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In Situ Studies of Limestone Dissolution in a Coastal Submarine Spring

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by

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Abstract

Limestone dissolution in karst environments is likely due to geochemistry of the water, the actions of microbial communities, and the effect of water flow. We explored the rate of limestone dissolution and will examine here the microbial communities associated with the limestone. A conduit within the brackish cave, Double Keyhole Spring, on the coast of central west Florida was the site of the experiment. PVC pipes (5cm x 16cm) were filled with crushed limestone that was screened to a 1.9cm – 2.54cm size range. There were three treatments (5 replicates each): Control - sealed autoclaved controls with limestone and conduit water; Low Flow – sealed at one end, with a screen on the other so water contacts the limestone but cannot flow through; High Flow – screen mesh at both ends to allow the flow of conduit water over the limestone in the tube. After 9 months, the samples were retrieved. The Controls showed a loss of 0.33% ± 0.10, Low Flow samples showed a loss of 1.63% ± 0.71, and High Flow samples lost 2.28% ±0.29. Other studies in freshwater conditions found an average mass loss of 2.25% over the same time period under conditions similar to the High Flow sample in this experiment. Q-PCR and LH-PCR were used to estimate microbial density and species richness. The microbial community growing on the limestone samples were found to be significantly different from sediment or water column samples in both diversity and richness. The conclusion of this study is that the archaeal community growing on the limestone is the main biological driver of limestone dissolution in Double Keyhole Spring.

Chapter One: Introduction

Karst is a landscape dominated by limestone bedrock that has been shaped by corrosion due to water flow. Approximately 11.2% of the Earth's surface is karst, constituting about 15 million km^2 (Durr et al., 2005). The dissolution of karst limestone is driven by water. Carbon dioxide (CO_2) from the atmosphere and interstitial soil spaces dissolves in water and forms a chemical equilibrium between water and carbonic acid, a weak acid: $H_2O + CO_2 \leftrightarrow H_2CO_3$. The carbonic acid-laden water flows over karst, dissolving the limestone into calcium ions and carbonate ions: $CaCO_3 + H_2CO_3 \leftrightarrow Ca^{2+} + 2HCO_3^-$ (Buhmann et al., 1987). This process forms cave conduits and passages; CO_2-laden water flows into cracks and fissures in limestone rock, slowly widening them by corrosion (sometimes erosion) and eventually making them large enough to form caves (Palmer, 1991). Other acids such as sulfuric acid form by a reaction between hydrogen sulfide, oxygen and water, also causing limestone dissolution and cave formation. This sulfuric acid dissolves the calcium carbonate to form gypsum and carbon dioxide : $H_2SO_4 + CaCO_3 + 2H_2O \leftrightarrow (CaSO_4 \cdot 2H_2O) + CO_2 + H_2O$ (Duchene & McClean, 1989).

The dissolution rate of limestone to form cave conduits has been estimated to be 0.01-0.1cm of wall retreat per year on average (Palmer, 1991). However, this rate can be affected by temperature, salinity, and topsoil makeup. Higher temperatures decrease the solubility of carbon dioxide in water, which would reduce the dissolution rate of limestone, but warmer areas of the Earth can have more dense plant growth, which increases the CO_2 concentration in the soil, increasing the CO_2 concentration in the water driving the reaction (Palmer, 1991). Water salinity

can also affect the solubility of calcium carbonate. Salt water can dissolve more calcium carbonate than fresh water (Smith et al., 1968). Once water is saturated with dissolved calcium carbonate, no more calcium carbonate can dissolve, though there is an exception. Two bodies of water, independently saturated, but with different chemical properties can dissolve additional limestone when combined through the mixing effect (Plummer, 1975). Due to the dissimilar solubilities of calcium carbonate in water of different chemical properties, when they are mixed, the relative saturation of calcium carbonate in the solution is decreased, thus there is an increased capacity for limestone dissolution. Differences in CO_2 concentration, salinity, pH, temperature and ionic strength can result in an increased capacity for limestone dissolution when two saturated water bodies mix (Fig. 1).

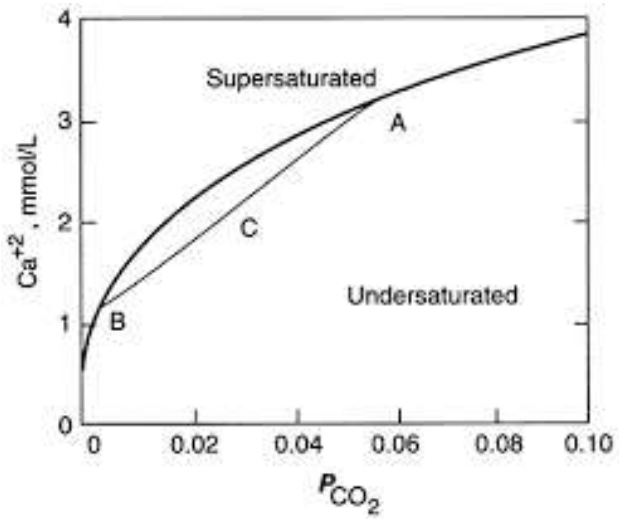


Figure 1: Effect of mixing water of different partial pressures of carbon dioxide on the solubility of calcium carbonate

Just as the dissolution rate has been extensively researched, so has the presence of microbes in limestone (Pronk et al., 2008; Sulu-Gambari, 2011; Gray & Engel, 2012). Flooded cave systems are known to be typically low nutrient environments (Saiz-Jimenez & Groth, 1999). This is mostly due to the lack of photosynthesis in the absence of sunlight. Cave

environments are mostly driven by autophagy microbes and some invertebrates consuming organic material that washes into the cave system during flood events (Saiz-Jimenez & Groth, 1999). Many cave organisms use this organic matter as their source of energy and organic carbon (Barr, 1985). However, detritus washed into the cave system provides a very limited energy source. The oligotrophic environment in most flooded karst caves harbors organisms with alternative energy strategies (Northup & Lavoie, 2001). Chemolithoautotrophs, which fix carbon dioxide and meet their energy requirements by oxidizing inorganic electron donors, are common (Gold & Margulis 1999). Methanogens and iron oxidizing microbes are also common residents in flooded cave environments (Barton & Northup, 2007). Since the cave systems are low in both organic carbon and oxygen, organisms suited for harsh environments are able to compete in the ecosystem. Many microbes in these extreme environments produce acids such as hydrogen sulfide as metabolic waste (Barton, 2010). This waste, in turn, makes the flooded cave environment even more harsh.

One alternative source of carbon for microbial metabolism is the calcium carbonate in limestone in which the cave is formed. The involvement of bacteria in the precipitation of calcium carbonate was first described in marine bacteria (Drew et al., 1913). Drew found that bacteria growing on coral reefs produce acid to dissolve the coral limestone in order to use the carbonate as a carbon source in this reaction: $H_2SO_4 + CaCO_3 \leftarrow\rightarrow Ca^{2+} + SO_4^{2-} + H_2O + CO_2$. Some bacteria use ammonia instead of sulfuric acid to utilize coral limestone. Numerous studies have been done on microbial weathering of carbonate rocks (Rodriguez-Navarro et al., 2012). Once the carbon has been released from the calcium carbonate it can be taken into the bacteria and fixed. One study found some microbial mats capable of boring into limestone bedrock in order to obtain the calcium carbonate for nutritive material (Lian et al., 2008). These studies

were mostly conducted in the laboratory environment under controlled conditions. Few studies have utilized the natural environment as the setting for researching the association of bacterial activity and limestone dissolution (Ascaso et al., 2002; Berthelin 1988).

There have been studies to link other types of microorganisms with limestone dissolution. For example, the role of microalgae growing on exposed surface limestone was studied and it was found that the limestone dissolution rate in Hongfeng Lake in China was directly proportional to the density of microalgae living there (Xie & Yanyou, 2013). Another research study investigated the effect of fungal mycelia and acidic excretion on limestone dissolution rate (Li et al., 2008). They found that the fungus of interest was not only using the limestone rock as an anchor to grow on, but the mycelia of the fungus were excreting an acid in order to dissolve the limestone and absorb the calcium carbonate. There have been studies of archaea in limestone caves but they focused on hydrogen sulfide-driven caves and other high acid cave environments. These studies have characterized the archaea species found in these environments as detritivores (Chelius & Moore, 2004; Chaudhary et al., 2009; Nold et al., 2010). However, studies of archaea in soil and groundwater have found potential to dissolve limestone (Danielopol et al., 2000; Hansel et al., 2008). Due to the difficulty of culturing archaea species in the lab, little work has been done on the process of archaeal limestone dissolution or what metabolic process might be used.

The focus of this study is on the role of microbial communities in the dissolution rate of limestone. The goal was to learn how much of an effect, if any, the microbial community has on the dissolution rate of limestone in a brackish, coastal cave of west-central Florida. An in-situ method was chosen in order to minimize human influence on the study and to account for unknown variables that may not be able to be recreated in a laboratory setting. The experimental

setup was designed in order to standardize all samples while maintaining natural conditions as much as possible. Certain nutrients were chosen to be measured as part of this study due to their association with microbial activity and limestone dissolution in previous studies (Portillo et al., 2008).

Chapter Two: Materials And Methods

2.1 Site Description

Double Keyhole Spring is located in a karst estuary approximately 1 km south of the town of Aripika on the shore of the Gulf of Mexico in central Florida. The large amount of brackish discharge (up to 2,576 L/s with an average of 918 L/s) flowing from Double Keyhole Spring forms the karst estuary. The hydrological, geochemical, and physical characteristics have been previously described (Menning, 2015). The cave is classified as a brackish, karstic cave with high iron content. This location was chosen because it has been thoroughly studied biologically and geochemically over the past seven years.

2.2 Experiment Setup

Crushed limestone mined from the same limestone formation containing Double Keyhole cave was purchased locally. This crushed limestone was sifted into a homogeneous size using a 2 sieve-sized apparatus. Rocks that passed through a 1 inch sieve but were caught on a ¾ inch sieve (1.9 cm – 2.54 cm) were used for measuring the rate of dissolution of limestone. Rocks that passed through both the 1 inch and the ¾ inch sieves, considered small-sized rock, were used for DNA analysis of the microbiological community growing on the limestone. Correctly sized rocks were vigorously washed in deionized (DI) water to remove dust and debris, then dried at 80˚ C for 72 hours to remove all moisture. The rocks were cooled then immediately weighed into approximately 150 gram samples. Once weighed, the rock portions were placed into PVC pipes (5 cm x 16 cm) and prepared for one of three sample treatments (5 replicates each):

- **Control** - sealed autoclaved controls with limestone and conduit water,
- **Low Flow** – sealed at one end, with a screen on the other so water contacts the limestone but cannot flow through,
- **High Flow** – screen mesh at both ends to allow the flow of conduit water over the limestone in the tube.

All treatment tubes and their contents were autoclaved before deployment to avoid contamination. Five similarly selected rock portions were used as a control to account for any mass lost in the washing and transportation of the rocks in the treatment tubes; these Washing Controls underwent all conditions other than being exposed to the cave conditions. This included the full pre-treatment washing regime, autoclaving, and transportation to and from the study site. A 10 gram portion of the small-sized rock was placed in a plankton net pouch and included in each Low Flow treatment tube with the dissolution samples in order to collect the microbial community found growing on the limestone for DNA analysis. Plankton net pouches were not included in the High Flow treatment tubes to avoid water flow restriction. Additional water samples were collected in 50 ml sulfuric acid washed glass bottles for chemical analysis of total carbon, total dissolved nitrogen, and phosphate concentrations in the water.

The microbial community itself was examined as a part of this study. A DNA fingerprinting method was used to estimate the diversity of the microbial community as well as compare it to other microenvironments in the cave conduit. By using a combination of quantitative polymerase chain reactions (q-PCR) and length heterogeneity PCR (LH-PCR), microbial relative abundance, species richness, and absolute abundance were estimated for each microenvironment. Abundance and species richness are commonly used measurements in

ecological studies. In addition, the microbial community of the study site has previously been studied using LH-PCR, thus the results of this study can be compared to previous research. The procedures used for the PCR reactions were chosen because the study site has been researched extensively with similar methods, thus baseline data was already available that could be compared to the results of this study in order to validate the methods used. An extensive history was available on water conditions, flow rate, salinity, chemistry and even microbial community diversity thanks to previous investigation of the environment in Double Keyhole Spring (Menning, 2014).

The 15 completed treatment tubes were then randomly bundled together to avoid bias, using zip-ties. Five extra, identically made tube bundles were constructed and deployed at the same time in order to allow further research. All six tube bundles were attached to a scaffolding in an array. The complete array was deployed upright, across the flow with tubes parallel to flow in order to maximize water and bacterial exposure. The array was placed approximately 25m into the Double Keyhole cave conduit at a depth of 4.5m (Fig. 2).

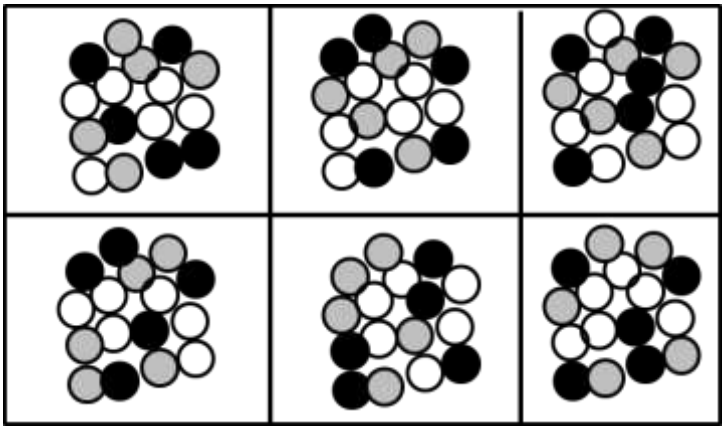


Figure 2: Depiction of the dissolution tube array

2.3 Limestone Dissolution Experiment

2.3.1 Sample Collection

All samples were collected by scientific divers using closed-circuit rebreathers under the auspices of University of South Florida Scientific Diving Program. The tube bundles were exposed to the natural conditions of Double Keyhole Spring for approximately nine months (August 26, 2014 to May 26, 2015). The tube bundle was cut from the array and the tube ends were covered in plastic sheets to prevent loss of material and captured water. Once the tubes were removed from the cave conduit, the captured water was decanted into clean, dry 500ml Nalgene bottles in order to collect water chemistry data. All samples were transported back to the lab at 4˚C.

Water samples were collected from the passage near the tube array in acid washed glass bottles for the purpose of testing water chemistry. Bottles needed to be acid washed to avoid any contamination by organic carbons in order to get accurate measurements of the water samples. These were transported on ice and kept at 4˚C until they were processed to test the water chemistry of groundwater flowing through Double Keyhole Spring at the time of sample extraction.

2.3.2 Sample Processing

All rock samples were gently washed for 5 minutes in DI water to remove the accumulated biofilm and debris. The rock samples were then placed in a drying oven for 72 hours at 80˚C to remove all moisture. This time and temperature was chosen by first washing a test group of rocks, drying them, and weighing them until no more weight change was observed.

Once dry, each rock sample was individually weighed and recorded. The pretreatment and post treatment weights were compared and the difference recorded.

The samples collected for water chemistry were tested in order to determine the concentration of total organic carbon, total nitrogen, and phosphorus using a Hach DR3900 Benchtop VIS Spectrophotometer (Hach, USA). Sample chemistry was measured within 24 hours of collection in order to prevent contamination and metabolism of carbon, nitrogen, and phosphate collected. Test-N-Tube kits were used to measure the three elements in question (Hach, USA). Water samples for the beginning time point in August of 2014 were stored at -20˚C until all samples were collected. Data for the total organic carbon concentration was only available for samples collected at the end of the experiment because water samples stored for long periods become contaminated with hydrocarbons. Containers used longer than approximately 48 hours leach hydrocarbons into water samples. In addition, the pH of the water for chemical analysis was measured using pH paper at the time of chemical analysis.

2.4 Microbial Community Analysis

2.4.1 Sample Collection

Samples collected for DNA analysis consisted of water column samples from the cave conduit, sediment samples from the conduit floor and the rock samples placed in plankton net pouches, each done in replicates of five. The water column samples were collected when the limestone dissolution tubes were deployed in August of 2014 and when they were extracted in May of 2015 at mid-passage near the tube array using sterilized 500ml Nalgene bottles, initially filled with sterile, deionized water, but inverted and filled with inert gas three times to remove any residual fresh water. The sediment samples were collected using 2ml Eppendorf conical vials

scraped along the conduit floor at random locations around the tube array at the time the limestone dissolution tubes were deployed and when they were collected.

The rock samples designated for DNA analysis were removed from their netting bags. The samples were then shaken for 60 seconds in sterile, autoclaved bottles filled with seawater. The seawater used had first been autoclaved then pulled through a two micron filter using a vacuum filtration system to ensure no contamination. The water wash was collected and passed over a 2 micron filter again to catch all the microorganisms present on the rock. The sample-laden filters were stored at -20˚C until they were processed.

2.4.2 Sample Preparation

The microorganisms caught on the filter papers were removed from the filters using a sterile DNA-free spatula in pH7 phosphate buffer saline. The samples were mixed by vortexing in a 15ml conical tube and the solution was removed to an Ultraclean Fecal DNA Kit tube (moBIO, USA). The filters were washed in this way two times. The DNA extractions were performed using these kits as described by Menning (2014). The concentration of clean environmental DNA was found using a Thermo Scientific Nanodrop 2000 Spectrophotometer (Fisher Scientific, USA).

2.4.3 Quantitative Polymerase Chain Reaction

To estimate the absolute abundance of archaea, bacteria, and microbial eukaryotes, quantitative PCR was conducted on all the DNA samples using SYBR Premix Ex Taq II (Takara, USA) and Reelplex^2 Mastercycler (Eppendorf, USA). The universal primers for each domain are listed in Table 1. The abundance was estimated by comparing the cycle threshold (Ct) values generated from the standard sample curve created from a pure DNA sample of a known

organism with a known DNA concentration. This data was used to extrapolate the estimated abundance of sample by a standard curve. This method was validated by Menning (2015).

Table 1. List of primers used for all PCR reactions with associated data.

Primer	Sequence (5'-3')	Variable Region Covered	Annealing Temperature (˚C)	Positive Control	Source
Archaea					
A1098F	CNGGCAACGAGCGMGACCC	7-8	50˚C	*Sulfolobus salfataricus*	Reysenbach and Pace 1995
UA1406R	ACGGGCGGTGWGTRCAA				Baker et al, 2003
Bacteria					
27F	ACACTTTCATCCTGGCTAG	1-2	50˚C	*Escherichia coli*	Lane, 1991
355R	GCTGCCTCCCGTAGGAGT				Giovannoni, 1991
Eukaryote					
1961F	TGGTGCATGGCCGTTCTTAG	5-6	50˚C	*Caenorhabditis elegans*	Modified from Sogin and Gunderson, 1987
2532R	CGGTGTGTACAAAGGGCAGGG				Modified from Sogin and Gunderson, 1987

2.5 Length Heterogeneity Polymerase Chain Reaction

In order to estimate species richness and relative abundance of archaea, bacteria, and microbial eukaryotes in the microbial samples, length heterogeneity PCR was conducted. An ABI 3130 four capillary Genetic Analyzer (Applied Biosystems, USA) was used. Ten nanograms of DNA were used in each PCR reaction with a universal primer for each domain (Table 1). The universal primers were selected to amplify two variable regions of the target 16S or 18S rRNA genes. The forward primers included a 56/FAM fluorescent tag for fragment detection. The samples were analyzed in their replicate sets of five along with a positive control (DNA from a pure culture, see Table 1), a negative control with no DNA included, and a blank sample with neither DNA nor primers to verify the absence of DNA contamination. Length heterogeneity PCR conditions have been described by Menning (2014). The ABI 3130 genetic analyzer produced electropherograms that were analyzed by Gene Mapper v4.0 (Applied Biosystems,

USA). The expected amplicons contain two conserved flanking regions and two internal variable regions. Peaks generated from fragments of a size representing only the conserved flanking regions or less (250 bp for archaea, 300 bp for bacteria and microbial eukaryotes) were omitted from further analysis (Suzuki et al., 1998). These limitations were chosen because this was the expected target gene length for the 16S and 18S genes. To reduce background noise in the data, peaks were only used for analysis if they fell between 50 and 300 base pairs for Archaea and Bacteria and 50 and 250 for Eukaryota as well as constituting at least 1.5% of the total area for that domain. The total number of peaks from each electropherogram was used to calculate the species richness. The relative species abundance for each sample was determined by dividing the area of each individual peak by the total of all peaks in each sample.

2.6 Statistical Analysis

Some statistical analysis of the water chemistry data and the q-PCR data was performed using a standard T test with the program SPSS (IBM, United States). The significance for the fragment analysis data was calculated using the program Primer 7 (Primer-E Ltd, United Kingdom). Peaks on the electropherogram were used to estimate the species richness. This was done by counting the number of peaks on each elecgtropherogram. The relative abundance was also estimated by comparing the area under a single peak to the total area under all peaks. For the purpose of this experiment, species refers to a distinct peak on a sample's electropherogram. Each peak of each sample was compared to the peak with the same number of base pairs of all other samples in order to create a similarity matrix. This data included the DNA density for each sample in mg/µl from the q-PCR analysis in order to estimate the absolute abundance. This matrix was then interpreted into a three dimensional image with distance equating difference in

the samples. This 3D image was then compressed into a two directional figure by the Primer program in order to be included in this paper.

Chapter Three: Results

3.1 Limestone Dissolution Experiment

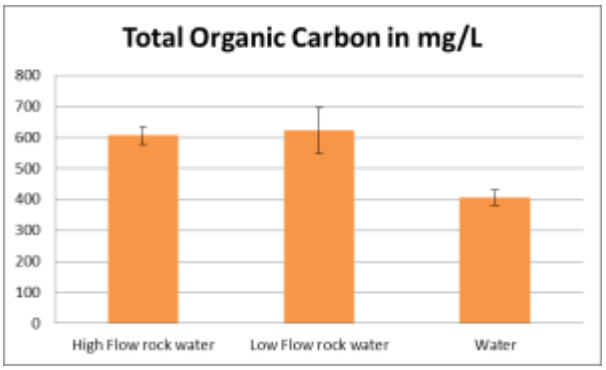
3.1.1 Water Chemistry

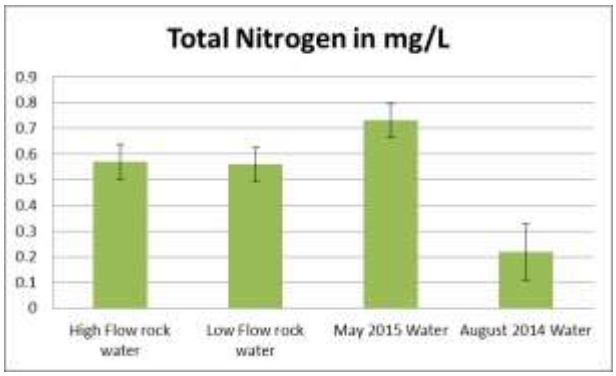
The total organic carbon levels for the water decanted from the dissolution tubes was significantly higher than the water column samples (Table 2). The water from the dissolution tubes had an average concentration of 624.2 mg/L and 607.40 mg/L, and the water column samples only contained 407.4 mg/L making the two sample sets significantly different ($p < 0.05$) (Fig. 3). Concentration of total dissolved nitrogen was highest in the water samples collected in May of 2015 and the lowest in August of 2014 (0.73 mg/L and 0.22 mg/L respectively). The samples collected by decanting the water trapped in the dissolution tubes had a concentration of dissolved nitrogen of 0.56 and 0.57 mg/L which were not significantly different ($p > 0.05$) (Fig. 3). The concetration of dissolved phosphate in the water samples decanted from the dissolution tubes was highly variable dependant on the amount of floculent matter captured by the plastic covering when the samples were removed from the cave system. The concentration of phosphate in water decanted from the dissolution tubes were still significantly higher than the water column samples ($p < 0.05$) and the Low Flow samples had the highest phosphate concentration (Fig. 3).

The measured pH of all the samples were indistinguishable from each other. The pH paper indicated all samples had an aproximate pH of 7.

Table 2: Water chemistry data for total dissolved carbon, nitrogen, and phosphate collected from the rock microenvironment and the water column at the beginning and end of the experiment

	Total Organic Carbon in mg/L			Dissolved Nitrogen in mg/L				Dissolved Phosphate in mg/L			
	May 2015			August 2014	May 2015			August 2014	May 2015		
	Water	High Flow rock water	Low Flow rock water	Water	Water	High Flow rock water	Low Flow rock water	Water	Water	High Flow rock water	Low Flow rock water
	383	583	538	0.1	0.8	0.6	0.5	0.61	0.14	0.68	1.52
	384	648	572	0.3	0.7	0.65	0.5	0.26	0.2	2.48	1.68
	429	588	611	0.3	0.7	0.6	0.55	0.45	0.28	1.49	9.62
	404	594	695	0.3	0.7	0.5	0.6	0.41	0.25	5.07	6.34
	437	624	705	0.1	0.8	0.5	0.65	0.53	0.2	1.58	6.98
Average	407	607	624	0.2	0.73	0.57	0.56	0.45	0.21	2.26	5.23
Standard Deviation	25	28	74	0.11	0.07	0.07	0.07	0.13	0.05	1.70	3.53





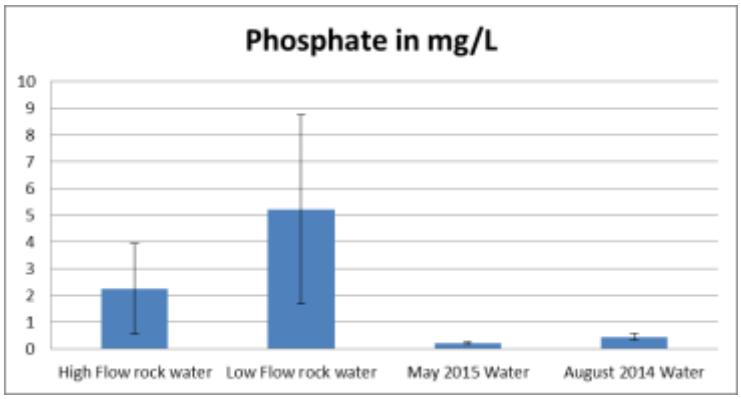


Figure 3: The total dissolved carbon, nitrogen and phosphate concentrations in 5 replicate water samples captured in the dissolution treatment tubes for both the High Flow and the Low Flow samples and the water column in the cave passage.

3.1.2 Dissolution

The rock in the High Flow treatment tubes lost significantly more mass than any other treatment; the High Flow treatment tubes lost an average of 3.428 g constituting a 2.28% ±0.29 loss. The Low Flow treatment tubes lost an average of 1.64 g constituting a 1.09% ± 0.71 loss and the Control tubes only lost 0.497 g, a 0.33% ± 0.10 loss ($p < 0.05$). The Washing Control group was not significantly different from the control group left in the cave (Fig. 4).

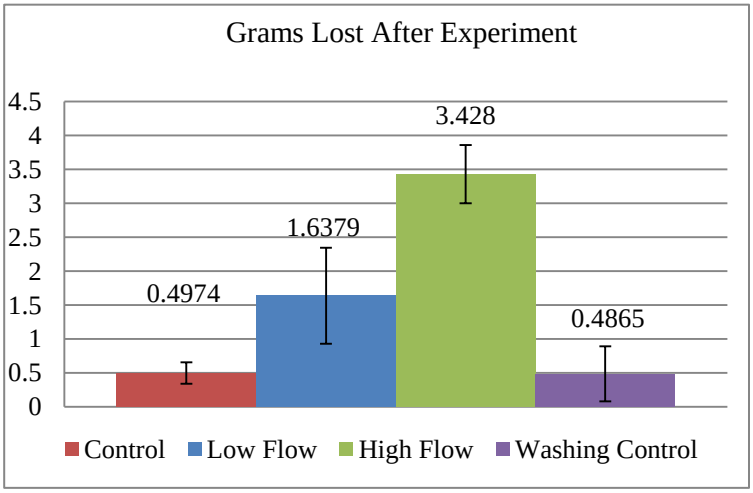


Figure 4: Mass of limestone dissolved by exposure to treatment conditions for nine months. Washing Control underwent the washing, sterilizing, and transportation regime but not exposure to any cave conditions.

Table 3: Mass in grams of limestone dissolved by exposure to the cave environment for nine months

	Starting mass	Ending mass	Grams lost	Average	Standard deviation
Control	150.9	150.513	0.387	0.50	0.16
	150.42	150.09	0.33		
	149.87	149.45	0.42		
	149.42	150.01	0.59		
	149.99	149.23	0.76		
Low Flow	149.7	148.48	1.22	1.64	0.71
	150.5	147.3	3.2		
	150.07	148.67	1.4		
	150.94	149.28	1.66		
	150.19	148.34	1.85		
High Flow	150.6	147.51	3.09	3.43	0.43
	150.09	146.46	3.63		
	150.49	147.3	3.19		
	150.64	146.46	4.18		
	149.34	146.29	3.05		

3.2 Microbial Community Analysis

3.2.1 Estimated Abundance

The rock samples had the highest relative abundance of archaea with this domain making up 91% of the total DNA found there. The water collected at the end of the experiment had the highest concentration of bacteria at 27% of its total DNA, and the sediment collected at the beginning of the experiment had the highest relative concentration of eukaryotic DNA at 1% (Fig. 5).

The data produced from PCR analysis of the DNA samples showed that the highest concentration of extractable DNA from all three domains was found in the rock samples (4.386 mg/ µL , 42.76 mg/ µL, and 0.062 mg/µL respectively). The smallest concentration in all three domains was found in the water sample at both the August and May time points (Fig. 6). The rock samples had significantly higher concentrations ($p < 0.05$).

3.2.2 Species Richness

Species richness data from length heterogeneity PCR analysis indicate that the average number of species for bacteria and archaea found in the water column at both beginning and end time points (26-23 and 24-22 peaks respectively) were higher than all other sample sets. The water column samples had the lowest richness in eukaryotic DNA. The rock samples had high richness for bacterial DNA (25 peaks) but the highest for eukaryotic DNA (17 peaks). The sediment samples' species richness fell between water column samples and the rock samples for all domains (Table 5).

Table 4: Concentration of DNA for each domain in each microenvironment in mg/µl as well as standard deviation for each sample set

	Bacteria	Standard Deviation	Archaea	Standard Deviation	Eukaryote	Standard Deviation
Beginning Sediment	0.59	0.18	2.67	1.32	0.03	0.04
Ending Sediment	1.49	0.25	13.25	4.78	0.02	0.006
Beginning Water	0.15	0.11	0.94	0.37	0.004	0.002
Ending Water	0.25	0.10	0.67	0.36	0.002	0.001
Rock	4.39	1.50	42.76	15.02	0.06	0.08

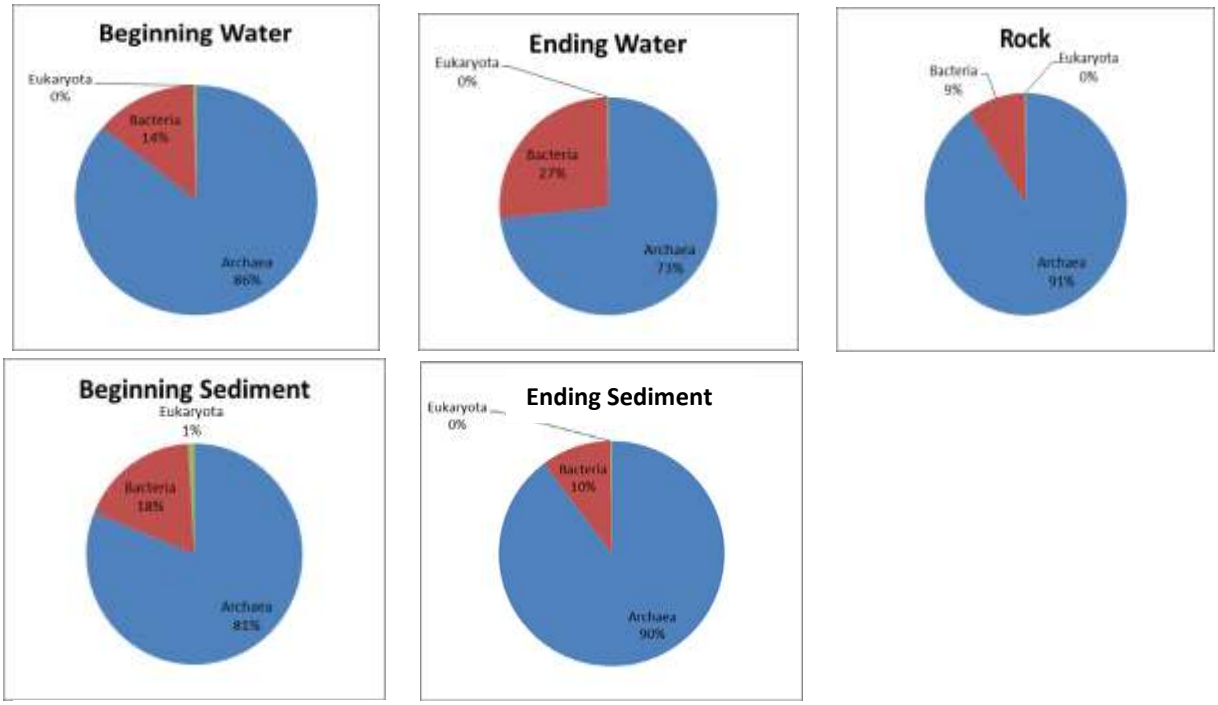


Figure 5: Relative abundance of each domain in the water, sediment, and rock samples from q-PCR data.

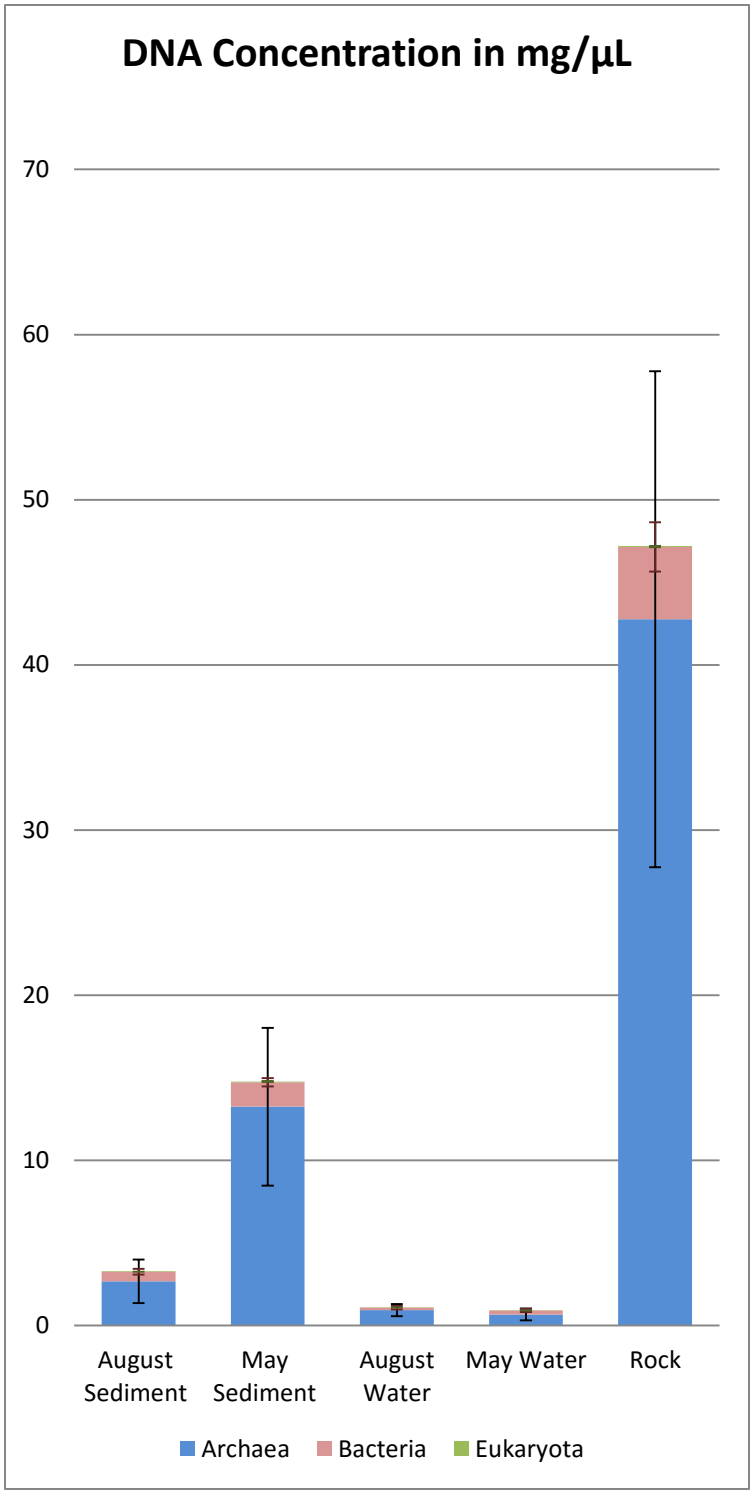


Figure 6: The abundance of DNA in mg/µl of each domains collected from each microenvironment and time point.

Table 5: Species richness for the three domains as peaks found by LH-PCR analysis. These estimations were calculated by counting the number of significant peaks of at least 1.5% of the total relative abundance found in each LH-PCR analysis and taking an average of every sample set of five.

LH-PCR peaks	Bacteria	Archaea	Eukaryotes
August ’14 Water	26	24	12
May ’15 Water	23	22	10
August ’14 Sediment	22	22	11
May ’15 Sediment	23	19	16
May ’15 Rock	25	19	17

Comparison of the percent abundance of species in each domain showed that many species were unique to a single microenvironment. Analysis of the bacterial DNA revealed only two species were unique to the rock microenvironment. These two species represented 12.48% of the bacterial DNA (Table 6). One bacterial species was found to be unique to the sediment microenvironment representing 10.75% of the bacterial DNA found there, and three species were found to be unique in the water column representing 8.89% of the bacterial DNA found there. Many bacterial species were present in two or all three microenvironments (Table 6). Analysis of the archaeal DNA revealed three species that were unique to the rock samples, representing 18.38% of the archaeal DNA found there (Table 7). Two archaeal species were found to be unique to the sediment samples representing 22.51% of the archaeal DNA in the sediment, and two species were unique to the water column samples representing 12.36% of the archaeal DNA in the water (Table 7). Analysis of the eukaryotic DNA only revealed one species to be unique to the rock samples. This single species represented 21.55% of the eukaryotic DNA in the rock samples. Three species were found to only live in the sediment samples making up 14.58% of the eukaryotic DNA in that microenvironment and three species were unique to the water column samples representing 13.39% of the eukaryotic DNA in the water column (Table 8).

Table 6: The relative abundance as a percentage of total of bacterial species identified by the number of base pairs detected by LH-PCR analysis. Only species that made up at least 1.5% of the total were included in this table. Species with over 2% are colored. Yellow indicates the species was found in all microenvironments. Blue indicates that the species was found in the water column samples or sediment samples or both, but not the rock samples. Red indicates that the species was only found in the rock samples.

Bactarial relative abundance					
Base Pairs	'14 August Water	'15 May Water	Rock	'14 August Sediment	'15 May Sediment
52	1.97	0	2.13	0	0.14
57	0	2.94	2.36	10.61	6.28
58	24.21	15.54	0	6.82	0
59	0	0	0	10.75	0
60	0	0	4.42	6.85	10.24
76	7.00	0.19	0	1.31	2.27
85	0.02	0.12	3.48	0	0
86	0.06	0	8.18	0	1.35
87	0	0	2.07	0	0
89	0.97	0	0	3.22	1.25
95	3.47	0	0	0	0
96	0.15	0.89	2.63	3.28	1.37
97	0	0	10.41	0	0
105	5.41	0	0	0	0
106	1.65	3.83	2.65	5.21	10.46
107	1.05	0	0.06	3.95	3.66
108	0	2.76	0.87	2.15	0.93
111	1.80	0.23	0	2.84	0
116	0	0.83	3.33	0	6.78
119	7.58	0	1.36	3.25	1.97
120	0	0.69	6.89	0.66	0.12
122	6.67	0.88	0	0	0
123	3.44	0.05	4.62	0	1.53
125	0	0	5.81	0.12	0
126	0.62	8.55	0	0	0
127	2.10	0	1.30	2.73	1.38
129	0	2.26	1.08	0	0.29
145	0	0.41	2.14	0	0.25
150	0.06	0	0	2.29	0.60
152	0	1.10	0	0.30	3.68
153	0.81	4.02	0.10	0	0.60
156	2.98	0.01	0	0	1.06
163	0	0.03	0	3.29	0
173	0.50	0.98	0	1.65	0
285	0	0	1.71	0	0
317	0.17	4.67	0	0.02	0.20
318	0.16	5.45	0.22	0	0

Table 7: The relative abundance as a percentage of total of archaeal species identified by the number of base pairs detected by LH-PCR analysis. Only species that made up at least 1% of the total were included in this table. Species with over 2% are colored. Yellow indicates the species was found in all microenvironments. Blue indicates that the species was found in the water column samples or sediment samples or both, but not the rock samples. Red indicates that the species was only found in the rock samples.

Archaeal relative abundance					
Base Pairs	14 August Water	15 May Water	Rock	14 August Sediment	15 May Sediment
64	5.02	4.38	0.00	0.73	0.36
65	5.94	0.00	0.22	0.00	0.00
66	0.00	10.25	0.00	0.42	0.81
70	1.25	0.00	0.00	0.00	0.00
80	1.71	0.00	0.00	0.00	0.00
81	0.00	1.04	2.60	3.97	4.43
82	1.23	0.91	0.00	6.67	8.39
97	1.92	2.23	0.00	0.10	0.10
98	2.03	2.70	0.87	0.18	0.02
99	0.00	0.00	1.11	0.00	0.00
132	0.00	0.00	1.17	0.13	0.46
188	0.84	1.20	1.09	0.50	0.71
189	0.92	1.20	1.05	0.87	1.24
284	0.99	0.60	0.00	0.00	1.41
285	4.43	0.00	0.00	1.62	3.37
286	2.44	3.82	15.54	1.32	2.79
287	0.00	0.00	2.12	0.00	0.00
291	0.00	0.00	0.00	5.83	0.00
292	2.83	1.44	0.00	0.00	8.53
294	0.00	0.00	9.39	0.00	0.00
299	0.08	17.31	0.00	0.91	1.01
300	6.65	0.00	0.00	0.00	0.00
302	5.71	6.17	0.00	0.00	0.00
304	1.87	0.00	0.00	0.00	0.00
305	0.00	4.11	0.00	0.00	0.00
306	0.00	0.00	6.90	0.00	0.00
308	0.00	0.00	0.00	16.68	0.00
314	12.79	4.02	3.96	0.00	0.00
315	0.00	0.00	22.95	9.99	0.00
316	20.75	13.43	0.00	29.32	47.14

Table 8: The relative abundance as a percentage of total of eukaryotic species identified by the number of base pairs detected by LH-PCR analysis. Only species that made up at least 1% of the total were included in this table. Species with over 2% are colored. Yellow indicates the species was found in all microenvironments. Blue indicates that the species was found in the water column samples or sediment samples or both, but not the rock samples. Red indicates that the species was only found in the rock samples.

Eukaryotic relative abundance					
Base Pairs	'14 August Water	'15 May Water	Rock	'14 August Sediment	'15 May Sediment
65	0.00	0.00	0.91	0.00	2.22
84	0.00	0.00	0.68	0.00	1.87
158	0.00	0.00	0.00	0.36	1.08
352	0.00	0.00	0.00	1.20	0.00
361	1.60	1.45	0.03	0.00	0.00
362	10.87	0.00	0.00	0.00	0.00
363	8.59	0.33	0.00	0.27	3.36
364	7.08	0.00	1.65	2.84	5.08
365	10.00	3.33	1.37	6.22	2.55
367	3.61	5.96	0.13	0.00	0.00
368	9.47	28.42	3.46	0.00	28.49
369	0.00	0.00	3.68	3.13	22.38
370	0.00	0.00	0.00	3.30	0.00
371	10.66	25.41	3.83	11.98	6.41
372	13.03	19.64	2.76	16.06	3.20
373	0.98	0.19	0.00	3.93	0.95
375	1.16	0.79	0.00	0.00	0.00
376	1.69	4.06	0.00	0.32	0.18
378	0.00	2.45	0.00	0.00	0.00
386	0.00	0.00	0.00	0.00	2.52
388	0.00	0.00	0.00	11.28	2.43
389	4.32	0.00	27.91	10.61	0.00
390	0.00	0.00	21.55	0.00	0.00
391	4.09	0.00	0.15	0.00	0.00
416	0.00	0.00	1.59	0.00	0.00
447	0.00	0.00	9.17	0.00	0.44
448	0.00	0.00	7.10	0.00	0.77
493	2.52	0.00	0.00	0.00	0.00

3.2.3 Multi-Dimensional Scaling Plots

Multi-dimensional scaling (MDS) plots produced from estimated absolute abundance and relative abundance data for each site indicate that the microbial community that grows on the limestone rock is less than 20% similar to those that grow in the water column and in the sediment in the cave conduit of Double Keyhole Spring. These plots also indicate that the microbial community from the sediment are between 20% and 40% similar to each other at the two time points and the water column samples are at least 20% similar to each other at the two different time points (Fig 7).

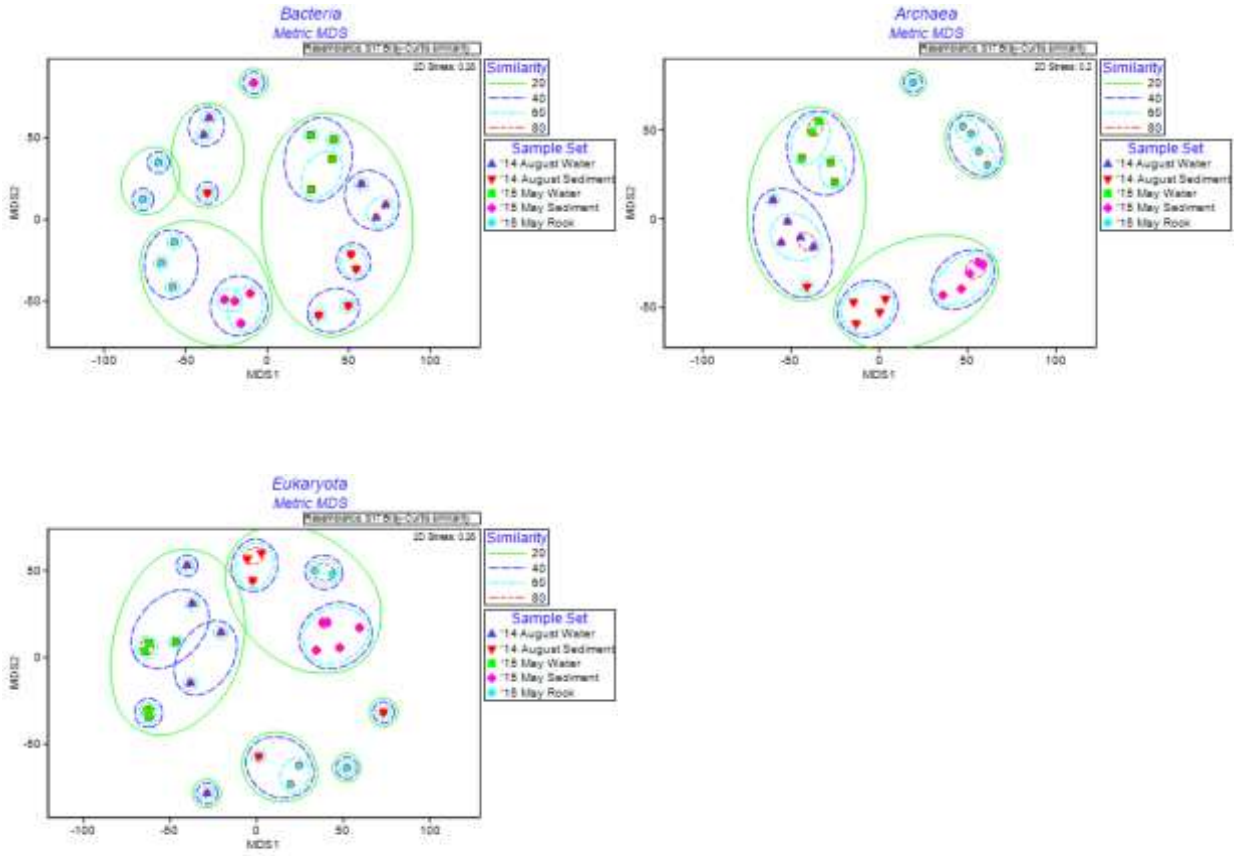


Figure 7: Multi-dimensional Scaling plot of bacterial, archaeal, and microbial eukaryotic communities collected from the water column, sediment, and rock samples. The five replicates of each sample set are depicted by particular colors and shapes. Colored circles around the samples indicate the percentage of similarity between.

Chapter Four: Discussion

4.1 Limestone Dissolution

The purpose of this study was to discover the influence microbial communities have on the dissolution rate of limestone in flooded cave systems. The results of the study suggest that microbial activity might contribute significantly to the dissolution rate of the limestone in Double Keyhole Spring. The Low Flow samples lost as much as 70% more mass than the control samples, an average of 1.14 grams per sample (Fig. 4). Since the Low Flow samples were exposed to minimal water flow, this loss of mass can be attributed to be mostly due to the microbial community growing on the samples. The High Flow samples lost more than the Low Flow samples. The High Flow samples were exposed to the microbial community as the Low Flows were in addition to the known corrosive action of water flow, thus this result was expected. A possible additional difference between the High Flow and the Low Flow samples might have been that the Low Flow samples could have been more nutrient limited than the High Flow samples due to the reduction in water flow. It is possible that the restriction of water flow through the Low Flow sample tubes prevented new nutrients from reaching the microbes growing in the Low Flow sample tubes, resulting in different microbial communities in the two sample types.

The mass of limestone lost over the 9 month treatment was significantly more in the High Flow and the Low Flow samples than both types of controls (Fig. 4). The mass of limestone lost in the High Flow tubes was comparable to other limestone dissolution studies (Liu et al., 2005). The High Flow samples lost an average of 2.28% total mass and another study found an average

dissolution rate of 2.79% (Mulec & Prelovsek, 2015). This rate is on the lower end of the range of measured dissolution rates for limestone. This is because the faster dissolution rates were measured in caves filled with water with high concentrations of hydrogen sulfide or sulfuric acid. Double Keyhole Spring is a less chemically corrosive cave system and thus is consistent with similar ordinary caves. The comparable pH found in the dissolution tubes and the water column supports that simple corrosion from acidity does not drive the dissolution rate in the Low Flow samples. The High Flow samples only lost an average of 1.79g more than the Low Flow samples. Assuming the microbial communities in the High Flow and the Low Flow samples were similar, the difference in limestone loss between the High Flow and the Low Flow samples suggests that 52.2% of the limestone loss might not be due to corrosive water flow. Instead this 52.2% could be due to the microbial activity of the community growing on the rock (Fig. 4). Assuming that most of the mass lost in the Low Flow treatment tubes was due to microbial activity, it suggests that the difference in mass lost between the High Flow samples and the Low Flow samples, 1.79g, can be attributed to water corrosion, a known cause of limestone dissolution.

It is possible that the Control samples lost as little limestone mass as they did because the water trapped in the sealed treatment tubes became saturated with calcium carbonate quickly. The saturated water would be unable to dissolve any more limestone over the experiment, no matter how long the exposure time. This possibility could be tested with an additional experiment involving a much larger volume of sterilized cave water flowing over limestone at a flow rate similar to the natural cave environment.

Extensive testing was performed to ensure that all measured loss of mass was due to the treatment exposure instead of unintended factors such as transportation, washing, processing, or

deployment in the cave system. The experimental samples lost significantly more mass than all the control treatments, validating the experimental design. The Low Flow tubes had approximately half the dissolution of the High Flow tubes. This indicates that as much as 50% of the limestone dissolution could be due to microbial activity. The Low Flow samples lost 1.64 g which was 47.8% of the 3.43 grams lost by the High Flow samples. Alternatively, it could be that in the Low Flow samples, the carbonate saturation was higher than High Flow due to the slower water turn-over, leading to the lower dissolution.

Because the effect of the minimal water flow cannot be disregarded from the results, it can be concluded that up to approximately 50% of the limestone dissolution occurring in Double Keyhole Spring cave is due to exposure to microbial activity (Fig. 4).The High Flow treatment tube results had similar loss of mass compared to other studies.

The water chemistry results indicate that the microbial growth on the limestone has a significant influence on the chemical conditions surrounding the limestone. The concentration of dissolved phosphate in the water decanted from the dissolution sample tubes was over ten times higher than the water collected from the cave passage (Fig. 4). The limestone itself in Florida has a high concentration of phosphate. The limestone bedrock in which Double Keyhole Spring is located has a dissolved phosphorus concentration of 0.1ppm. This is double the average concentration in the continental United States (Odum, 1958). Thus it is likely that the high concentration of phosphate in the samples decanted from the treatment tubes is from the limestone itself. The difference in phosphate concentration between the High Flow and Low Flow rock water samples supports that water flow in the Low Flow samples was indeed less than in the High Flow samples (Fig. 3). Phosphate released from the limestone rock was washed away faster in the High Flow samples. It is possible that water saturated with dissolved calcium

carbonate was also washed away in a similar manner. If the Low Flow samples didn't have as high a flow rate, the higher calcium carbonate saturation in the water around the rock would cause the limestone to dissolve slower.

The concentration of dissolved nitrogen was significantly higher in the water decanted from the dissolution tubes compared to the water column sample collected at the same time, but lower than the concentration in the water column sample collected at the beginning of the experiment (Fig. 3). It is possible that if samples from the rock microenvironment were analyzed at the beginning of the experiment along with the August water samples the concentration would have followed the same pattern, but the available data is inconclusive.

The concentration of total organic carbon (TOC) was significantly higher in the water decanted from the dissolution tubes than the water samples (Fig. 3). This was not unexpected because of the dense layer of organic matter growing on the sample rocks and in the treatment tubes. The higher concentrations of TOC in the tube samples suggest a higher rate of organic activity and stimulation of microbial growth in the High Flow and Low Flow dissolution tubes. The TOC concentration found in the water column is consistent with the groundwater in the area (USGS, 2006).

Additional data for the water chemistry of Double Keyhole Spring that can affect the dissolution rate of limestone were taken from a previous study at the same site. Continuous seasonal monitoring of the cave system revealed an average salinity of approximately 18 ppt. The salinity changes daily with the tidal flow of the estuary with high salinity 30 ppt during high tide to as low as 10 ppt during low tide after heavy rainfall. The salinity may affect the microbial community. Only organisms that can tolerate large changes in salinity can survive in Double Keyhole Spring (Menning, 2014). This kind of constant change can limit the biodiversity as only

certain species are able to tolerate such a wide range of salinity (Gerdes et al., 1985). However, this kind of regular ecological disturbance can also lead to higher species richness by not allowing a single species to dominate (Girvan et al., 2005). A stable environment allows the single species best equipped to thrive in that particular environment to dominate, but constant change means a continuous flux of which species is best able to handle the momentary environment. This is supported by the estimated relative abundance data in this study.

4.2 Estimated abundance

The estimated microbial abundance of the rock samples was significantly higher than any of the sediment or water samples. The methods used were specific to the domain of life (archaea, bacteria, microbial eukaryotes) so the separate values are discussed here. The abundance of archaeal DNA was significantly higher than that of any other domain in all five sample sets (Fig. 6). Previous investigation into the PCR primers used for this study showed that the archaeal primers were approximately nine times more effective at replicating archaeal DNA then the eukaryote primers and bacterial primers (Menning, 2014). Taking this bias into account, the estimated abundance of archaea was still at least ten times more abundant than bacterial DNA and more than one hundred times more abundant than eukaryote DNA in all sample sets and time points (Table 9).

Table 9: Conversion of estimated DNA concentration for archaeal DNA to accommodate for primer affinity bias.

		Pre-conversion					Post Conversion				
DNA in ng/μL	Sample Number	1	2	3	4	5	1	2	3	4	5
	August 2014 Sediment	37.03	34.14	22.76	18.37	8.04	4.11	3.79	2.53	2.04	0.89
	May 2015 Sediment	120.77	157.70	164.69	66.27	86.83	13.42	17.52	18.30	7.36	9.65
	August 2014 Water	13.08	7.51	7.41	9.98	4.11	1.45	0.83	0.82	1.11	0.46
	May 2015 Water	9.40	3.28	3.61	9.81	4.07	1.04	0.36	0.40	1.09	0.45
	Rock	256.51	450.14	221.58	492.06	504.04	28.50	50.02	24.62	54.67	56.00

These findings are consistent with a previous study of the microbial communities of Double Keyhole Spring. Few eukaryotic organisms can thrive in the harsh cave environment. Eukaryotic microbes are less able to survive in the harsh environment because of the lack of primary production and low oxygen saturation (Gonzalez et al., 2003). The abundance of archaea in soils has previously been documented at between 2%-10% (Bates et al., 2010). The percentage abundance in the cave environment was between 73% in the water column and 90% in the rock samples, suggesting that archaea are more able to survive the microenvironmental conditions of the cave environment than bacteria or microbial eukaryotes. Archaeal communities have been well documented to be adapted to low nutrient environments (Pace, 1997). The highest relative density of archaeal DNA was found in the rock samples. Ninety percent of the DNA found from q-PCR analysis was archaeal. It can be hypothesized from this that archaea are responsible for some or even most of the microbial activity that is contributing to the increased dissolution rate of the limestone rocks. With such a high density of archaea and a significantly higher density living on the limestone itself, it can be inferred that the presence of limestone promotes the survivability of archaeal species. A more detailed DNA analysis would be required to know if the archaeal species found in Double Keyhole Spring are capable of metabolizing calcium carbonate and if they are utilizing the limestone as a resource.

The estimated microbial abundance found in the sediment samples in this study is consistent with previous work (Menning, 2014). The significant difference between the two sediment samples could have been due to natural seasonal changes. In the late summer, when the first sediment samples were taken, there is high water flow through the Double Keyhole Spring cave system because of high summer rainfall (Menning, 2014). This high flow has been found to wash out microbial communities in the sediment. Contrarily, when the sediment samples were

collected at the end of the experiment in May, there was little rainfall, thus there was lower flow in the cave system. This lower flow allows the microbial community enough residence time in the cave system to reproduce in larger numbers (Battin et al., 2009).

4.3 Species Richness and Relative Abundance

Species richness was varied between sample types. The water samples had the highest number of estimated bacterial and archaeal species but the lowest in eukaryotic species. The rock samples had the lowest number of estimated bacterial species, but the number of estimated eukaryotic species was significantly higher. The reason for such differences could be that the different microenvironments provide different ecological niches. The lower diversity in bacterial species can indicate the rock microenvironment is less suitable for bacterial growth, or it could mean there are a few species of bacteria that dominate the rock surface to the exclusion of others. I hypothesize that the lower diversity of bacterial species on the rock is more likely due to domination by a few species. Only three species make up 25.48% of the estimated absolute abundance of bacterial DNA (Table 3). The species with 86, 97, and 120 base pairs in their 16S ribosomal LH-PCR fragments were only found in the rock samples and could be responsible for dominating that microenvironment. Eight species of bacteria were found in the water and sediment samples but not the rock samples.

There was only one eukaryotic species exclusively found in the rock samples, but that single species, with 390 base pairs in the 18S ribosomal code, constituted 21.55% of the total eukaryotic abundance. The species with 389 base pairs made up 27.91% of the estimated absolute abundance in the rock samples, but that species was also found in high abundance in the water and sediment samples. A total of seven eukaryotic species were found in all three microenvironments. The rock samples had a low absolute abundance of eukaryotic DNA, but a

high relative abundance with three species making up over 50% of the eukaryotic biomass in the rock samples (Table 4). The microbial eukaryote abundance was expected to be low because in the absence of light they must either be predators or grazers as they cannot photosynthesize. Previous studies have found *Allovahlkampfia spelaea*, a protozoa, to be common in flooded caves (Czerwik-Marcinkowska, 2013). This type of protozoan eats single celled organisms like the archaea and bacteria growing on the limestone. *Allovahlkamfia spelaea* has a documented 18S ribosomal gene length of ~400 base pairs. This is very close to the estimated number of base pairs of the unique species found on the rock samples at 390bp. This supports the hypothesis that the rock microenvironment is distinct from the other microenvironments in the cave system.

The results of archaeal DNA analysis showed that the most abundant archaeal species found in the rock samples were also found in the sediment samples. The archaeal species represented by the 315 base pair fragment in its 16S ribosomal DNA code made up 22.95% of the archaeal DNA found in the rock samples and 9.99% in one of the sediment sample sets. In addition, the species represented by the 286 base pair DNA fragment made up 15.54% of the archaeal DNA found in the rock samples but was also at least 1.3% of all other sample types. This indicates that the archaeal species most able to thrive in the rock microenvironment are ubiquitous to the cave environment, but may be best suited to living on the limestone (Table 5). Only three archaeal species were found to be able to live in all three microenviroments.

Oligotrophic environments such as flooded caves often have oligocultures dominated by only a few species (Adetutu & Ball, 2014). As microbiomes of brackish karst caves are only beginning to be studied, it is hard to know if the findings in Double Keyhole Spring are unique or characteristic for the environment. Another study noted the presence of *Allovahlkampfia spelaea* in a cave in Slovenia and other aquatic karst environments. It is possible that this or a

similar species is one of the dominant eukaryotic species found in Double Keyhole Spring.

Studies of bacterial communities in flooded caves are much more extensive than studies of eukaryotic or archaeal species. *Proteobacteria, Actinobacteria,* and *Cytophagales* are the most common phylotypes found in flooded caves (Barton et al., 2004). These phyla of bacteria have been described as dwelling in groundwater and soils (Selje & Simon, 2003), thus it can be conjectured that the species found in Double Keyhole Spring belong to these phyla. Further research and DNA analysis is needed to know the exact archaeal species that live in Double Keyhole Spring.

4.4 Multi-Dimensional Scaling Plots

Examination of the MDS plots of the communities of all five sample sets shows spatial variation between sites generally grouped by collection microenvironment. The sediment samples mostly grouped together, the water samples generally grouped together, and the rock samples generally grouped together. The exception to this pattern is the plot of the eukaryotic samples. This plot was less clear but this result was expected as previous analysis of the eukaryotic DNA in Double Keyhole Spring was similarly mixed (Menning, 2014). This is most likely due to the very low abundance of eukaryotic DNA in the system.

The MDS plot of the archaeal DNA showed a clear dissimilarity between the rock samples and all other samples. These samples were found to be less than 20% similar to any other samples. In addition, the water and sediment samples collected at the beginning of the experiment were more similar to each other than the water and sediment samples at the end. All of this indicates that the rock microenvironment supports a different biodiversity of archaea than the water or sediment (Fig. 6). Because the archaeal community on the rock is so different from the water column and the sediment it can be hypothesized that the difference is due to the

limestone itself. If the three species of archaea that were only found in the rock samples are capable of dissolving the limestone, it would mean these species would be contributing to the increased dissolution rate of the limestone when exposed to the microbiology of Double Keyhole Spring. DNA sequencing is required to know if these species are capable of such limestone dissolution.

The MDS plots for eukaryotic DNA and bacterial DNA are less clear than the archaeal DNA plot. However, they still indicate that the community found in the rock samples is distinct from the water and sediment, and they are more similar to the sediment than the water (Fig. 6). This information is consistent with other studies that have found the microbiome living on limestone karst is similar to soil samples of the area (USGS, 2006). It is likely that the bacterial community living on the limestone in Double Keyhole Spring is at least partially responsible for the increased rate of dissolution because previous research has indicated that many bacterial species are capable of not only dissolving limestone, but also using the calcium carbonate as a nutrient source (Subrahmanyam et al., 2012). It is unlikely that the distinct eukaryotic community found in the limestone samples is responsible for an increased dissolution rate. Instead, the difference is more likely due to the availability of prey items for organisms like protozoa to feed on in the rock microenvironment as opposed to the water or sediment microenvironments.

The differences in community between the two sediment samples and between the two water samples could be due to seasonal variation in the environment (Fig. 6). Temperature difference alone can cause a dramatic change in the microbial community. Previous studies of Double Keyhole Spring found the average temperature in late summer is as high as 24.7 ˚C, and the temperature in spring as low as 23.6˚C (Menning, 2014). Other seasonal changes include pH,

which can be higher in the late summer; DO, which is generally lower in the spring than the summer; salinity, which is higher in spring than the summer, and flow rate. These characteristics of the water can affect the microbial community in sediment and water column (Table 10).

Table 10: Historical values for Temperature in Celsius, dissolved oxygen in mg/L, pH, and Salinity in ppt (Menning, 2014)

	Temperature	Dissolved Oxygen	pH	Salinity
Summer	24.7	0.2	7.8	19.4
Spring	23.6	0.4	7.2	15.4

It is possible that the only part of the limestone dissolution in Double Keyhole Spring is directly caused by the direct metabolism of bacterial, archaeal, or eukaryotic microbes. Part of the dissolution is due to the acidity produced by the microbial community and the metabolic processes, and limestone dissolution is merely a side effect. However, the measured pH in the dissolution tubes was indistinguishable from the water column so this is unlikely to have been a driving factor in this experiment. It is possible that a thin layer of more acidic water exists between the microbial mat and the limestone, which would have been impossible to measure in this study, but the measured pH in the dissolution tubes was not significantly acidic. The significant difference in water chemistry between the water collected from the dissolution tubes and the water column in the cave conduit supports the hypothesis that microbial action and not water chemistry is driving the dissolution. In order to know if the increased rate of dissolution is an unintended result of acidity or if the limestone is being dissolved in order to use the calcium carbonate as a metabolite, Next-Generation sequencing analysis such as Illumina would be required. Proteomic analysis of the microbial community growing on the limestone of Double Keyhole Spring might reveal a capability, or lack thereof, to dissolve limestone through enzymatic production of acids or other carbonate-utilizing metabolic pathways.

Chapter Five: Conclusions

The major findings of this study are that:

1. The water immediately surrounding the rock is significantly different chemically from the water column.
    a. This is most likely due to the microbial community.
    b. The chemistry surrounding the limestone probably drives the limestone dissolution.
2. Microbial activity appears to be responsible for up to approximately 50% of the limestone dissolution in Double Keyhole Spring.
    a. The Low Flow samples lost 70% more mass than the controls.
    b. The Low Flow samples lost 50% of the total mass lost in the High Flow samples.
3. The archaeal community living on the limestone appears to be the main biological driver of limestone dissolution.
    a. The rock community was more similar to sediment in eukaryotic DNA analysis but more similar to water in bacterial DNA analysis.
    b. The rock samples were most unique in archaeal DNA analysis.
    c. Further research would be required to know exactly what species of archaeal live on the limestone in Double Keyhole Spring.

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Chapter Seven: Appendix

Table A-1: The initial, pretreatment weight of all the prepared rock treatment tubes. Bundle 6 was used for this experiment. Tubes labeled A are control tubes, tubes labeled B are Low Flow Tubes, and tubes labeled C are High Flow tubes.

Bundle 1		Bundle 2		Bundle 3	
Tube Identity	Weight (g)	Tube Identity	Weight (g)	Tube Identity	Weight (g)
1A1	149.93	2A1	150.76	3A1	149.27
1A2	150.36	2A2	150.09	3A2	149.64
1A3	149.20	2A3	150.49	3A3	150.47
1A4	150.56	2A4	150.88	3A4	150.47
1A5	150.31	2A5	150.83	3A5	149.28
1B1	150.06	2B1	149.40	3B1	150.93
1B2	149.03	2B2	149.65	3B2	150.48
1B3	150.66	2B3	149.78	3B3	150.91
1B4	149.55	2B4	149.48	3B4	150.24
1B5	149.45	2B5	149.42	3B5	149.74
1C1	147.15	2C1	151.70	3C1	149.47
1C2	150.17	2C2	150.65	3C2	151.00
1C3	151.34	2C3	149.52	3C3	149.51
1C4	148.00	2C4	149.43	3C4	151.73
1C5	149.32	2C5	150.85	3C5	150.09
Bundle 4		Bundle 5		Bundle 6	
Tube Identity	Weight (g)	Tube Identity	Weight (g)	Tube Identity	Weight (g)
4A1	150.68	5A1	150.16	6A1	150.09
4A2	150.16	5A2	149.46	6A2	150.49
4A3	150.96	5A3	150.27	6A3	149.87
4A4	149.38	5A4	150.24	6A4	149.42
4A5	149.59	5A5	150.65	6A5	149.99
4B1	150.21	5B1	150.55	6B1	149.70
4B2	149.21	5B2	149.48	6B2	150.56
4B3	150.88	5B3	149.49	6B3	150.07
4B4	150.73	5B4	149.25	6B4	150.94
4B5	150.44	5B5	149.86	6B5	150.19
4C1	151.35	5C1	150.30	6C1	150.60
4C2	149.92	5C2	151.18	6C2	150.09
4C3	151.17	5C3	149.12	6C3	150.49
4C4	149.14	5C4	150.22	6C4	150.64
4C5	151.82	5C5	149.46	6C5	149.34