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Nicole Geerdes
University of Northern Iowa

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THE SEARCH FOR LIFE IN EXTREME ENVIRONMENTS: MEASURING DIVERSITY AND QUANTITY OF ARCHAEA IN WIND CAVE NATIONAL PARK

A Thesis Submitted

in Partial Fulfillment

of the Requirements for the Designation

University Honors

Nicole Geerdes

University of Northern Iowa

December 2021

This Study by: Nicole Geerdes

Entitled: The search for life in extreme environments: Measuring diversity and quantity of Archaea in Wind Cave National Park

has been approved as meeting the thesis or project requirement for the Designation

University Honors

12/13/2021	_Dr. Marek Sliwinski_____
Date	Honors Thesis Advisor
_____	_____
Date	Dr. Jessica Moon, Director, University Honors Program

Abstract

Archaea have the potential to play an essential role in their environment at the base of the food chain. In particular, most of the known species in the phylum Thaumarchaea are autotrophs that can fix their own carbon using energy from ammonia oxidation, that is, they produce their own organic molecules from carbon dioxide by burning nitrogen-based fertilizer. Since their discovery, it has been difficult to learn more about these microbes because they do not grow well in pure laboratory culture. Therefore, much of what is known about them comes from analyzing environmental DNA samples. This allowed researchers to discover that Archaea are ubiquitous in soils and are found even in seemingly barren environments such as the subterranean lakes in Wind Caves National Park. This cave system is one of the most complex in the world and provides an analog for some of the other extreme environments that exist in the solar system. By utilizing molecular techniques such as qPCR and Sanger sequencing of cloned PCR products, we quantified the relative number of archaea in the cave system, as well as identified some of the archaeal species based on their 16S rRNA genes. Our qPCR results revealed that there are fewer archaeal 16S rRNA genes inside of the cave than near the natural entrance to the cave. Our preliminary results of the archaeal clone library identified only thaumarchaeal species suggesting this phylum is the most numerous Archaea in the cave system. In terms of spatial distribution, archaeal DNA was found at all levels of the cave including near the surface and in the deep subterranean cave lakes. Future studies will be able to use our collection of environmental DNA samples to quantify and analyze species diversity using additional molecular techniques such as NGS (next generation sequencing).

Introduction

Archaea is the most recently defined phylogenetic domain, added alongside Bacteria and Eukarya in 1977. At the time, little was known about this group of microbes, except that the identified members were strict extremophiles (Woese & Fox, 1977). By using DNA techniques targeted towards Archaea, researchers have since discovered that this elusive group of microbes is actually ubiquitous. Not only can they be found in environments that are extremely hot or acidic, but also in moderate environments such as lakes, oceans, soils, and even on human skin (Brochier-Armanet et al., 2008; DeLong, 1998; Probst et al., 2013).

Initial analysis of Archaea suggested that the domain be divided into two phyla: Euryarchaeota, which included the methanogens and those related, and Crenarchaeota, which included the thermoacidophiles and those related (Woese, 1990). More recent genetic analysis led to the addition of the phylum Thaumarchaeota, a group whose members had branched off of the already established Crenarchaeota phylum (Brochier-Armanet et al., 2008). The three thaumarchaeal subgroups, 1.1a, 1.1b and 1.1c are of special interest because they have been previously identified within cave environments (Chelius and Moore, 2004; Pester et al., 2011; Spang et al., 2010; Zhao et al, 2017).

In 2005, the first Thaumarchaeota was grown in pure culture. A member of group 1.1a., *Nitrosopumilus maritimus*, was isolated from a tropical saltwater tank at the Seattle Aquarium (Konneke et al., 2005). Since then, a few additional Thaumarchaeota have been successfully isolated or grown in highly enriched cultures. The most recent species, *Nitrosarchaeum koreense*, was obtained from agricultural soil using a combination of isolation techniques (Jung

2018; Jung et al., 2011). The ability to obtain pure cultures of archaeal species allowed researchers to begin unraveling the metabolism of the different phylogenetic groups identified through environmental DNA sampling. Many of the cultured Thaumarchaeota are chemolithoautotrophs; they use energy from ammonia oxidation to fix carbon dioxide into an organic carbon source (Zhang et al., 2010; Bernhard, 2010; Jung et al., 2018; Konneke et al., 2011, Tourna et al., 2011). The metabolism of these Thaumarchaeota led researchers to believe that these microbes could play an important role as primary producers, pointing to their ecological significance (Leininger et al., 2006; Daebeler et al., 2012).

Most microbes living in nature are recalcitrant to laboratory cultivation; instead researchers utilize DNA techniques to measure the abundance and diversity of microbial communities (Lloyd et al., 2018). Using such techniques, Ochsenreiter et al. (2003) discovered that the relative abundance of soil Archaea is between 0.5% to 3%. In a study of Wind Cave National Park by Hershey et al. (2018) which focused on sampling of the deep lakes through water filtration, they found approximately 4% of the microbial community is Archaea. A different research group studying Wind Cave focused on the Rainbow Falls region and showed that the majority of archaeal sequences were Thaumarchaeota (Chelius and Moore, 2004).

Caves in other parts of the world have also been studied. Zhao et al. (2017) sampled the Cambrian-dolomite Heshong Cave in China at eight sampling sites (S8-S1), ranging from the cave entrance to ~200 m into the cave, respectively. In some samples they found that the abundance of Archaea was two-fold greater than Bacteria, suggesting that Thaumarchaea perform most of ammonia oxidation in the Heshong cave. Four of these sampling sites were used to generate16S rRNA gene libraries, which revealed differences in archaeal communities. The

diversity was greatest across the phylum Thaumarchaeota in S1 and S2, the two samples farthest from the entrance. These samples had sequences that belonged to all three groups (1.1a, 1.1b, 1.1c). S5 had sequences in two of the three groups, and S8, the sample nearest the entrance, had sequences in only the 1.1b group. All 4 sample sites had sequences that were placed in group 1.1b, making it the most frequently found.

The specific microbes within an environment depend on many physical and chemical characteristics. Both pH and nitrogen concentration affect the thaumarchaeal community composition. Phylogenetically different microbial communities are found in acidic environments compared to environments with basic pH values (Nicol et al., 2008). Additionally, ammonia oxidizing archaea have a higher affinity for ammonia than their competing ammonia-oxidizing bacteria (Martens-Habbena et al., 2009). This means that in low nutrient environments with less ammonia availability, such as deep cave sediment, they have the potential to be more successful.

Methodology

Sample Collection

Samples were collected into sterile containers and transported back to the lab at room temperature. In the lab, samples were stored in a refrigerated cold room at 4°C. For each of the cave lake samples, sediments were separated from the cave water by centrifugation for 10 min. at 20,000 rcf, which was sufficient to produce a clear supernatant. The resulting pellets were used for DNA extractions. Samples of paloefill (dry cave sediment) and mineral crusts were used directly for DNA extractions.

DNA extraction

DNA was extracted from a 0.20-0.25 g subsample of each pelleted cave sediment using either the DNeasy PowerBiofilm kit (Qiagen, 24000-50) or the DNeasy PowerLyzer PowerSoil Kit (Qiagen, 12855-100) following the manufacturer's protocol. To determine whether the DNA was of sufficient purity for downstream molecular analysis, extracted DNA was then tested as template in endpoint PCR using GoTaq Green MasterMix (Promega, M7122) in 25 µL reactions. Each PCR reaction was 12.5 µL of GoTaq Green MasterMix, 9.25 µL nuclease free water, 1.25 µL 8mg/mL BSA, 1 µL 10 mM forward primer, 1 µL 10 mM reverse primer and 1µL DNA template. The PCR touch-down cycling protocol for both primer pairs A109F-519uR and 515F-806R was as follows: 95℃ for 2 minutes followed by 20 cycles of 95℃ for 30 seconds, 62℃ (decreasing 0.5℃ per cycle) for 30 seconds and 72℃ for 90 seconds followed by 25 cycles of 95℃ for 30 seconds, 52℃ for 30 seconds and 72℃ for 90 seconds. A final elongation step of 10 minutes at 72℃ was then followed by a 12℃ hold. DNA extractions were quantified on a Biotek Synergy H4 Microplate reader using Quantifluor dsDNA system (Promega, E2671) and analyzed using the Gen5 version 1.10 software. A negative control and positive control were also processed through the DNA extraction kits. The negative controls ensured that positive results were not caused by contaminants from the DNA extraction. The positive control represented the possible human contaminants that could have been obtained during sampling. Further description of the positive and negative controls can be found in Table 1.

qPCR

A standard curve-based qPCR assay was performed to quantify the relative amount of archaeal 16S rRNA genes and bacterial 16S rRNA genes using primers A109F-519uR and 27F-519uR, respectively. 10 µL reactions were run on a Bio-Rad C1000 Touch Thermal Cycler, consisting of 5 µL ThermoFisher 2x PowerUp SYBR Green Master Mix (Thermo Fischer Scientific., A25742)

0.5 µL of each 10 µM primer, 3 µL of nuclease free water and 1 µL of template. Cycling conditions consisted of: 50℃ for 2 minutes, 95℃ for 2 minutes, followed by 40 cycles of 95℃ for 15 seconds, 55℃ for 15 seconds, 72℃ for 90 seconds. Following qPCR cycling, a continuous melt curve was performed. Results were analyzed using QuantStudio Version 1.5.1. All qPCR reactions were completed in triplicate following MIQE guidelines (Bustin et al., 2009). A sample including *Escherichia coli* genomic DNA was amplified with 27F-519uR primer pair to generate bacterial standards, and WI211, a plasmid constructed previously, was amplified using the A109F-519uR primer pair to generate archaeal standards following the previously described endpoint PCR protocol (Swanson & Sliwinski, 2013a). Prior to performing serial dilutions of PCR amplicons, PCR product was purified using the Wizard SV Gel and PCR clean-up kit (Promega, A9281). Purified PCR products were quantified using the Biotek Synergy H4 microplate reader with the Quantifluor dsDNA system (Promega, E2671) and analyzed using the Gen5 version 1.10 software before performing serial dilutions for standard curve analysis.

PCR cloning and Sequencing

Generation of PCR products was achieved using the same methods for end-point PCR described above with archaeal specific primer forward primer A109F and 519uR reverse primer. Before cloning, the PCR amplicons were visualized on a 1.5% agarose gel to confirm that a singular discrete band was present at the expected size of 386 nucleotides. PCR products were then purified using the Wizard SV Gel and PCR clean-up kit (Promega, A9281). Cloning of the PCR product was performed using the pGEM-T easy vector system (Promega, A1360) following the manufacturer's protocol. Transformants were generated using the manufacturer’s suggested electroporation protocol for the GenePulser Xcell (Bio-Rad). Initial blue-white screening of transformants was performed and presumptive positive transformants were further analyzed

using colony PCR. To prepare template for colony PCR, sterile pipette tips were used to transfer a small amount of each colony into 25 µL of DNA grade water followed by incubation at 95℃ for 10 minutes. 1 µL of the incubated colony mixture was used as template for end-point PCR using the previously described protocol with an annealing temperature of 48℃ for primer pair m13F-SP6. A subset of chosen colony PCR products were then purified using the Wizard SV Gel and PCR clean-up system (Promega, A9281) in preparation for Sanger sequencing. Sequencing was performed by the Iowa State University DNA facility using their stock universal 21M13 Primer ("U") on an Applied Biosystems 3730xl DNA Analyzer. Sequences were aligned using BLASTn analysis (https://blast.ncbi.nlm.nih.gov/). A pairwise comparison of WCNP sequences to known thaumarcheal clades 1.1a, 1.1b and 1.1c was also completed using BLASTn.

Results

Sampling

To collect samples at Wind Cave National Park (WCNP) Figure 1, we conducted a field expedition over the first few weeks of May 2021. In brief, a variety of scientists and undergraduates formed groups to explore different cave areas over multiple days to collect replicate samples from the five sites as described in Table 1 and pictured in Figure 1. Representative pictures of samples which were collected can be seen in Figure 2. Two of these trips were to the deepest part of the cave. For a more detailed cave map, please visit the NPS interactive map (https://www.nps.gov/subjects/caves).

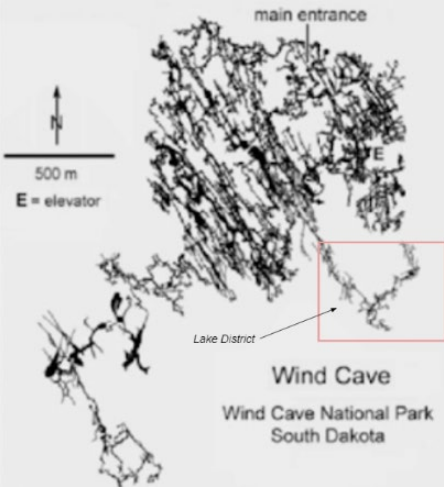


Figure 1. Wind Cave Map
The Lake District on the map represents the area within the lower cave system which contains What The Hell lake and Calcite lake.

Sample	Sample Site / Cave Location	Description	Label on Sample Tube from Field Expeditions
OUT1[1]	Outside Natural Entrance	Outside Cave System	#1 Outside Natural Opening Wind Cave National Park, Geerdes 5/14/21
OUT2[1]	Outside Natural Entrance	Outside Cave System	#2 Outside Natural Opening Wind Cave National Park, Geerdes 5/14/21
RSR1	Rat Scat Room	Dry upper cave	9 MKS 5/12/21
RSR2	Rat Scat Room	Dry upper cave	8 MKS 5/12/21
RSR3	Rat Scat Room	Dry upper cave	7 MKS 5/12/21
RSR4	Rat Scat Room	Dry upper cave	6 MKS 5/12/21
RSR5	Rat Scat Room	Dry upper cave	5 MKS 5/12/21
RSR6	Rat Scat Room	Dry upper cave	4 MKS 5/12/21
RSR7	Rat Scat Room	Dry upper cave	3 MKS 5/12/21
RSR8	Rat Scat Room	Dry upper cave	2 MKS 5/12/21
RSR9	Rat Scat Room	Dry upper cave	1 MKS 5/12/21
RSR10	Rat Scat Room	Dry upper cave	14 MKS 5/12/21
RSR11[1]	Rat Scat Room	Dry upper cave	15 MKS 5/12/21
RSR12[1]	Rat Scat Room	Dry upper cave	16 MKS 5/12/21
SL1	Silent Lake	Wet sediment from upper cave	3 silent lake 5/10/21 Perez
SL2	Silent Lake	Wet sediment from upper cave	3 silent lake 5/10/21 Perez

SL3	Silent Lake	Wet sediment from upper cave	2 Flow pool 5/10/21 Perez
SL4	Silent Lake	Wet sediment from upper cave	1 bottom flow pool 5/10/21 Perez
WTH1	What The Hell Lake	Wet sediment from lower cave	Calcite Raft near peninsula #1 Geerdes 5/11 WTH, 5A NRG p. 2-3
WTH2	What The Hell Lake	Wet sediment from lower cave	Shore Opp. Peninsula WTH #1, 4A 5/11 NRG p. 2-3
WTH3	What The Hell Lake	Wet sediment from lower cave	Before waterfall #1 Geerdes 5/11 WTH, 2A NRG p. 2-3
WTH4	What The Hell Lake	Wet sediment from lower cave	WTH Standing Pool to the R. peninsula #1, 1A 5/11/21 NRG p. 2-3
WTH5	What The Hell Lake	Wet sediment from lower cave	WTH Standing Pool to the R. peninsula #2, 1B 5/11/21 NRG p. 2-3
WTH6	What The Hell Lake	Wet sediment from lower cave	Before waterfall #2 Geerdes 5/11 WTH, 2B NRG p. 2-3
WTH7	What The Hell Lake	Wet sediment from lower cave	Shore Opp. Peninsula WTH #2, 4B 5/11 NRG p. 2-3
WTH8	What The Hell Lake	Wet sediment from lower cave	Calcite Raft near peninsula #2 Geerdes 5/11 WTH, 5B NRG p. 2-4
CL1	Calcite Lake	Wet sediment from lower cave	7 5/14/21 Calcite Perez
CL2	Calcite Lake	Wet sediment from lower cave	3 5/14/21 Calcite Perez
CL3	Calcite Lake	Wet sediment from lower cave	1 5/14/21 Calcite Perez
CL4	Calcite Lake	Wet sediment from lower cave	2 5/14/21 Calcite Perez
CL5	Calcite Lake	Wet sediment from lower cave	4 5/14/21 Calcite Perez
CL6	Calcite Lake	Wet sediment from lower cave	5 5/14/21 Calcite Perez
CL7	Calcite Lake	Wet sediment from lower cave	6 5/14/21 Calcite Perez
CL8	Calcite Lake	Wet sediment from lower cave	9 5/14/21 Calcite Perez
CL9	Calcite Lake	Wet sediment from lower cave	8 5/14/21 Calcite Perez
NEG-	Negative control	Sterile sand and water mixture	
HUM+	Human contaminant control	Sand and water handled scientists	

Table 1. Sample Descriptions

[1] DNA samples extracted with DNeasy PowerLyzer PowerSoil Kit (Qiagen, 12855-100), all other samples were extracted with DNeasy PowerBiofilm kit (Qiagen, 24000-50).

Negative control consisted of 200 µL of water from a sterile sand and sterile water mixture.

Human contaminant control consisted of the same negative control spiked with human microflora.

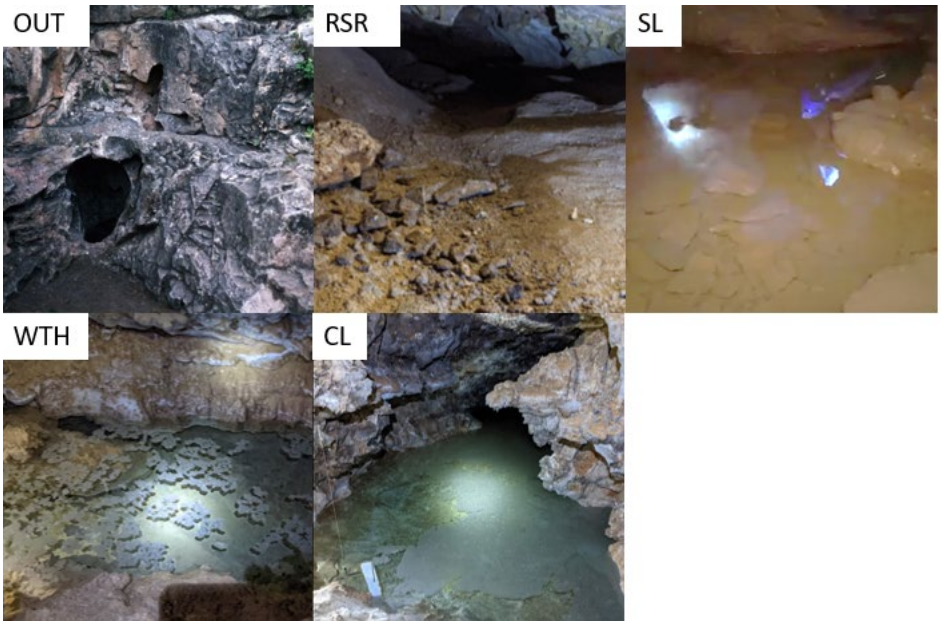


Figure 1. Sampling Sites
The sampling sites are designated as: OUT (outside natural entrance), RP (rat pack area), SL (silent lake), WTH (what the hell lake), CL (calcite lake). Samples outside the natural entrance were collected from the soil next to the natural opening to Wind Cave. The rat pack area and silent lake are considered part of the upper cave system. What the hell lake and calcite lake are considered part of the lower cave system.

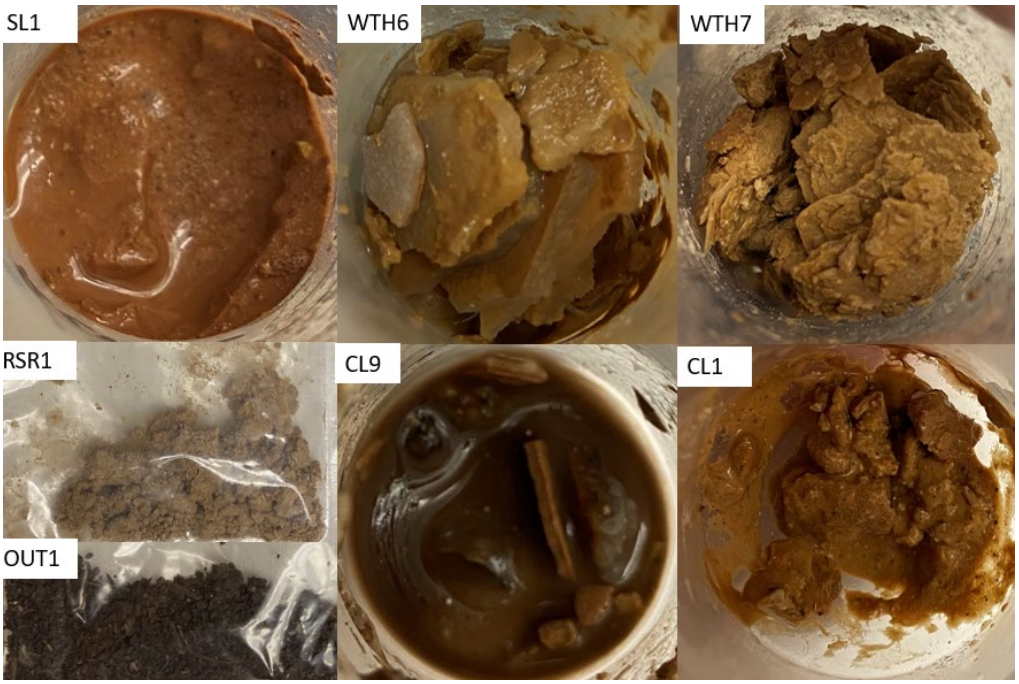


Figure 2. Images of sediment pellets and dry samples
The sample labels are designated as: OUT (outside natural entrance), RP (rat pack area), SL (silent lake), WTH (what the hell lake), CL (calcite lake). For scale, the diameter of the conical centrifuge tubes in the images is approximately 27 mm.

DNA extraction and amplification

DNA was extracted from all samples and quantified using Abs 260/280. The DNA concentration was typically below the limit for accurate results which is 10 ng / μL (data not shown). However, all DNA samples were at concentrations high enough to be detected by end-point PCR with a standard polymerase, Promega GoTaq. The negative control consisted of sterile water mixed with sterile sand and was processed at the same time using the same reagents as the cave samples. After an aliquot of the negative control was taken for DNA extraction, the same beaker of sterile sand / water was handled in the lab to add human contaminants typical of the collection process in Wind Cave. I rubbed my fingers through the sand to add human epidermal cells, and I rinsed my mouth with drinking water, which was added to the beaker to add buccal cells and human microflora. Most of the environmental DNA samples produced dark bands visible on an agarose gel stained with ethidium bromide, while a minority produced band intensities that were barely above the limit of detection (Figure 4). All environmental DNA samples produced bands that were distinct from the negative control which did not produce a visible band. Some samples showed extra bands that do not match the expected size. We have observed this type of pattern in preliminary tests with these primers when there is a low concentration of target template. For instance, when using these archaea-specific primers in a reaction with only non-target bacterial DNA template (*E. coli*) bands were produced as shown in Figure 3.

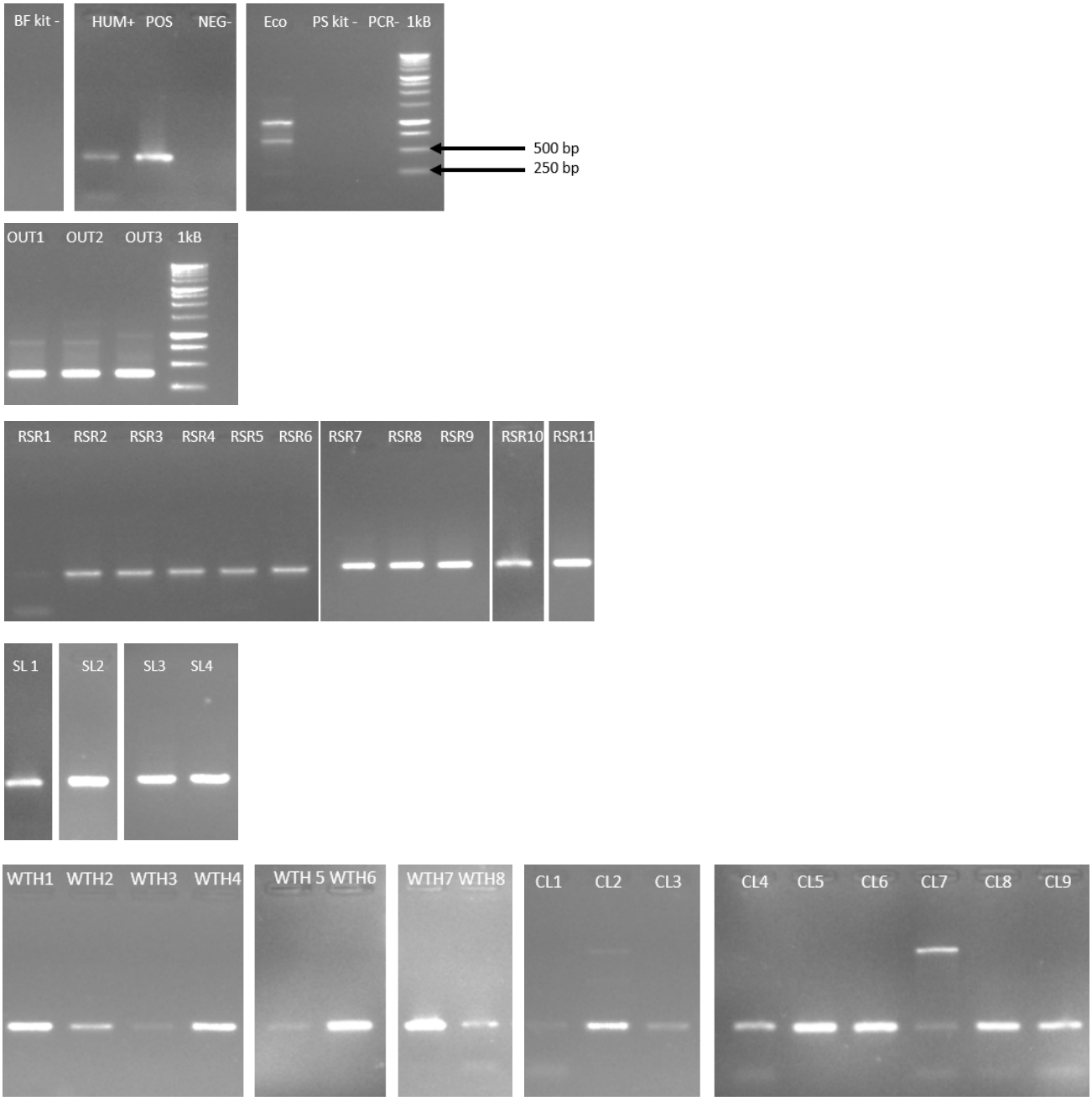


Figure 3. Endpoint PCR using archaeal-specific primers A109F-519uR

All environmental DNA templates amplified archaeal sequences. Unaltered gel images are in Supplemental Figures. Labels used in this figure are described in detail in Table 1. Additional labels are: (Eco) = E. coli DNA as template in PCR to check for specificity of primers; (PS kit -) = DNA grade water extraction via DNeasy PowerLyzer PowerSoil Kit (Qiagen, 12855-100) to ensure kit reagents are not contaminated; (BF kit -) = DNA grade water extraction via DNeasy PowerBiofilm kit (Qiagen, 24000-50) to ensure kit reagents are not contaminated; (PCR -) = negative PCR control of DNA grade water to ensure that PCR reagents are not contaminated; (POS) = positive PCR control of previously amplified DNA.

Relative abundance of archaeal 16s rRNA genes

We selected a subset samples to measure the number of microbial templates in different areas of Wind Cave National Park (WCNP). The samples chosen for qPCR analysis were the following: OUT2, RSR2, SL1, WTH6, WTH8, CL9, HUM+ (see Table 1 for descriptions). The standard curve produced for the archaeal qPCR analysis had a R^2 value of 99.59% (Figure 4). The sample collected outside the cave had more Archaea than any of the cave samples, suggesting a decrease in abundance of Archaea within the cave system (Figure 5). The standard curve produced for the bacterial qPCR analysis had a R^2 value of 99.48% (Figure 6). Similarly, the sample collected outside the cave had more Bacteria than any of the cave samples. When comparing upper cave samples to lower cave samples, there were more copies of both archaeal and bacterial 16s rRNA genes in the upper portion of the cave, suggesting microbial life abundance decreases in the deepest portions of the cave. Interestingly, the human contaminant sample showed bacterial numbers similar to some of the environmental samples, while the archaeal numbers were lower than any of the environmental samples.

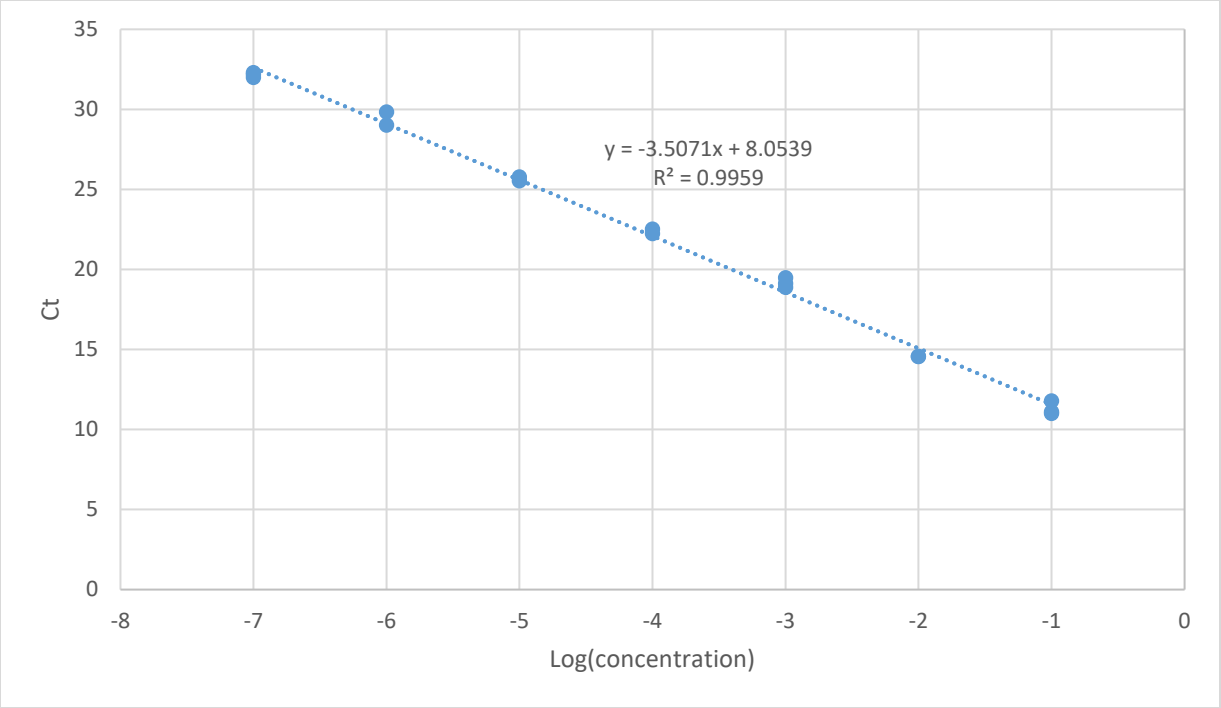


Figure 4: Archaea qPCR Standard Curve

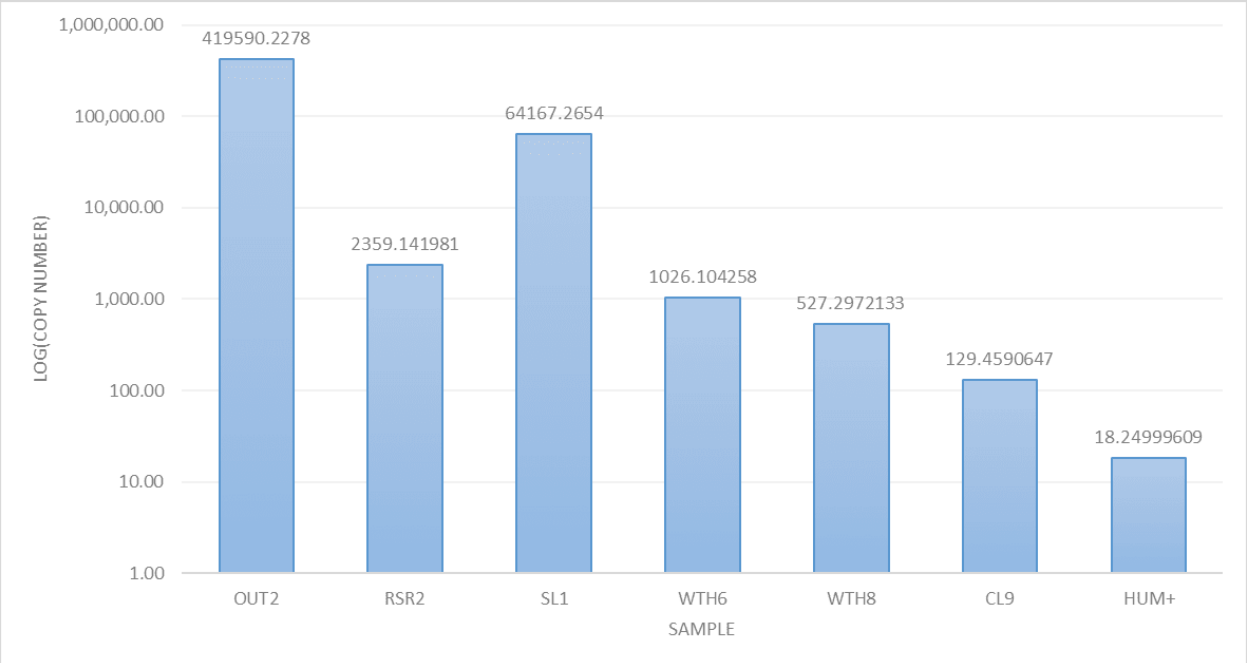


Figure 4. Comparison of archaeal 16S rRNA copy numbers in Wind Cave
See Table 1 for sample descriptions.

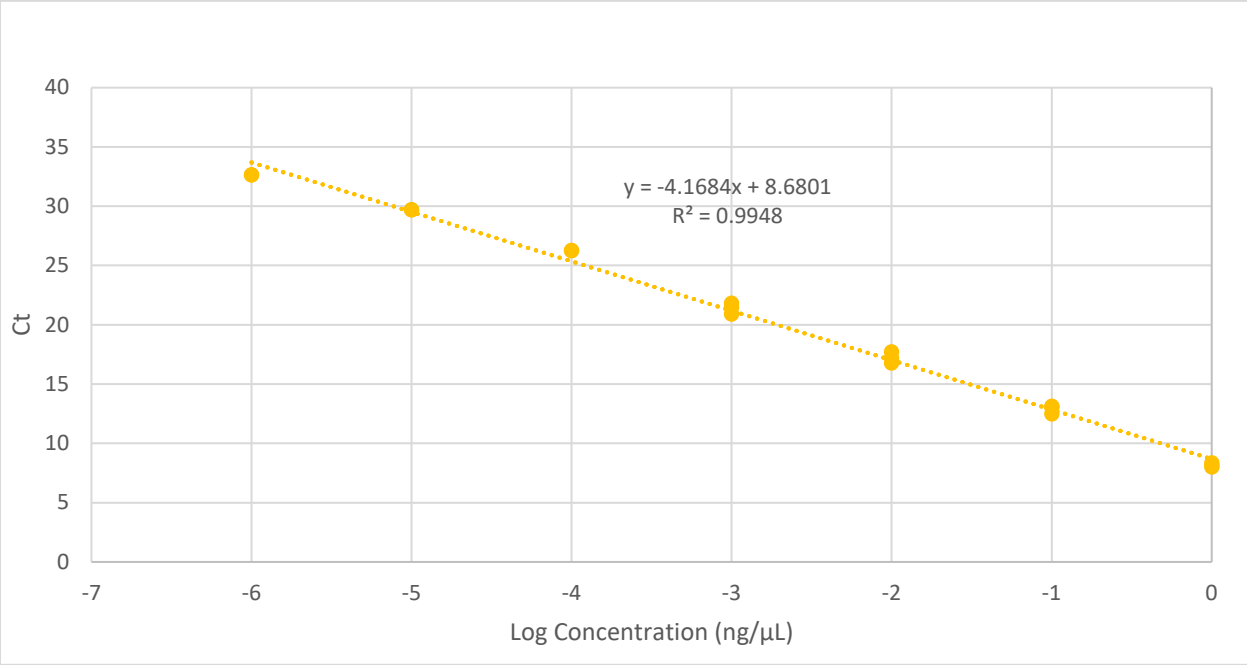


Figure 5. Bacteria qPCR Standard Curve

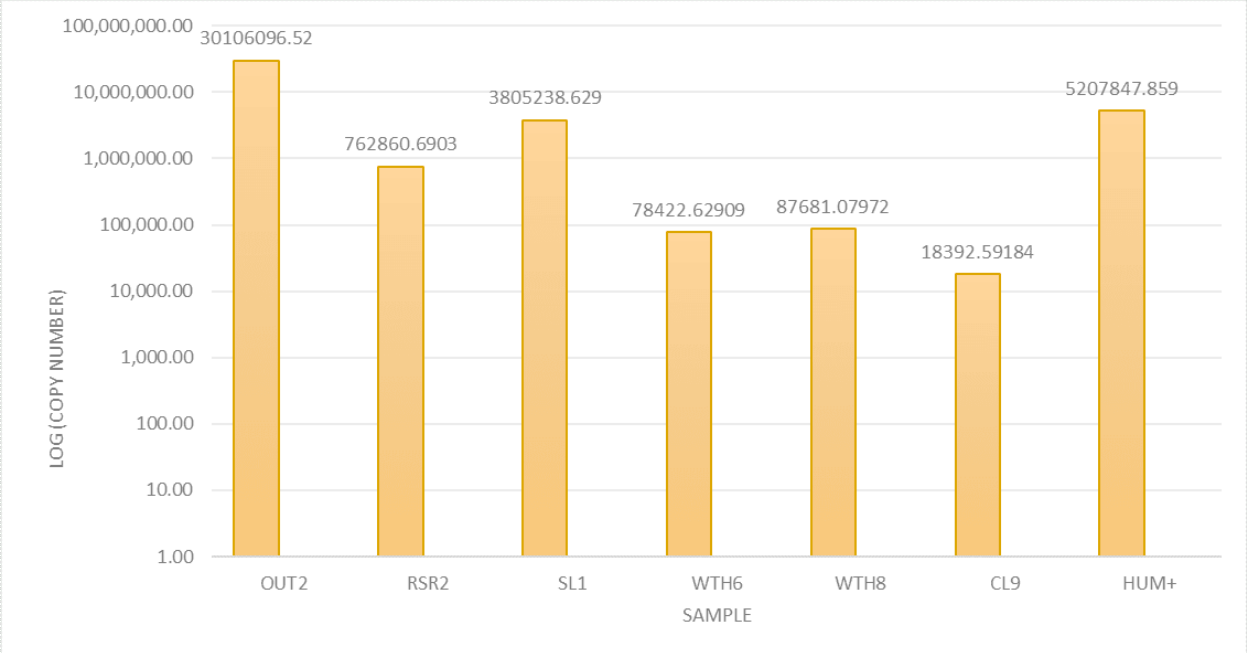


Figure 6. Comparison of bacterial 16S rRNA copy numbers in Wind Cave
See Table 1 for sample descriptions.

Archaeal Community Composition

To measure archaeal species diversity, we created a clone library using a subset of our PCR products that were amplified with A109F-519uR. The environmental DNA samples included: RSR3, SL2, WTH1, CL1. BLAST results showed that all clones had 100% sequence identity to known Thaumarchaeota. BLAST pairwise alignment results also showed that clones from calcite lake and silent lake were most closely related to thaumarchaeal clade 1.1b. Clones from WTH lake and the rat pack area were most closely related to thaumarchaeal clade 1.1a.

	Percent (%) Identity		
Clone	1.1b [1]	1.1a [2]	1.1c [3]
RSR3	81.1	90.7	79.4
SL2	87.6	78.9	85.7
WTH1	81.7	89.9	81.2
CL1	93.8	79.4	83.8

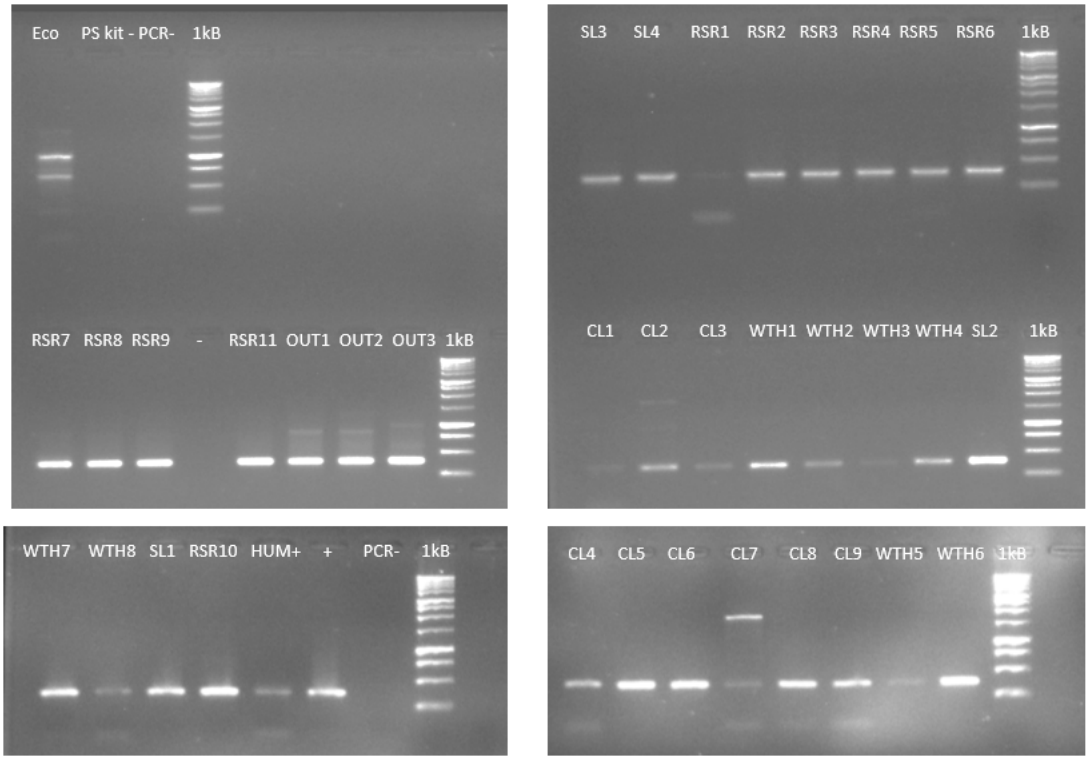
Table 2: BLASTn results for pairwise alignment analysis
[1] aligned to GenBank Accession U62811.1
[2] aligned to GenBank Accession NR_102913.1
[3] aligned to GenBank Accession GQ141959

Discussion

Quantitative analysis using qPCR showed that the abundance of microbial life in the cave decreases in comparison to microbes living at the surface. One possible explanation is that there are fewer and/or less energetic nutrients within the cave. This is in line with the observation that the deepest part of the cave is inhabited by the fewest microbes.

All archaeal clones were closely related to known Thaumarchaeal species. As found in previous research completed in WCNP and other caves, Thaumarchaota is present and likely dominates the archaeal community (Hershey et al., 2018; Chelius and Moore, 2004; Zhao et al., 2017). It is of notice, that CL clone had 99% identity to WCA6 (GenBank Accession No. AY217525.1) an uncultured Thaumarchaeota from WCNP as well as S4-1 (GenBank Accession No. KF053861) an uncultured Thaumarchaeota from Heshang Cave (Chelius and Moore, 2004;). RP clone had 100% identity to clone N10-D10 (GenBank Accession No. HE796303) found in an alpine lake as well as 99% identity to clone 135 (GenBank Accession No. FJ718987) isolated from South Dakota mine water. SL clone had 100% identity to clone HSC-1-12 from Heshang Cave (JX436832), as well as 100% identity to FL0428A_S89 (GenBank Accession No. FJ716363), another cave isolate. WTH clone had 99% identity to clone WCA19 (GenBank Accession No. AY217520), and 98% identity to WCA51 (GenBank Accession No. AY217530.1) and WCA17 (GenBank Accession No. AY217521.1), all isolated from WCNP. From this data, it is likely that Thaumarchaea make up a large percentage of the total archaeal community. Despite this, further clone library development and sequencing must be done to determine community composition at each sampling site to completely understand the diversity within the phylum throughout WCNP.

Supplemental Figures



Supplemental Figure 1: Unaltered Endpoint PCR using archaeal-specific primers A109F-519uR

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