, the class of Gemmatimonadetes. This is a new class of organism that was initially discovered in a sewage treatment plant. Analysis of the abundance of various members of the

proteobacteria (Fig 2B) revealed no significant differences in the abundances between treatments: Alphaproteobacteria were the dominant group in all four conditions. Weighted UniFrac analysis of this data indicated that these communities are not significantly different, and actually share many of the same OTUs, as indicated by UniFrac metric values that were close to zero.

### *Analysis of nifH diversity*

In order to examine the diversity of nitrogenase genes present in our samples, extracted DNA was then used to amplify a portion of the *nifH* gene using primer pair UEDA19F and UEDA407R. These sequences were then aligned against a database of *nifH* genes downloaded from the FunGene Functional Gene Pipeline & Repository (<http://fungene.cme.msu.edu/index.spr>). Trees were then built using FastTree and visualized using iTOL (Fig 3). We expected to see reductions in pH selecting for particular types/classes of nitrogenase genes, which would be shown by clustering of those particular *nifH* sequences. However, what we see is that each condition has *nifH* sequences distributed across the tree, indicating that for each condition, there is a large diversity of *nifH* sequences. This is not surprising given that we see no significant changes in the community composition over the study period. Perhaps if we had given the soil samples more time or increased the mannitol concentration to facilitate turnover, we would have seen selection for a particular group/class of nitrogenase genes. However, in our study, we were unable to see any significant alteration of both community structure, and metabolic capability (as indicated by diversity of *nifH* gene sequences). Interestingly, when we used BLAST to assign these *nifH* genes to a particular group, we found that the 90% of the total

sequences obtained from all the conditions had blast hits to genes involved in bacteriochlorophyll synthesis. Either our primers do not effectively target all *nifH* genes (which is unlikely given that these degenerate primers have been used in the past to examine *nifH* diversity) or, our treatment of the soil has altered the community in such a way that we have selected for organisms expressing *nifH* genes that are not actually involved in nitrogen fixation.

### *Community Enrichments*

In order to isolate some of the organisms that we expected to become enriched, we generated nitrogen free medium (NFM) with 25mM HOMOPipes adjusted to the appropriate pH with H<sub>2</sub>SO<sub>4</sub> for the enrichment of aerobic nitrogen fixing bacteria as well as nitrogen free sucrose medium (NFSM) with 25mM HOMOPipes adjusted to the appropriate pH with H<sub>2</sub>SO<sub>4</sub> for the enrichment of anaerobic nitrogen fixers. From our liquid enrichment cultures, we also generated solid media by the addition of agar. After several days, we isolated slimy clear colonies on our NFSM medium for all pHs. Similar looking organisms were obtained on our NFM. Interestingly, for our pH 3 treated soil, we obtained pink colonies (Fig 4) that had a cyst-like morphology. We speculate that this may represent *Azotobacter* species based on its pigment production and morphology. However, due to time constraints, we were unable to sequence the 16SrDNA in order to identify them.

## Figures

**Fig 1A. Community Comparison of clone libraries.** Analysis of relative taxon abundance of various classes of organisms present in the 16S clone libraries of treated soil samples. Proteobacteria represents the major species present. No significant change in community composition could be detected.

**Fig 1B. Analysis of proteobacteria composition.** Analysis of the relative abundances of different groups of proteobacteria. No real pattern emerges with regards to differences in community composition. In those samples treated with pH buffers, we see the deltaproteobacteria gaining dominance.

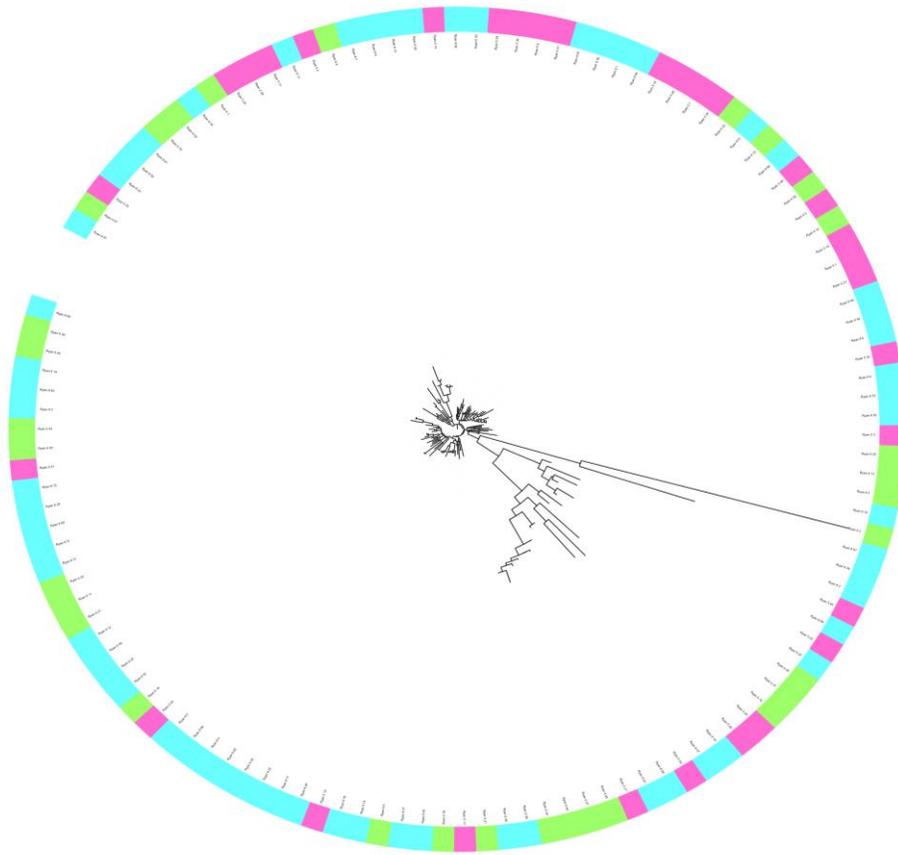
**Fig 1C. Unweighted UniFrac analysis of 16S clone libraries.** The top panel shows a dendrogram indicating that the pH 3 treated soil was distantly related to the community present in the untreated and pH 7 treated soil. The pH 5 treated soil was most distantly related. The bottom panel is a test of significance between pairs of environments. Most environments were not statistically significant between environments. A p-value of .06 for the comparison between pH 3 and untreated soil suggests a statistically significant difference in community composition.

**Fig 1D. Weighted UniFrac Analysis of 16S libraries.** The unweighted UniFrac analysis demonstrated pH 3 treated soil was most closely related to the pH 7 treated soil. Again, pH 5 treated soil was most distantly related. . The bottom panel is a test of significance between pairs of environments. When the relative abundance of organisms was taken into account, these communities were not statistically different with regards to their community composition.

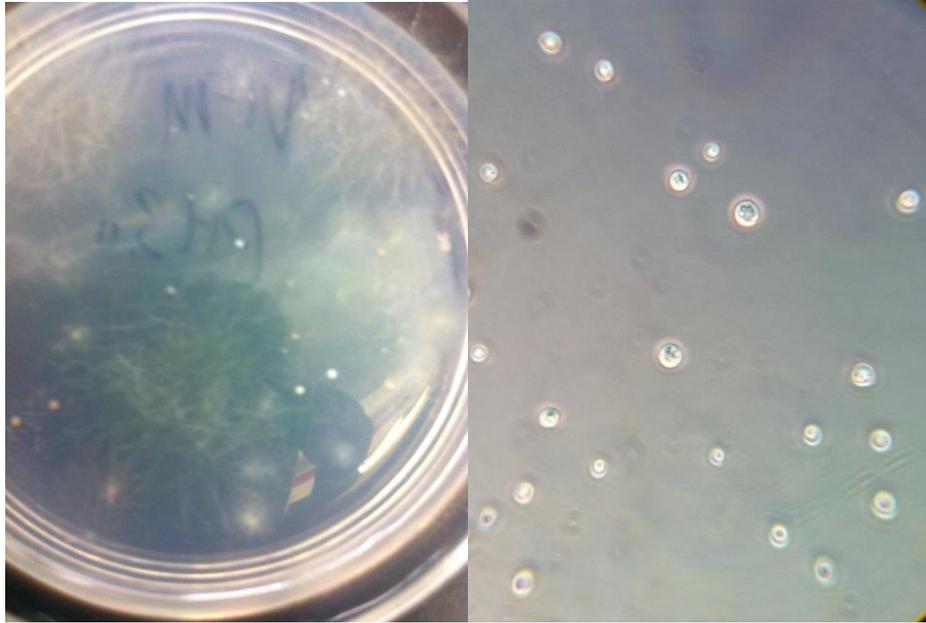
**Fig. 2A Relative abundance of taxa from 454 analysis.** Analysis of 454 data indicated that unclassified bacteria dominated the samples. Again, no significant pattern can be detected among treatments. Interestingly, Gemmatimonadetes was found only in pH 3 treated soil samples.

**Fig.2B Relative abundance of *Proteobacteria* from 454 analysis.** The relative abundance of proteobacteria in the sample was analyzed as well. Alpha proteobacteria were the dominant species present in the 454 analysis.

**Fig. 2C Weighted UniFrac analysis.** The top panel shows a dendrogram demonstrating that pH 3 treated soil was most distantly related from the other samples. However, analysis of the Unifrac metric values, shown in the table below indicates that these communities are not actually significantly different from one another. The UniFrac metric values, which are very close to zero, indicate that all of these samples actually share many OTUs, indicating that their community compositions are very similar.



**Fig 3. Analysis of nitrogenase diversity.** The figure above shows a tree of *nifH* sequences obtained from our clone libraries. Colors here represent various samples: Green indicates pH 5 treated soil, pink indicates pH 3 treated soil, and blue indicates untreated soil. pH 7 treated soil was left out of the analysis because it yielded no useable reads.



**Fig4. Images of enriched organisms.** Shown on the left is the pink colony obtained during enrichment for aerobic nitrogen fixers (pH 3). The panel on the right demonstrates the morphology of the organism at 100x

### *References*

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