

## **Inventory of Wind Cave Biota: Microbial Diversity**

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### **Introduction**

The aim of this study was to examine the microbial diversity within the sediments and soils of Wind Cave at Wind Cave National Park in South Dakota. Broadly defined, the microbes include prokaryotic (Bacteria, Archaea) and eukaryotic (algae, protozoa, and fungi) organisms that are difficult to observe with the naked-eye and less than 100  $\mu\text{m}$ . This report builds on and borrows the background materials submitted in the interim report submitted June 30, 2007 (Moore *et al.* 2007).

Findings from previous investigations at Wind Cave have pointed to detritus based communities of vertebrates and invertebrates, with the carbon being photosynthetically derived (Peck 1959; Jesser 1998; Moore et al 1996; Moore and de Ruyter 2000). Work by Lipari (1997) used direct counts, fluorescent stains and microscopy to study culturable bacteria and fungi. A later study by Chelius and Moore (2004) utilized a molecular biological approach to describe the microbial structure of cave sediment which had been only partially represented by the culturable fraction. From this work it was possible to infer a corresponding ecosystem. The successful application of these molecular techniques to the Rainbow Falls sampling site laid the groundwork for this project.

The purification of DNA from soil and sediment samples, cloning of said DNA and its sequencing could be applied to a larger number of collection sites which would yield information on biodiversity and tourist impact.

### ***Project Goals***

We examined sediment and soil samples from outside and within the cave, and on and off the well-traveled tour and caver routes to meet the following goals:

- 1) Determine the diversity of the microbial (Bacteria) community in Wind Cave in relation to geological gradients and features.
- 2) Determine if tours are having impacts on the microbial community in Wind Cave.

### **Site Locations**

Samples were collected from 9 sites within Wind Cave and 1 site outside the cave within 2 meters of the Natural Entrance (see Figure 1, Map of Biological Sample Sites in Wind Cave). With the exceptions of sites PK 11 and UYA2, all of sites had been sampled for microbial and faunal densities in previous studies (Moore et al. 1996, Jesser 1998, Moore and de Ruyter 2000, Chinn 2001, Horton 2005).

The 9 sites within Wind Cave were categorized by cave level to fit the geologic gradient/features criterion and by proximity to the tour route to fit the tour impact

criterion laid out in Goals 1 and 2, respectively. The site outside the natural entrance was used as a reference site to separate soil bacteria from cave bacteria.

The names of the sites used for sampling were corrected from the previous year end reports, where Sites 5, 6 and 7 were transposed and Site 2 is Pillar/Juice room. Sample identity was confirmed from the original collection tube. Table 1 is the corrected table of the sampling sites, with the status of the analysis for each site.

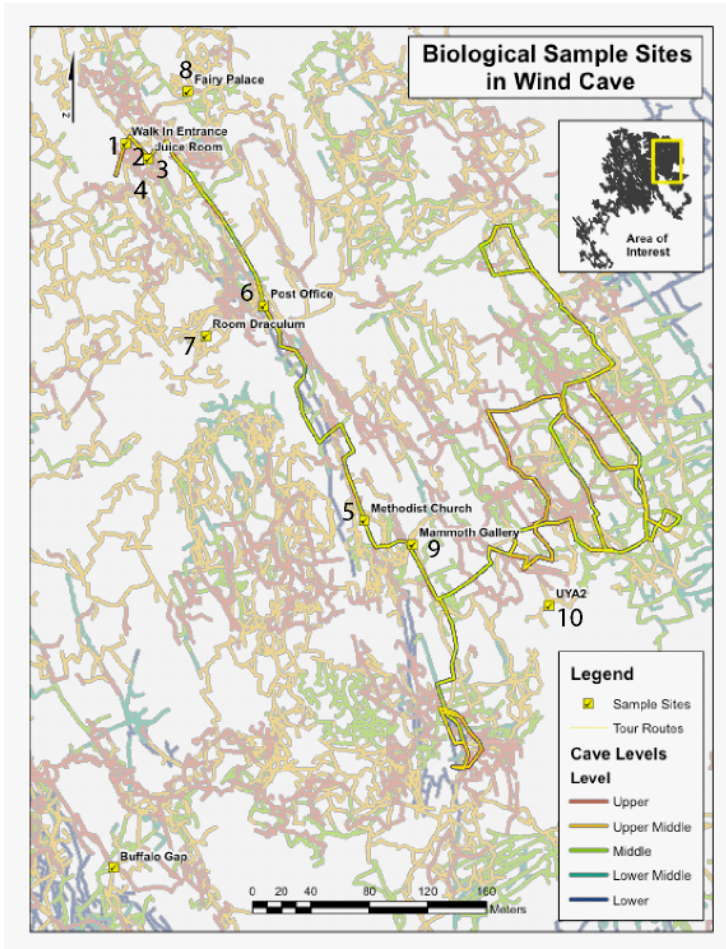


Figure 1. Map of Wind Cave sampling sites. To scale representation of the tunnel system within Wind Cave. Colors of tunnels indicate depth and numbers indicate the sampling sites used in this work.

**DNA Extraction:** DNA was extracted from the soil samples using a Soil DNA extraction kit (MoBio, CA). For each small scale extraction, 0.5 g of soil was used following the manufacturers instructions with the exception that the sample was subject to two rounds of heating at 65°C for 5 minutes each with 5 minutes of bead beating at room temperature. The final elution of 50µl was used directly in PCR.

## Materials and Methods

### *Sampling Soils and Sediments*

Samples were collected aseptically using sterile wooden tongue depressors with sediments being placed into sterile 15 ml tubes. If possible, 5 g of soil was collected from each location and stored at -80 °C until processed. Sediment was collected on and off trail locations to facilitate paired comparisons. Five sediment samples, collected at least 0.5 m apart at random locations within a site were used to compare spatial variability.

### *Microbial Diversity*

The diversity of bacteria was determined from DNA sequences. These DNA sequences were amplified from regions of bacterial 16S rDNA, obtained from the soil samples using the techniques described below.

Table 1. A listing of sample sites that includes the level of the cave that they are located in, their proximity to the tour route, and the date that samples were collected.

<i>Sample Site</i>	<i>Cave Level</i>	<i>Tour Route</i>	<i>Collected</i>	<i>PCR Dilution</i>	<i>Analysis and Status</i>
<b>1. Walk-in Entrance</b>	Outside	N.A.	7/21/05	1:100	DNA, PCR, DGGE, Cloned, Sequenced
<b>2. Pillar/Juice room</b>	Upper	On	7/21/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>3. Juice Room</b>	Upper	Off	7/21/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>4. PK 11</b>	Upper	Off	7/21/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>5. Methodist Church</b>	Upper/Middle	On	7/21/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>6. Post Office</b>	Upper/Middle	On	7/21/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>7. Room Draculum</b>	Upper/Middle	Off	7/21/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>8. Fairy Palace</b>	Upper/Middle	Off	8/11/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>9. Mammoth Gallery</b>	Upper/Middle	Off	8/11/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced
<b>10. UYA2</b>	Upper/Middle	Off	8/11/05	1:10	DNA, PCR, DGGE, Cloned, Sequenced

*Polymerase Chain Reaction (PCR) Amplification of Bacterial 16S rDNA:* Primers used for amplification were PRBA338F Bacteria V3 region (338-358) 5' # AC TCC TAC GGG AGG CAG CAG 3'. PRUN518R Universal V3 region (534-518) 5' ATT ACC GCG GCT GCT GG 3' with # indicating a GC clamp attached to the 5, end of the primer (5' CGC CCG CCG CGC GCG GCG GGC GGG GGG GCG GGG GCA CGG GGG G 3'). This region contains one variable loop of the rRNA. Each PCR amplification was performed in 50  $\mu$ l with 250 nM primers, 200  $\mu$ M dNTPS, 1X PCR buffer and 2.5 U GoTaq polymerase (Madison, WI). Amplifications were performed in an Eppendorf Mastercycler with the following conditions, initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94°C for 30 s, 55 °C for 30 s and 72°C for 30 s with a final extension at 72°C for 7 min and held at 4°C. PCR products were confirmed by electrophoresis on 3% agarose gels before further analysis. Serial dilution of template was used to determine the concentration required for efficient PCR.

Primers used for amplification of the longer amplicon were 27f (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492r (5' GGT TAC CTT GTT ACG ACT T3'). Amplification was set up as above with touch down cycling conditions as follows 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 60°C for 60 s (with a 0.5°C decrease per cycle )and 72°C for 150 s, 20 cycles of 94°C for 30 s, 55°C for 60 s and 72°C for 150 s with a final extension at 72°C for 7 min and held at 4°C. The PCR products for this primer combination were approximately 1200 base pairs in length and were confirmed by electrophoresis on 1% agarose gels.

*Denaturing Gradient Gel Electrophoresis. (DGGE):* DGGE was carried out in a CBS vertical electrophoresis unit (DGGE-1001, Del Mar, CA). PCR products were resolved on a 7.5% polyacrylamide gels in 1X TAE (40 mM Tris, 20 mM acetate 1 mM Na<sub>2</sub>EDTA) in a denaturing gradient of 40-60% (40% formamide and 7M urea representing 100% denaturants). Electrophoresis was performed at 250 V for four hours after an initial low starting voltage of 25V for 20 min, in 1X TAE maintained at 60 °C. Gels were stained with either ethidium bromide (0.5  $\mu$ g/ml) or Sybr Gold (1:10000, Molecular Probes, Eugene, OR) and visualized on a UV trans-illuminator.

*Band Excision:* The PCR products from the different sample sites were excised from the polyacrylamide gels following denaturing gradient gel electrophoresis. If the lanes on

the gels possessed distinct bands corresponding to a single species or strain, the bands were excised from the gel, the DNA extracted, purified, reamplified and sequenced for identification. If the bands within the lanes were in fact a combination of several distinct products, then further dilution and separation was warranted. To separate the products further, each lane corresponding to one sample site was divided into 26 equal segments, from the top to the bottom of each lane. Each segment was excised with a sterile scalpel and DNA was extracted by addition of 0.2ml of sterile water and freezing/ thawing two times. This was used as template for PCR and these products were run on a second DGGE gel. Major bands in each lane were noted and excised and underwent the same process to extract the DNA from the acrylamide. The process of excising bands, extracting and amplifying the DNA, and running a DGGE gel was repeated as needed.

*Cloning of 16S DNA fragments:* PCR products were concentrated on Millipore Montage spin filters and cloned into pGEM-T Easy vector (Promega). Ligations were transformed into JM109 cells and selected on LB amp/IPTG/Xgal plates. Transformants were screened for insertions using vector-based primers for PCR followed by agarose and DGGE gels. Second round PCR with T7 and SP6 primers was used to generate product that could be purified and sequenced.

*DNA Sequencing:* Sequencing was performed by Polymorphic DNA Technologies (Alameda, CA). This facility utilizes robotic liquid handling and automated capillary electrophoresis DNA sequencing to provide cost effective high throughput sequencing.

### ***Physical and chemical characteristics of soils and sediments***

We compared inorganic nitrogen (Ammonium and Nitrate) levels using 2M KCl extractions (Giblin *et al.* 1991). Total organic matter content for each soil will be measured using the loss on ignition (LOI) method (Davies, 1974). Soil pH was measured using a 5:1 solution of water:soil that was measured after 1 hour. Soil texture was measured by shaking soils with sodium hexametaphosphate, separating sand with a 53 $\mu$ m sieve, and taking hydrometer measurements of the soil solution initially and after two hours to determine silt and clay content (Gee and Or 2002).

### ***Statistical Analysis***

Density linkage cluster analysis of sampling sites was performed using the SAS/STAT software using the percentage of represented bacterial groups within each sampling site.

## **Results and Discussion**

Samples were collected from the indicated sites, DNA extracted, amplified by PCR and analyzed by DGGE and sequencing. The different concentrations of DNA and contaminants that might inhibit PCR required differential dilution of samples. Higher concentrations of bacteria and DNA templates would be expected in the soil sample from outside the cave system than inside the cave, requiring a greater dilution to obtain PCR products. The results confirm this, and are consistent with previous results comparing

outside soils to cave sediments that employed plate counts and direct counts of bacteria (Moore *et al.* 1996, Jesser 1998, Chinn 2001, Horton 2005).

Analysis by DGGE revealed that the most complex banding patterns were found with samples taken from either outside the cave, (Site 1) or were closer to the entrance (Figure 2). Sites that were either off trail or further from the entrance displayed banding patterns of less complexity. This was not unexpected but many of the cloned fragments could not be assigned an identity. Although the DGGE gels provided a qualitative examination of the bacterial diversity from the apparent banding patterns, sequencing bands that could be separated and cloned easily did not give an accurate representation of the bacterial diversity within these samples. Furthermore, the separation of individual bands on DGGE gels is determined by base substitutions within the amplicon of interest. This sensitivity is a result of differential melting of the double stranded DNA, which is effectively tethered at one end with the GC clamp. Unfortunately there are physical constraints to the dimensions of the gel apparatus in which this separation occurs and amplicons that can be resolved are relatively short. Consequently, sequence information generated from these PCR products is also concise, making identification more difficult. With these shortcomings it was decided to switch to a set of primers (27f and 1492r, Lane 1991) which generate a longer amplicon and had been successfully used in identification of bacteria from Wind Cave in an earlier study (Chelius and Moore, 2004). As some sequenced data had already been generated for Sites 1-4 the new strategy was applied to the remaining six sites.

Samples for sequencing were grouped together to take advantage of robotic sequencing from 96 well plates which was more cost efficient than single sample sequencing. Sites 1-4, which were cloned from DGGE bands were sent as the first group.

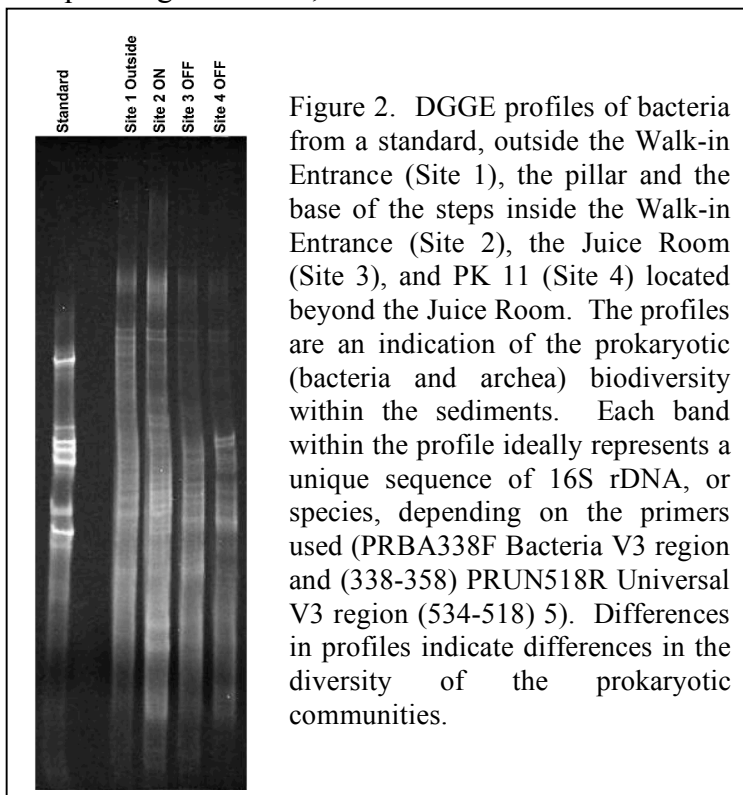


Figure 2. DGGE profiles of bacteria from a standard, outside the Walk-in Entrance (Site 1), the pillar and the base of the steps inside the Walk-in Entrance (Site 2), the Juice Room (Site 3), and PK 11 (Site 4) located beyond the Juice Room. The profiles are an indication of the prokaryotic (bacteria and archaea) biodiversity within the sediments. Each band within the profile ideally represents a unique sequence of 16S rDNA, or species, depending on the primers used (PRBA338F Bacteria V3 region and (338-358) PRUN518R Universal V3 region (534-518) 5). Differences in profiles indicate differences in the diversity of the prokaryotic communities.

Changing to a larger PCR fragment to facilitate more accurate identification of species was tested with Sites 10, with 96 samples sent from this site. For Sites 5 and 6 a total of 96 samples were sequenced (48 each) and for Sites 7, 8 and 9 a total of 192 samples were sent. The sequence data is supplied on a supplemental compact disc (CD). A metafile is included at the end of this report and on the CD describing the data and how it may be accessed (Appendix 1).

When an analyzed sample returned a DNA sequence (>98% of samples submitted) the sequence was compared to those at the

database of the National Center for Biotechnology Information (NCBI) using the BLAST program. Samples that shared 91% or greater homology to database entries were so characterized.

The ClustalW2 program running interactively on the EMBL-EBI website was used to check if sequences were identical (sharing >97% homology) to other sample sequences and to examine phylogram trees based on obtained sequences. Using this program to search for repeated clones was invaluable as it returns a table of scores of similarity between sequences. Characterizing the clones from Sites 5 and 6 manually had suggested that some sequences had been repeated, which was not unexpected due to the nature of the cloning strategy used. However, score tables for these sequences obtained from the ClustalW2 program did not indicate any repeated sequences using the 97% homology cut off. From all the samples from Sites 5 through 10, no repeated sequences were found within each sampling site. Consequently there was no need to plot rarefaction curves for this data as all curves would have displayed a 45 degree slope. Although this investigation has looked at several hundred cloned DNA sequences, it does not represent an exhaustive study. In terms of sampling it would have been easy to increase the number of clones sequenced from each area by approximately ten fold. Financial and time constraints did not facilitate such an extended study. Undoubtedly, if the sample sizes from each site had been increased sample richness would have decreased and rarefaction curves of the diversity would begin to level out. Sequences determined for Sites 1 to 4 were chosen from DGGE banding patterns and picked for the greatest possible diversity and would not be suitable for rarefaction analysis.

Sequenced species fell into fourteen general classes of bacteria. The percentage of clones in each class was calculated for each site examined). Grouping the sequences in this manner facilitates comparison between the sites studied. The groups contain many species but do have some general qualities, some of which are outlined below in Table 2.

There was considerable variety in the groups of bacteria found within the cave and the first hypothesis to be tested was whether humans had a direct impact on the

Table 2. A listing of sample sites and the percentage of clones in each bacterial class at each site.

SITES	1	2	3	4	5	6	7	8	9	10
Actinobacteria	0.0	19.2	11.1	0.0	6.3	47.9	4.2	2.1	8.3	33.0
Bacteroidetes/Chlorobi	13.0	3.8	0.0	0.0	0.0	0.0	2.1	0.0	2.1	0.0
Chlamydiae/Verrucomicrobia	17.4	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0
Chloroflexi	0.0	3.8	0.0	7.7	0.0	2.1	0.0	0.0	0.0	0.0
Fibrobacteres/Acidobacteria	21.7	3.8	27.8	0.0	31.3	6.3	14.6	43.8	8.3	12.5
Firmicutes	0.0	0.0	0.0	0.0	0.0	12.5	4.2	0.0	0.0	0.0
Gemmatimonadetes	4.3	7.7	22.2	0.0	0.0	0.0	0.0	4.2	4.2	4.2
Nitrospirae	0.0	0.0	5.6	0.0	0.0	0.0	4.2	4.2	2.1	2.1
Planctomycetes	0.0	0.0	0.0	0.0	4.2	0.0	4.2	4.2	6.3	0.0
$\alpha$	13.0	15.4	11.1	7.7	4.2	8.3	16.7	2.1	41.7	20.8
$\beta$	4.3	0.0	5.6	0.0	14.6	4.2	14.6	8.3	4.2	2.1
$\gamma$	0.0	11.5	11.1	46.2	10.4	4.2	10.4	16.7	8.3	18.8
$\delta$	0.0	0.0	5.6	7.7	10.4	0.0	10.4	0.0	12.5	6.3
Unclassified	26.1	34.6	0.0	30.8	16.7	14.6	14.6	14.6	2.1	0.0

microbial communities found. Looking at the communities found on and off trail, no significant differences were apparent. However, the presence of absence of tourists is not the only variable in this analysis. Geographical location, in terms of depth and geology almost certainly play an important role in the microbial communities present.

It is interesting to note that Sites 2, 6 and 10 have higher proportions of actinobacteria, which as a group are often associated with decomposition of organic matter and this may relate to the higher levels of nitrate found in these soils. The high proportion of  $\alpha$ -proteobacteria in Site 9 was a trend not found in other sites within the cave, though this soil sample did have the greatest proportion of silt. The  $\alpha$ -proteobacteria contain one-carbon compound metabolizing members, the Rhizobia, that fix nitrogen, in addition to phototrophic bacteria. Site 3, the Juice Room, with sample taken off trail has a higher percentage of clones of Fibrobacteres/Acidobacteria and Gemmatimonadetes groups, which may suggest that there are bacteria utilizing cellulose and minerals as an energy sources respectively.

Its is likely that the presence of human beings traveling on the tour routes and smaller animals found near the entrance do indeed track additional nutrients into the cave. Nevertheless, to utilize this supplemental energy, opportunistic bacteria must compete with established fauna that are suited to the temperature, pH, moisture and mineral content present in the different locations within the cave. Small distances within the cave, in terms of feet and inches can represent very different microenvironments, particularly when considering how veins of minerals can be present or absent or whether water passes through a particular location. Using the percentages of the different groups of bacteria found at each site a cluster analysis was performed to determine which sites grouped together in terms of bacterial diversity. Site 1 was unlike any of the other sites and did not group with any of them (see Figure 3).

From the cluster analysis there is no readily discernable dichotomy into Sites that are on or off trail. This gives weight to the proposition that there has not been a discernable effect on the microbial population caused by humans walking on the trail routes. It is interesting that the two sites that are most similar are Sites 9 and 10. Both areas are off trail in the upper/middle section of the cave and the most similar to these two areas is Site 7. Why the next most similar is Site 3, which is from the Upper part of the cave but is still off trail is difficult to speculate whereas Site 8, which is also off trail and upper/middle is more distant than Site 5 which is on trail.

To aid in the characterization of each of the sites, physical and chemical properties of the sediment taken for the species analysis was performed (Table 3). The range of the pH of the sampled sediments was just over one unit and were a little more alkali than had been noted in a previous study (Chelius and Moore, 2004). However, there was a large range in the nitrate concentrations, up to three orders of magnitude, with the highest levels recorded in Pillar/Juice room and Post Office. Concentrations of nitrate in these areas were even higher than from soil taken outside the cave at the entrance.

Statistical analyses were employed to determine if any of the chemical and physical characteristics were affecting the observed diversity (Figure 4). No correlations could be found with texture class, or nitrate and ammonium concentration. Conversely, a striking correlation was found with percentage organic matter and diversity (Figure 4, bottom left panel).

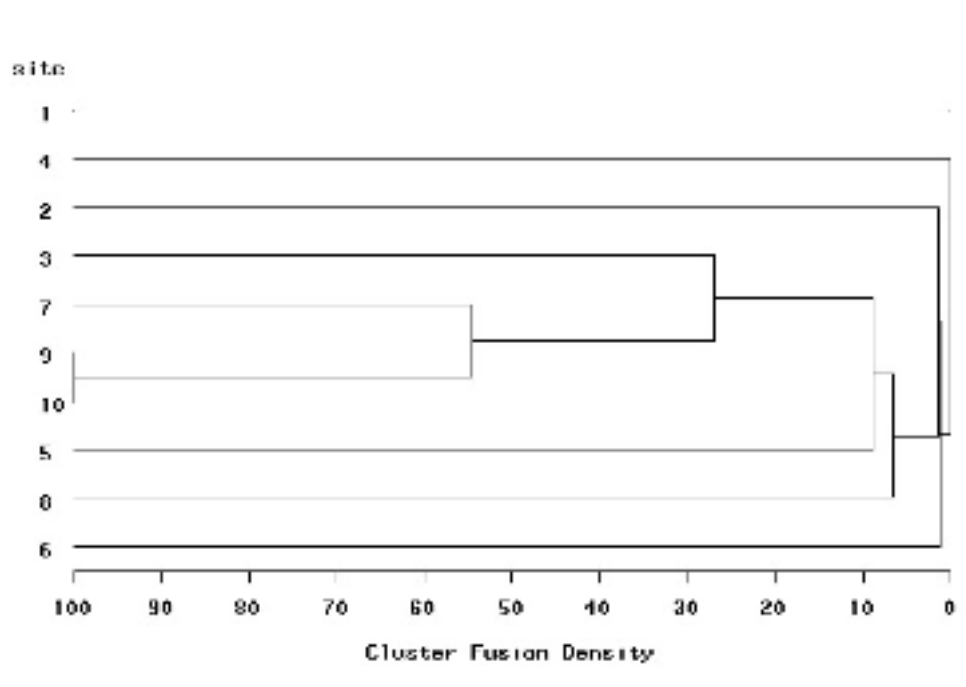


Figure 3. Cluster analysis of sampling sites. Density linkage cluster analysis is using the percentage of represented bacterial groups within each sampling site. The cluster procedure was performed by the SAS/STAT software. Sampling sites that are diversified to a similar extent, branch later from the common root.

Table 3. Physical and chemical characteristics of sampling sites within Wind Cave.

Site	Avg pH	µgNO <sub>3</sub> -N/g dry soil	µgNH <sub>4</sub> -N/g dry soil	Avg %OM*	Texture class
1 Walk in entrance	8.129	12.007	3.253	10.6089	clay loam
2 Pillar/Juice on	9.042	130.396	1.016	6.1095	sandy loam
3 Juice Room off	9.248	15.009	0.518	3.4895	clay loam
4 PKII off	9.214	0.739	0.949	8.2394	clay loam
5 Meth Church on	9.036	0.984	0.362	5.7111	loam
6 Post Office on	9.254	35.251	0.960	7.1323	sandy clay loam
7 R. Drac. off	9.150	0.318	0.736	2.5577	loam
8 FP off	9.140	0.307	1.097	4.7661	sandy loam
9 MG off	9.169	0.346	0.389	2.3706	loam
10 UYA2 off	8.485	47.132	0.645	3.5194	sandy loam

(\* The numbers for avg % organic matter are an order of magnitude higher than previously observed. No experimental errors have been found for this but we believe that the relative magnitudes of values from one site to another are correct).

Data from site 1 was omitted as this area was outside of the cave and skewed the association, but within the cave itself, microbial diversity was inversely related to the presence of organic matter. Points that are towards the y-axis on the left have lower

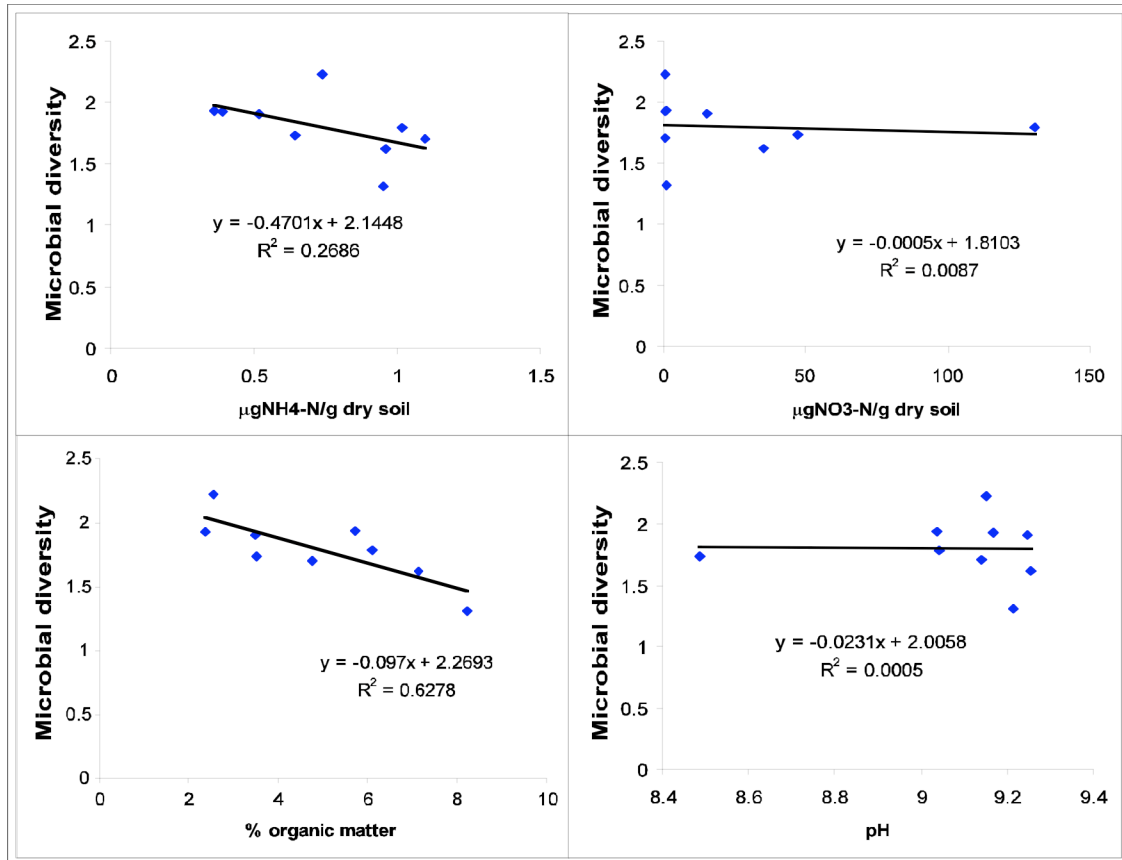


Figure 4. Microbial diversity versus physical and chemical characteristics presented in Table 3. Microbial diversity was defined as a variation of the Shannon Weiner Index,  $H$ , using the percentages

of clones,  $p_i$ , in each  $i$  class ( $i=1, \dots, 10$ ) presented in Table 2 as follows:  $H = -\sum_{i=1}^{10} p_i \ln p_i$ .

amounts of organic matter, more bacterial diversity and are off trail. Site 4, despite being off trail has a higher proportion of organic matter than might be expected but it follows the trend by displayed decrease bacterial diversity.

Finding this negative correlation between the level of organic material and diversity of bacteria present raises some interesting questions. However, this association was subtle and is coupled to the geographical location of the sampling site. This had been suggested earlier in the analysis when Site 4 segregated with Sites 2 and 6 by cluster analysis. In light of the higher organic material content found at Site 4, it was not surprising that it did not associate with other sites that were off trail.

Earlier work had demonstrated that bacterial biomass increases with increased organic inputs (Jesser 1998, Moore and de Ruiter 2000, Horton 2005). Moreover, the sediments from remote regions of the cave were found to host assemblages of microbes characteristic of marine sediments and deep subterranean strata (Chelius and Moore 2004). Our results indicate that the introduction of organic material, presumably from exogenous sources may have a detrimental effect of the natural assemblages of microbes in the cave.

## Conclusions

This study has provided an interesting snapshot into the microbial communities found within Wind Cave. Our initial investigations using DGGE gels indicated that individual sediments did contain dissimilar populations as judged from the differentially migrating PCR products. When these products were cloned and sequenced in the next phase of the work, differences could be noted but it was difficult to postulate what factors were affecting the species diversity. Cloning of longer PCR products from the remaining collections sites in conjunction with chemical analysis of the sediments has provided enough information to hypothesize that organic matter is a driving influence in bacterial diversity. To summarize, our study involved the following phases:

1. Samples collected from 10 Sites
2. DNA purified from samples
3. 16rDNA amplified from DNA
4. 480 clones sent for sequencing
5. Clone sequences phylogenetically analyzed and grouped
6. Physical and chemical characteristics of soil samples determined
7. Statistical analysis indicates diversity influenced by organic carbon

The cave is a dynamic environment. In addition to the movement of animals in and out of the cave, there are changes in the flow of the watercourses in response to surface precipitation and these are in addition to the human visitors to the cave. From these findings, bacterial communities are closely related to the organic material available but they are seemingly independent of nitrogen sources. The novel result is that the input of organic material had a negative effect on the microbial diversity when measured in terms of evenness. Although it is difficult to pinpoint the reasons for the variations in the organic material found at the various sampling sites it would not be unreasonable to assume that traffic from tours is one possible source, with energy input as lint, skin and hair.

A long term study, in which samples could be collected from the same sampling sites over a period of several years and analyzed in a similar fashion, would provide deeper insight into the impact of visitors to this National Park. One might predict that the impact of the tourist trails on the microbial diversity to be cumulative, with the continual introduction of new organic carbon into the ecosystem. This has ramifications for maintaining Wind Cave in a condition so that future generations can share the inspiration and enjoyment of this natural resource. Without the benefit of a more comprehensive study some measures could be employed to reduce the ecological footprint of the tours, including:

- I. Inform visitors of the delicate ecosystem within the cave.
- II. Request that guests contain grooming activities to the visitors center.
- III. Contain foodstuffs to designated areas.

Although this study was of limited size and scope it has provided valuable information regarding the impact of humans on this environment. Only through good tour practices and careful park management can the Park Service personnel at Wind Cave retain the authenticity of this ecosystem.

### Acknowledgements

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## **Appendix I. Wind Cave Meta Data File**

The CD contains the sequencing data obtained from the samples sites in Wind Cave.

Upon viewing the data CD ten folders, each one corresponding to a sampling site are identified, one through ten and by name. Each folder contains the DNA trace files, electropherograms (ABI files) and its corresponding unedited sequence files (SEQ files) for each sample submitted to and returned from the automated sequencer. Although these pairs of files share similar names they differ by the data they contain and the concomitant file type and extension.

The ABI files requires a file viewer, usually associated with software from the sequencing machine manufacturer, commercially available sequence analysis programs or freeware such as BioEdit. The ABI files are 200-400 kilobytes in size. When opened with an appropriate viewer the individual peak heights, width and intensities can be examined. The sequence of the individual bases determined by the software program can be verified against the raw data. An example from the folder for Site 1, Walk in Entrance, would be the first file, A04\_A02\_0298Aplate01\_P1.ab1. The file name has been created by the sequencing facility and is a function of the position of the well within the plate. Some file names also contain a designation JMGB, which was part of the coding used by the laboratory when sending multiple samples, for example from Site 10, UYA2, the file A01\_JMGB-53\_0352Aplate01\_P1.ab1.

The SEQ files can be opened directly with Microsoft Word or similar text editors. The text can be copied and pasted from the word processor into sequence analysis programs, such as those that can be found on the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The SEQ files are 1-3 kilobytes in size. From Site 1, Walk in Entrance, would be the first SEQ file is A01\_JMGB-53\_0352Aplate01\_P1. Often at the beginning of the SEQ file, letters other than the expected A,G, T or C can be found. These letters correspond to the initial detection of the sequencing fragments in which the machine is determining baseline values. When the threshold values have been set, only nucleotide bases are displayed.

The files are all marked with a date and time. These temporal markings indicate when each sample was analyzed by the sequencing facility.