Effect of biologically mediated sulfur oxidation on carbonate dissolution in submerged, fresh-water calcite caves

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ABSTRACT

The importance of carbonic acid to carbonate dissolution in submerged caves is well known (White, 1988). However, carbonic acid is not the only acid present in many cave environments. Acids generated microbiologically, such as sulfuric acid generated during sulfide oxidation (Hose et al, 2000, Macalady et al, 2006, Engel and Randall, 2011, Sarbu et al, 1996, Vlasceanu et al, 2000, Stoessel et al, 1993) can also be present in high concentrations. Yet, the extent to which such acids affect carbonate dissolution has yet to be quantified.

The present study sought to determine the amount of calcite dissolution due to proton generation from microbially mediated sulfide oxidation in submerged, fresh-water caves. Two columns, representing the cave environment, were constructed in lab; one column was inoculated with sulfide-oxidizing bacteria, whereas the second column lacked the bacteria necessary to conduct sulfide-oxidation, and therefore acted as a control. Because the sulfide-oxidizing bacteria obtained from the cave did not grow in the column, the two columns were treated as replicates to determine the appropriateness of the two column comparison system to eventually determine the extent of biologically mediated sulfide oxidation on calcite dissolution.

The cave system was also modeled using the reaction-path model PHREEQCI in order to gain quantitative insight into the amount of calcite dissolution due to both abiotic and biotic sulfide oxidation. The modeling results revealed no dissolution due to abiotic sulfide oxidation, but indicated a maximum amount of 228.8 mg/L calcite dissolution per mL of groundwater due to bacterial sulfide oxidation, demonstrating the potential for cave speleogenesis and enlargement due to biologically mediated sulfide oxidation.

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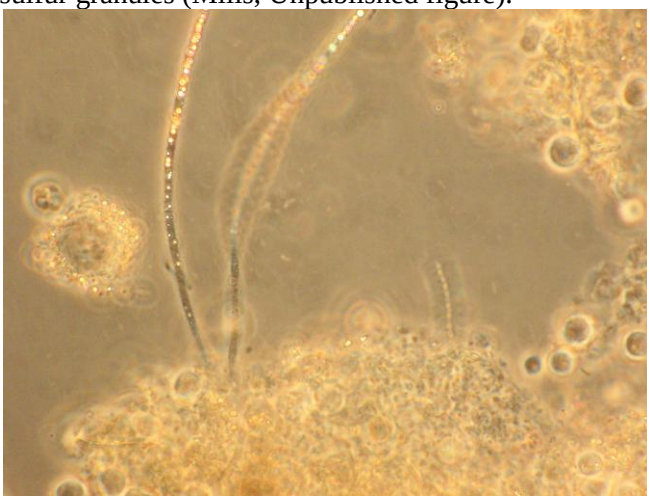
INTRODUCTION

The extent of microbially mediated sulfide-oxidation on limestone dissolution is a relatively new topic in the study of karst ecosystems and the extent to which dissolution occurs has yet to be quantified. Sulfur-oxidizing bacteria, however, have been a subject of study since the discipline of microbial ecology first formed. These bacteria were first extensively studied in sulfide-rich springs where large, white mats of filamentous bacteria were found (Dworkin, 2012). Each mat was composed of long filaments of bacteria full of sulfur granules (Fig. 1). Even at the time of discovery, Sergei Winogradsky was able to deduce that the deposition of sulfur granules was attributed to the oxidation of hydrogen sulfide to elemental sulfur. He then speculated on the chemoautotrophic nature of sulfide-oxidizing bacteria through his work with *Beggiatoa*, and their reliance on sulfur oxidation as an energy source (Winogradsky, 1889). Since then, mats of sulfide-oxidizing bacteria, identified by the internal deposition of sulfur granules, have been found in many ecosystems with high sulfide concentrations, including soils and in both fresh- and salt-water marshes (Ehrlich and Newman, 2009).

Figure 1: Filamentous bacteria *Beggiatoa*, full of shiny sulfur granules (Mills, Unpublished figure).



It was not until the 1980's, however, with the use of deep sea submersibles, that these sulfide-oxidizing microorganisms were found in aphotic ecosystems. Microbial mats dominated by *Beggiatoa* and *Thiothrix* were found surrounding high temperature, sulfidic hydrothermal

vents on the sea floor. As previously observed, the microorganisms used the oxidation of H_2S to element sulfur (S^o) as an energy source, but unlike the previous ecosystems studied, the subsequent deep sea hydrothermal vent ecosystems were entirely dependent on these sulfide-oxidizing, chemolithotrophic bacteria for energy and organic-matter production. Due to the lack of dependence on photosynthetic organic-matter, deep sea hydrothermal vents became one of the first aphotic ecosystems studied (Jannasch and Mottl, 1985).

Deep-sea hydrothermal vents, however, are not the only ecosystems based on chemo- rather than photo-autotrophic bacteria. Hypogenic cave communities, devoid of any light, are based on energy produced by chemoautotrophs as well. Many examples of these caves exist; from submarine caves in Italy to large caverns in Mexico (Bottrell et al, 1991; Hose et al, 2000; Sarbu et al, 1996; Vlasceanu et al, 2000; Mattison et al, 1998). Similar to the deep-sea hydrothermal vents, sulfide-oxidation, in which H_2S is oxidized to S^o, and, sometimes, to sulfate, is the energy source for carbon fixation, i.e. organic matter synthesis. In each cave, microbial mats were observed in areas with high H_2S concentrations. These areas can occur along the water-atmosphere interface in cave pools and streams, or more commonly, at the interface between the cave conduit and the limestone cave walls where sulfide rich groundwater seeps through the bedrock (Mattison et al, 1998). These mats contained large numbers of sulfide-oxidizing bacteria that could potentially have an impact on limestone dissolution and cave enlargement (Hose et al, 2000, Macalady et al, 2006, Engel and Randall, 2011, Sarbu et al, 1996, Vlasceanu et al, 2000, Stoessel et al, 1993).

Calcite, the main reactive mineral in limestone, is easily dissolved in the presence of H^+ ions in solution and is therefore sensitive to the acidity of natural water. Due to this sensitivity, acids, such as carbonic acid are usually considered to increase dissolution of calcite (White,

1988). The H^+ ions in solution react with calcite ($CaCO_3$), resulting in dissolution (Eqns. 1 and 2).

$$H_2CO_3 \leftrightarrows HCO_3^- + H^+ \quad (1)$$

$$CaCO_3\,(s) + H^+ \leftrightarrows Ca^{2+} + HCO_3^- \quad (2)$$

One of the products of sulfur oxidation (Eqns. 3 and 4) is sulfuric acid which ultimately dissociates to H^+ ions in aqueous solutions (Ehrlich and Newman, 2009):

$$H_2S\,(aq) + \tfrac{1}{2}\,O_2 \leftrightarrows S^o + H_2O \quad (3)$$

$$S^o + 1\,\tfrac{1}{2}\,O_2 + H_2O \leftrightarrows 2H^+ + SO_4^{2-} \quad (4)$$

An increase in calcite dissolution is hypothesized, due to the production of acid when sulfide oxidation, either biotic or abiotic, is present.

The extent of sulfuric-acid speleogenesis has a potentially large impact on the rate of cave enlargement. In several subaerial caves containing microbial mats, very low pH readings were measured within the water droplets hanging from the mats, indicating oxidation from elemental sulfur to sulfate and a resulting increase in H^+ ions (Hose et al, 2000; Sarbu et al 1996). The pH of the droplets (known as 'snottites') has been recorded to be between 0 and 1, yet, the cave streams remain near neutral. Such an observation indicates that the acidic water produced by the sulfur-oxidizing bacteria is quickly neutralized during calcite dissolution from the cave walls (Hose et al, 2000, Eqn. 2). Very few studies, however, have sought to quantify the extent and the rate of calcite dissolution due to microbially mediated sulfide oxidation.

One recent study conducted in the sulfide-rich, saline portion of the Edwards Aquifer in Central Texas found increased amounts of calcite dissolution in the presence of sulfide-oxidizing bacteria when compared to abiotic calcite dissolution *in situ* (Engel and Randall, 2011). The Edwards aquifer is developed in limestone and includes abundant, filamentous, microbial mats

along the walls of the open-hole wells, which were determined to contain sulfide-oxidizing bacteria. The study used microcosms suspended in the well holes, one only containing pure calcite chips and a second inoculated with sulfide-oxidizing bacteria, to determine the effect of biologically mediated sulfide-oxidation on dissolution. The amount of dissolution was determined by the microcosm weight before and after inoculation and characterized visually by scanning electron microscopy to determine the amount of etching on the calcite chips. While the rate for biotic calcite dissolution was not reported by the authors, the study was the first to show direct evidence for an increased amount of calcite dissolution due to sulfide oxidizing bacteria in limestone aquifers.

Accelerated calcite dissolution due to microbial processes has also been demonstrated and successfully monitored in the laboratory (Jacobson and Wu, 2009). The study found a two-fold increase in calcite dissolution due to the microbially mediated conversion of ammonium to ammonia for nutrient uptake and subsequent biomass incorporation by heterotrophic bacteria. The conversion of ammonium to ammonia and subsequent nitrogen incorporation into microbial cell biomass, releases H^+ ions into solution, decreasing the pH and alkalinity. This acidifying reaction is coupled by the increased pCO_2 in solution due to rapid respiration attributed with fast microbial growth, resulting in additional dissolved carbonic acid. By comparing an abiotic batch reactor containing growth medium and pure calcite chips with a biotic batch reactor inoculated with the heterotrophic bacteria, the amount of calcite dissolution due to microbial growth and ammonia uptake was determined. The amount of calcite dissolution was monitored by noting the amount of etching along the calcite surface using scanning electron microscopy (SEM), monitoring changes in chemical parameters such as pH, alkalinity, and calcium concentrations over time, and by differences in the weight of the calcite chips before and after

microbial incubation. The study proved the feasibility of measuring the rate and amount of calcite dissolution in the laboratory even in the presence of weak acid formation due to microbial growth and substrate uptake.

Up to this point, most aphotic caves with abundant chemoautotrophic bacteria that have been studied were either subarerial or located in saline waters. However, submerged, freshwater caves have been shown to contain extensive microbial mats as well, mainly composed of sulfide-oxidizing bacteria (Franklin et al, 2005). The Wekiwa Springs cave system located in central Florida is a submerged, freshwater, cave with profuse bacterial mats growing on the walls, floor, and ceilings of the water-filled conduit (Fig. 2). The groundwater seeping through the limestone cave walls originates from the Floridan aquifer system and contains a sulfide concentration of as much as 1-3 mg/L, however, sulfide is not usually detected in the flowing cave water (Mills, unpublished results). The removal of sulfide is credited to the presence of abundant sulfur-oxidizing bacteria, often existing in microbial mats as thick as 10 cm. Little detritus from the surface has been found in the aphotic zone due to the large discharge of water exiting the cave, however, invertebrate organisms such as amphipods, isopods, and crayfish have been found, indicating the main organic carbon source for the cave ecosystem is the sulfide-oxidizing bacteria (Franklin et al, 2005).

Figure 2: Image of microbial mats from the Wekiwa Springs cave system. The mats are long, white, and filamentous. Note the divers hand at right for scale (Mills, unpublished figure).

Research Questions

The presence of sulfide-

oxidizing bacteria in fresh water cave systems has been well documented and the potential for biologically mediated calcite dissolution has been widely hypothesized. Yet, the extent to which sulfide oxidizing bacteria contribute to calcite dissolution has not been described in detail or quantitatively. This study sought to determine the extent of calcite dissolution due to sulfide-oxidizing bacteria by:

1) Mimicking the cave environment in a pair of batch reactors to determine:
    a. The extent of abiotic and biotic sulfide oxidation
    b. the amount of calcite dissolution due to sulfide oxidation
2) Using the modeling program PHREEQCI to:
    a. Produce an additional comparison to the abiotic batch reactor
    b. Theoretically model the extent of calcite dissolution based on the parameters of the cave environment.

The work carried out in the study was completed in hopes of better understanding the extent of biospelogenesis in limestone caves and determine the potential for increased porosity in an important subsurface environment often relied on as water sources for human consumption.

METHODS

Study Site

No-Mount cave, part of the Wekiwa cave system is located in Wekiwa Springs State Park north of Orlando, Florida. The cave is initially fed by groundwater from the Floridan aquifer system which consists of a sequence of limestone, dolomitic limestone, and dolostone layers. The Floridan aquifer is confined by the overlying Hawthorn formation composed mostly of lower permeability clay, silt, and sand beds (Miller, 1986). The submerged entrance of the cave lies along a horizontal bedding plane at the bottom of a large, natural spring basin. There is a small photic zone at the entrance followed by a narrow opening into a larger aphotic cavern that penetrates down into the limestone bedrock at a small angle. The aphotic zone is dominated by

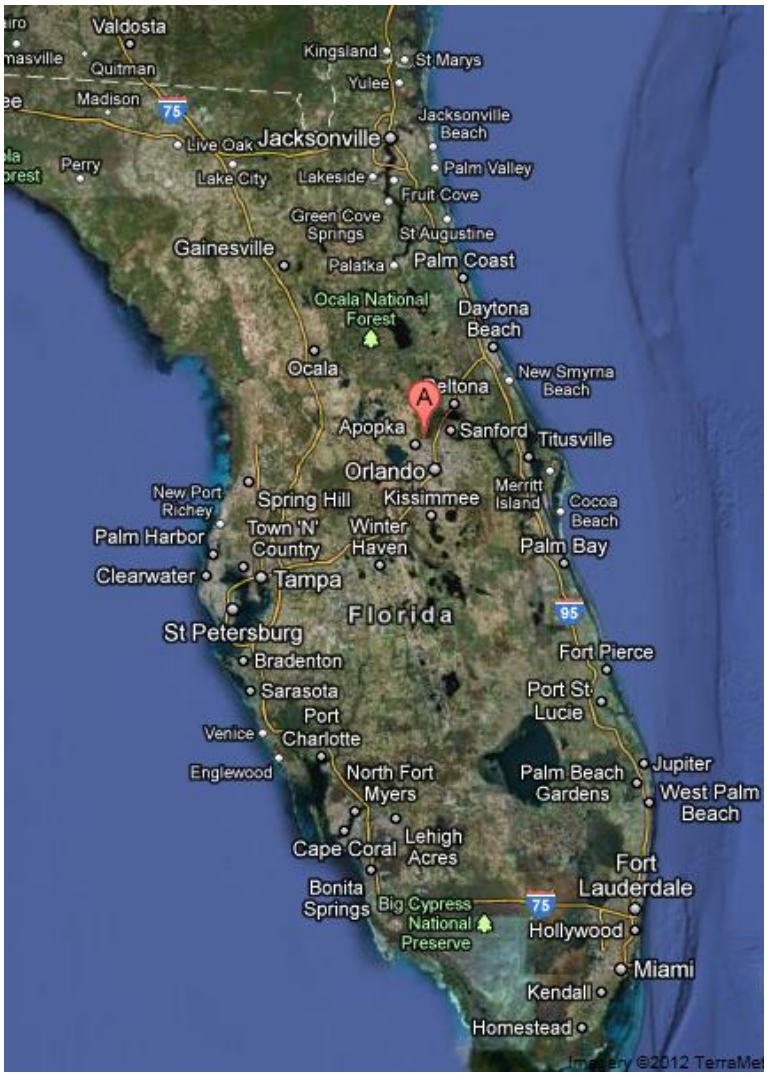


Figure 3: Satellite image of the Wekiwa Springs Cave location (red flag). The cave is in the Wekiwa Springs State Park near Orlando, Florida at 28° 42.69'N, 81° 29.49'W.

large, filamentous bacterial mats which support an ecosystem of small invertebrates and can only be accessed by specially trained SCUBA divers (Herman et. al, unpublished manuscript).

Although the layered limestone, dolomitic limestone, and dolostone of the Floridan formation is composed of calcite, dolomite, and high-magnesian calcite, calcite is considered the predominant and most reactive mineral in the formation. Additionally, the formation contains insoluble clastic grains including sand (predominantly quartz) and silts and clays (predominantly illite and kaolinite). Trace amounts of apatite (a calcium phosphate mineral) have been found in the limestone formation (Miller, 1986). Due its abundance and great reactivity, however, all modeling and calculations for the present study will be based on calcite.

Batch Reactors

To determine the effect of biologically mediated sulfur oxidation on calcite dissolution, two columns were constructed to simulate the environmental and biological conditions in the submerged No-Mount cave (Fig. 4). One column, termed biotic, was inoculated with sulfide-oxidizing bacteria on top of crushed limestone, both of which were obtained from the cave. A second column was left un-inoculated to serve as a control. The chemical composition (pH, alkalinity, Ca^{2+}, HS^-, Cl^-, NO_3^-, SO_4^{2-}) of the discharge from each column was determined from samples collected twice during each week over a continuous four week period. The composition of the discharge water was compared between the two columns to determine the effect of sulfur oxidation on carbonate dissolution.

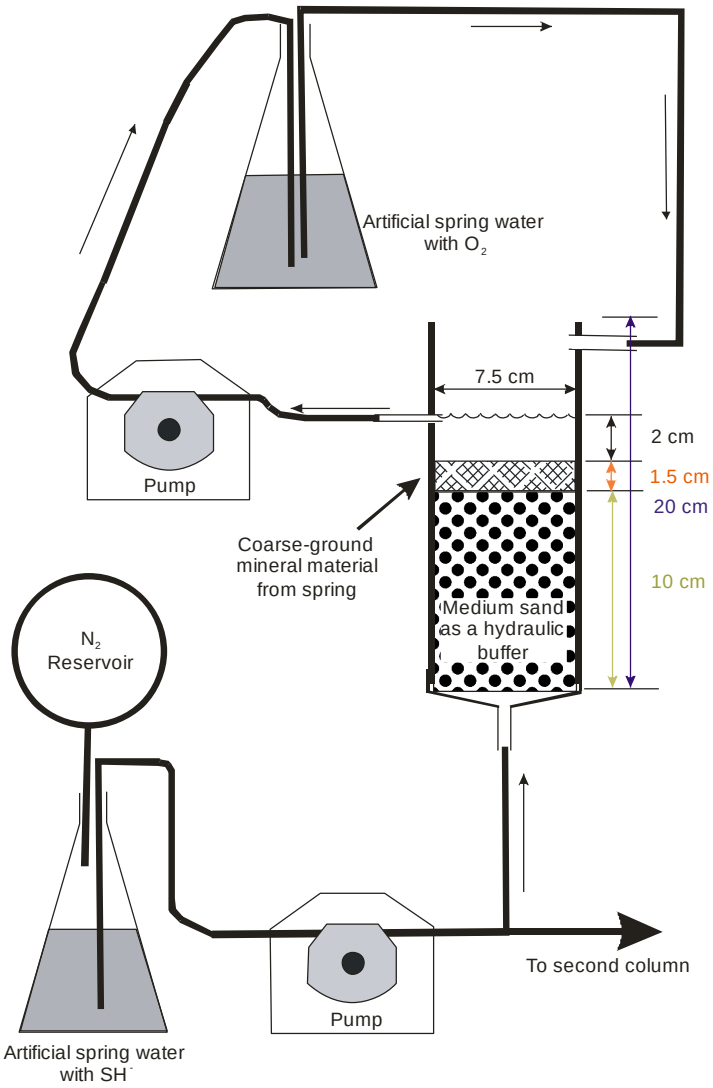


Figure 4: Schematic of a single batch reactor. Artificial groundwater from the same reservoir was pumped to both columns. There were two separate reservoirs for the flowing cave water in the head space above each column (Mills, unpublished figure).

Each column was constructed of 7.5-cm diameter PVC pipe (Schedule 40), about 20 cm in length. The bottom was filled with a 10-cm layer of 0.5-1mm grain-size sand on top of which was placed a 1.5 cm

layer of 0.5-1mm crushed carbonate obtained from the Wekiwa caves. The sand acted as a hydrological buffer to ensure uniform flow through the column, while the carbonate imitated the limestone cave walls and created the interface between the groundwater and cave water where abiotic and microbiological sulfide-oxidation could occur.

For both columns, water was pumped from the bottom of the column through the sand and carbonate layers at a discharge rate of about 4.6×10^{-4} mL/s, resulting in a 9.5-day residence time of water in the sediment layer of the column and a 30.5-hr residence time within the limestone layer. The chemical composition, pH, and temperature of the water is similar to that of the groundwater seeping through the cave's limestone bedrock walls (Table 1).

In order to reduce oxygen saturation in the artificial groundwater, the solution was sparged with N_2 gas to displace the oxygen and a headspace of N_2 left above the groundwater reservoir. A mylar balloon provided makeup N_2 at ambient pressure that displaced water as it was pumped from the reservoir. This ensured the dissolved oxygen concentrations were low enough to accurately reflect the low oxygen concentrations observed in the groundwater surrounding No-Mount cave.

Table 1: Chemical Composition of Groundwater. The chemical composition is similar to that measured in wells around Wekiwa cave. There is an excess of Na^+ in order to create an equal charge balance. Na_2SO_4, $NaNO_3$, NaCl and NaS were used to generate the various anion concentrations.

	Concentration (mg/L):
Cl^-	4.98
NO_3^-	0.573
SO_4^{2-}	16.1
Na^+	12.1
Dissolved O_2	0 ppm
HS^-	0.78
pH	5.37

Table 2: Chemical Composition of the cave water. The chemical composition is essentially the same as the groundwater; however, no sulfide was added. This generates a 0 mg/L base line for which additional sulfur oxidized either abiotically or biotically can be compared.

	Concentration (mg/L):
Cl^-	4.97
NO_3^-	0.630
SO_4^{2-}	16.2
Na^+	11.0
Dissolved O_2	Equilibrated with atmosphere
pH	8.5

In the head space above the crushed carbonate, water was pumped through the column, across the surface of the carbonate, at a velocity of 3.03 mL/s resulting in about a 2.0 cm layer of water on top of the calcite layer with a residence time of about 30-seconds. The chemical composition of the artificial cave water was the same as the artificial groundwater, except sulfide was left out (Table 2). The difference of pH results from the addition of Na_2S to the artificial groundwater solution. The Na_2S raises the pH to about 8.5 as the sulfide dissociates from sodium to form HS^- in solution, resulting in an increase in OH^- ions. Since no measurable amounts of sulfide have been observed in the Wekiwa cave water, it was assumed that all sulfide from the groundwater was used by the sulfur oxidizing bacteria resulting in a 0 mg/L breakthrough of sulfide into the cave.

About 50 mL of water was collected from the artificial cave water reservoir for analysis twice a week from each column for 4 continuous weeks. The samples were taken via a glass tube inserted into the cap covering each cave-water reservoir. Some constituents (pH, alkalinity, and Ca^{2+}) were measured immediately after collection. Others (Cl^-, SO_4^{2-}, and NO_3^-) were determined on refrigerated samples within two weeks of sample collection. Samples for thedetermination of dissolved sulfide were fixed with Zn-acetate as described by Otte and Morris (1994) and were analyzed within a week of collection. The chemical composition of the water samples from the two different columns was compared over time. By comparing the water chemistries from the columns, the impact of sulfur oxidizing bacteria on carbonate dissolution can be estimated.

Chemical Analysis

For each water sample, pH, temperature, alkalinity, and Ca^{2+} concentration was determined when the water sample was collected. The pH was measured using a pH electrode.

Alkalinity was determined through the Inflection Point Titration Method as described by USGS (2006). Calcium concentrations were determined by titrating the water sample with EDTA as described in Standard Methods (USPHS, 1989). For better color change resolution at the titration end point, 0.4 g of Erichrome blue indicator, instead of 0.2 g indicator, was used per titration.

The anions Cl^-, SO_4^{2-}, and NO_3^- were determined by ion chromatography within 2 weeks of sampling. Sulfide concentrations in Zn-acetate preserved samples were measured using the colorimetric method described by Otte and Morris (1994) and Cline (1969) within one week of each sampling.

Calcium concentrations and alkalinity were used to determine the saturation index with respect to calcite. The chemical speciation model WATEQ (Ball and Nordstrom, 2001) was used to determine saturation indices for all water samples. WATEQ is a computerized model of simultaneous equilibria amount solutes and mineral phases. Computation is based on thermodynamic data and results in a quantitative description of aqueous speciation and saturation indices with respect to mineral phases for an input water sample.

The saturation index is an indicator of how far the water sample is from equilibrium with respect to calcite and is calculated based on the pH, alkalinity, and calcium concentrations using:

$$SI_c = \log \frac{\gamma_{Ca2+}[Ca^{2+}]\gamma_{HCO_3-}[HCO_3^-]K_2}{a_{H+}K_c} \quad (4)$$

where SI < 0 indicates that calcite dissolution will occur, SI = 0 indicates the solution is at equilibrium with respect to calcite and no further dissolution or precipitation will occur, and SI > 0 indicates that calcite precipitation will occur. The saturation index was compared between water samples from the two columns to determine if sulfur oxidation increases the capability of the system to dissolve calcite and whether the saturation index changes over time based on the

expected accumulation of sulfate in the cave water due to continuing abiotic or biotic sulfide oxidation.

Reaction Path Modeling

PHREEQCI (Parkhurst and Appelo, 1989) is a reaction-path model based on thermodynamic data and equations to calculate the equilibrium state of a natural water of known composition with respect to the surrounding geologic setting (*i.e.* the minerals in the rock in contact with the natural water). The program is able to calculate the resulting chemical composition of aqueous solutions after the mixing of two solutions, after equilibrating with a gas phase, and after equilibrating with various minerals. In this study, PHREEQCI was used to model a variety of scenarios, including the column system in the laboratory and the cave system *in situ* with both abiotic and biotic interactions. The scenarios are described below along with explanations of assumptions used during modeling.

a. Modeling the abiotic column

Modeling the abiotic column helped to demonstrate that the modeling capabilities under various assumptions could accurately reflect a physical experimental system. If the results obtained from the modeling accurately reflected the chemical concentrations obtained from the abiotic batch reactor, the subsequent modeling results for the abiotic cave system could be assumed to reasonably reflect the actual cave environment. Each scenario was input into PHREEQCI as a series of steps, which are outlined in Table 3 for the abiotic column.

The amount of calcite dissolved in each reaction step was approximated using the calcite dissolution rate reaction first described by Plummer et-al (1979), the approximate surface area of contacted calcite, and the residence time of the solution with the calcite layer. The rate of reaction is defined by:

$$R = k_1[H^+] + k_2 k_{CO_2} P_{CO_2} + k_3 - k_4[Ca^{2+}][HCO_3^-] \quad (5)$$

where, k_1, k_2, k_3, k_4 are calculated at T = 288K by:

$$\log k_1 = 0.198 - \frac{444}{T} \quad (6)$$

$$\log k_2 = 2.84 - \frac{2177}{T} \quad (7)$$

$$\log k_3 = -5.86 - \frac{317}{T} \quad (8)$$

Table 3: Steps, assumptions, and explanations for the laboratory abiotic column PHREEQCI modeling scenario.

Step 1: Artificial groundwater in contact with calcite layer	a) Solution 1: 1 mL artificial ground water defined using the average of pH, alkalinity, Cl^-, NO_3^-, SO_4^{-2}, HS^-, and Na^+ of all *in vitro* groundwater solutions made (Table 1) b) Equilibrium 1: Artificial groundwater allowed to equilibrate with calcite until SI = 0. Based on the rate calculated (eqn. 4), average residence time, and exposed surface area calcite would dissolve until equilibrium was reached.
Step 2: Resulting groundwater solution mixing with artificial cave-water and equilibrating with an oxygenated atmosphere	a) Solution 1 saved after step 1 was used as the groundwater solution b) Solution 2: 1,000 mL artificial cave water defined using averages of pH, alkalinity, Cl^-, NO_3^-, SO_4^-, and Na^+ for all *in vitro* cave water solutions made (Table 2) c) Oxygenated atmosphere simulated with 9 mg/L dissolved O_2 in the artificial cave water d) Mix 1: Groundwater solution and artificial cave water mixed in a 1:1 ratio such that 1 mL of groundwater mixed with 1000 mL of artificial cavewater.
Step 3: Mixed groundwater/cave water solution allowed to react with a layer of calcite and equilibrate with ambient lab pCO_2	a) Solution 3 saved after step 2 b) Reaction 2: Solution 3 was allowed to react with 0.2717 mol calcite, as was determined using the rate for calcite dissolution, residence time in the column headspace, and surface area of the exposed calcite. c) The resulting solution was equilibrated with a pCO_2 of $10^{-3.3}$ atm (Jacboson and Wu, 2009).
Step 4: Resulting 'circulating' cave water allowed to react with calcite layer in varying SI values based on results measured from the abiotic column *in vitro*	a) Solution 4 saved from step 3 b) Reaction 3: calcite was allowed to react with the solution to varying SI values.

$$\log k_4 = -7.56 + 0.016T - 0.64 \log P_{CO_2} \quad (9)$$

and the $[H^+]$, $[Ca^{2+}]$, and $[HCO_3^-]$ are the concentrations of these ions in the water sample of interest.

From Eqn. 4, a rate expressed in mmol/cm^2/s is obtained for calcite dissolution. In order to determine the amount of calcite dissolved, the residence time of the ground water solution in the calcite layer was determined using:

$$Tr = \frac{V\varphi}{q} \quad (10)$$

where the residence time is related to the specific discharge, q, which is equal to the groundwater flow rate through the column, the volume, v, is equivalent to the volume of the calcite layer, and the porosity, φ, was determined for the calcite layer (φ = 0.75).

Finally, the surface area of the calcite in contact with 1 mL of groundwater was estimated. An average grain diameter of 0.75 mm was used to determine the total surface area of calcite in the 1.5-cm-thick calcite layer. It was assumed that the calcite grains were arranged in the typical rhombohedra packing, where each grain is in contact with a total of 12 other grains. Using the equation for specific surface area, M of open-packed sediment grain from Taylor et-al, 1990:

$$M = \frac{\pi}{\left(\frac{1}{\sqrt{2}}\right)d} \quad (11)$$

where d is the diameter of the average grain, an estimate for the total amount of reactive calcite surface in the calcite layer was obtained by multiplying the specific surface area by the total number of calcite grains. The total amount of reactive surface area calculated was 1.23×10^9 mm^2.

The total amount of calcite (in number of moles) dissolved was then calculated from the rate, residence time, and exposed surface area and used in the model scenario. Mainly due to the

high reactive surface area exposed to dissolution, the amount of estimated calcite dissolution due to the artificial groundwater was high and it was assumed that the solution would have sufficient residence time and amount of exposed calcite to reach a saturation index of 0.

A similar procedure was used to determine the total amount of calcite dissolved after the ground water solution and artificial cave water were mixed and exposed to an oxygenated atmosphere. However, the residence time was calculated for the headspace above the calcite layer and the surface area of calcite was approximated as the thin top layer of calcite grains in the column. It was assumed that only half of the calcite grain surface area was exposed to calcite dissolution with respect to the circulating cave-water solution now occupying the headspace above the calcite layer.

The modeling results were then compared to the results obtained from the circulating water in the two abiotic columns *in vitro*. For each column and each sampling period, a saturation index was calculated based on the measured pH, alkalinity, and Ca^{2+} concentration. The saturation index obtained was then set in PHREEQCI to obtain theoretical values for pH, alkalinity, and Ca^{2+} concentration for the corresponding time step and column. This allowed for a direct comparison between the results obtained from the abiotic column *in vitro* and the results obtained from the PHREEQCI modeling.

b. Modeling the abiotic cave environment

The abiotic cave environment was modeled based on the assumptions made in the abiotic column modeling scenario and previous groundwater and cave-water data collected from No-Mount cave (Table 4).

c. Modeling the biotic cave environment

Table 4: Outline of modeling scenarios and assumptions used for the abiotic cave modeling scenario.

Step 1: Groundwater in contact with calcite	a) Solution 1: Ground water concentration as defined by well-water samples obtained from the surrounding limestone aquifer (Table 5). b) Equilibrium 1: the solution was equilibrated with a pCO_2 of $10^{-2.5}$ atm, a typical value for a limestone aquifer. c) Equilibrium 2: the solution was allowed to equilibrate with the calcite cave walls until SI = 0. It is assumed that the ground water is in contact with calcite for a long enough residence time to equilibrate completely.
Step 2: Groundwater mixing with circulating cave-water	a) Solution 1: solution obtained after the ground water equilibrated with calcite b) Solution 2: cave-water concentration as defined by measurements taken in the cave system (Table 6). c) Mixing 1: The mixing ratios were varied to determine the amount of groundwater needed to effect calcite dissolution.
Step 3: Resulting circulating cave-water dissolving calcite	a) Solution 3: solution obtained after step 2 b) Reaction 1: no calcite dissolution will occur based on the negative rate of reaction determined.

For the biotic cave environment, several different scenarios were run each using slightly different assumptions with respect to the degree of biotic sulfide oxidation. The composition of the groundwater measured in the Floridan aquifer surrounding No-Mount cave (Table 5) and the cave water in No-Mount cave (Table 6) were used as the defined solutions in the model. The

Table 5: Analyzed water from a 62.5 m deep ground-water well in the limestone aquifer surrounding the Wekiwa cave system. A triplicate of samples was analyzed for the site (Herman et al, unpublished manuscript).

	Concentration (mg/L)
Cl^-	5
NO_3^-	0.3
SO_4^{2-}	16
Na^+	9
Dissolved O_2	0.1
HS^-	1.3
pH	8.2
HCO_3^-	147.2
Ca^{2+}	791.3

Table 6: Analyzed cave-water from the deepest point within the aphotic cave zone in No-Mount Cave. A triplicate of samples was analyzed for the site (Herman et al, unpublished manuscript).

	Concentration (mg/L)
Cl^-	13
NO_3^-	4.3
SO_4^{2-}	18
Na^+	10
Dissolved O_2	0.5
HS^-	0
pH	7.4
HCO_3^-	182.2
Ca^{2+}	306.4

general scenario for each model is outlined in Table 7 along with the variations

Currently, no stoichiometry or rate of reaction has been formulated for sulfide-oxidizing bacteria; however, based on a series of assumptions, the amount of biological sulfide oxidation due to sulfide-oxidizing bacteria was approximated. While there is no published reaction stoichiometry, the estimated energy obtained by the microorganisms during oxidation has been determined. For the partial-oxidation from sulfide to S^o, a total of 177.31 kJ/mol is gained while the complete oxidation from sulfide to sulfate yields a much greater energy yield of 744.49 kJ/mol for sulfide-oxidizing bacteria found in a geothermal, limestone well in Vulcano, Italy (Amend et al, 2004). Based on the high amounts of energy obtained and assuming there is no limitation on bacterial growth and hydrogen sulfide was the limiting reagent in all reactions, it was assumed that all hydrogen sulfide will be oxidized to either sulfur or sulfate. This assumption is consistent with the lack of measurable sulfide in No-Mount cave water.

The first scenario, termed "high-extreme", assumed the bacteria would fully oxidize

Table 7: Assumptions for each step in the biotic cave environment scenarios.

Step 1: Groundwater solution in contact with calcite	a) Solution 1: Groundwater composition sampled from a well located near No-Mount cave in the limestone aquifer (Table 5). b) Equilibrium 1: solution equilibrates with respect to calcite. It is assumed that the water is in contact with calcite for enough duration to achieve SI = 0.
Step 2: Modeling Microorganisms	a) Three different biological sulfide oxidation rates were used: i) High extreme: complete oxidation from sulfide to sulfate ii) Low extreme: half oxidation from sulfide to sulfur granules iii) Mid-scenario: all sulfide oxidized to sulfur; half sulfur oxidized to sulfate
Step 3: Equilibrium with calcite and pCO_2	a) Equilibrium 2: resulting solution is assumed to be in contact with the calcite long enough to equilibrate to SI = 0 b) Equilibrium 3: solution is equilibrated to a cave pCO_2 of $10^{-2.5}$ atm.

sulfide to sulfate generating the maximum amount of acidic protons during complete sulfide oxidation (Eqns. 3 and 4). The second scenario assumed a more conservative stance where the entire sulfide concentration of the natural water was only partially oxidized to S^o and ultimately stored in the bacterial cell as sulfur granules resulting in production of OH^- anions and no acidic protons (Eqn. 3). The third scenario modeled was based on the combination of the two previous scenarios and observations of *Thiothrix* and *Beggiatoa* in the cave environment. In this scenario it was assumed that all the sulfide in the groundwater was oxidized to S^o and stored intercellularly, as has been observed in cells acquired from No-Mount cave (Herman et al, unpublished manuscript). Then, based on the low pH measurements recorded at the interface of the biofilms, it was assumed that half of the sulfur granules were subsequently oxidized to sulfate, generating acidic protons (Hose et al, 2000).

RESULTS

Batch Reactors

Despite repeated attempts, the sulfide-oxidizing bacteria obtained from No-Mount cave did not grow (Appendix 1). Therefore, a comparison between the biotic and abiotic column did not occur. A comparison, however, between the two abiotic columns over a 4 week period helped to demonstrate the feasibility and reproducibility of the column comparison system to be used to determine the effect of biologically mediated sulfide oxidation on calcite dissolution if growing cells were present on the calcite layer.

The two columns were compared over time by using the ratio of the analyte concentration in column 1 to that of column 2 (Fig. 5). The ratio gives an indication of how different the two columns were with respect to the major variables in calcite dissolution; pH, alkalinity, calcium,

and the saturation index. There was high variability for both alkalinity and calcium with relatively constant results for pH and the calculated SI.

With respect to the concentration of anions over the sampling period, only chlorine showed an increasing trend in concentration over time. Both sulfate and nitrate showed no relationship over time. Both columns showed the same trends in anion concentration, however, column 2 had slightly higher concentrations of all anions, especially as time increased.

Both columns showed calcite dissolution in the presence of abiotic sulfide oxidation and dissolution due to dissolved carbonic acid. Because no sulfide was detected in any of the column samples, it was assumed that all sulfide added to the artificial groundwater was chemically oxidized when exposed to oxygenated cave water at the calcite layer/circulating cave water interface. Like biotic sulfide oxidation, abiotic sulfide oxidation generates the same amount of H^+ ions in solution and subsequently can increase carbonate dissolution.

Visually, both cave-water reservoirs increased in turbidity over the entire sampling period despite repeated washings before the period began, indicating carbonate dissolution due to

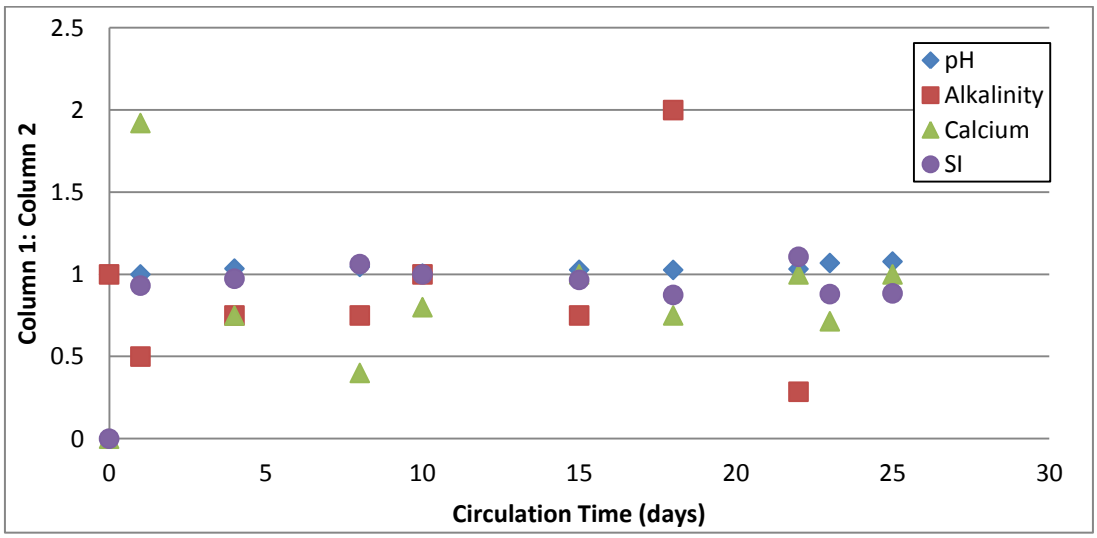


Figure 5: Ratio between Column 1: Column with respect to pH, alkalinity (mg/L), calcium (mg/L), and Saturation Index (SI) over the period of cave water circulation. Note the high variability with respect to alkalinity and calcium, but the relatively constant pH and SI values.

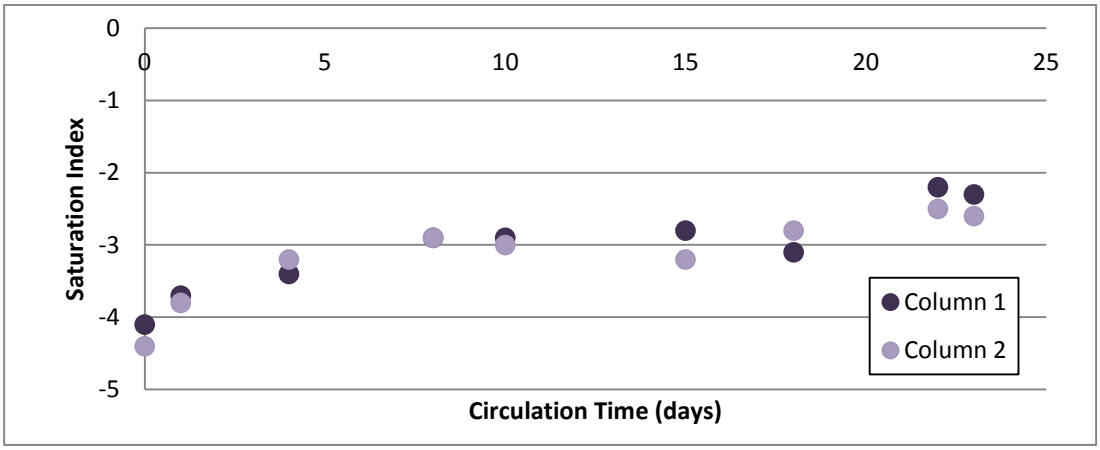


Figure 6: Saturation index over sampling period for both columns. There was a general increase towards equilibrium between the solution and solid phase calcite over time for both columns.

abiotic reactions. In addition, the saturation index increased towards 0 over time indicating calcite dissolution over the entire sampling period as the solution sought to equilibrate with respect to calcite (Figure 6).

Reaction Path-Model

a. Abiotic Column Modeling

The experimental column results were compared to the theoretical modeling results to determine how well the assumptions made in running the model reflected the physical experiment. For each water sample a saturation index was calculated. The saturation index was then used in the abiotic column modeling to determine the expected pH, alkalinity, and calcium

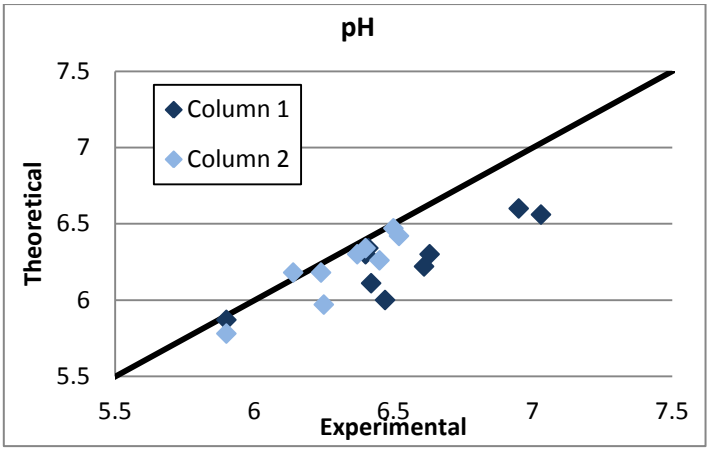
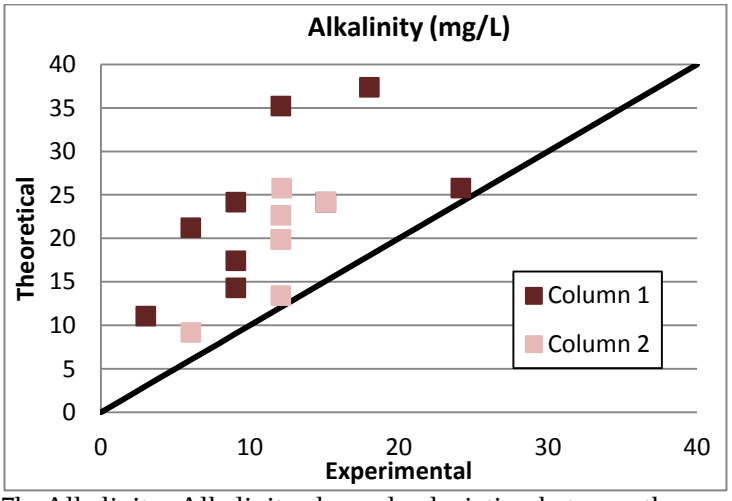
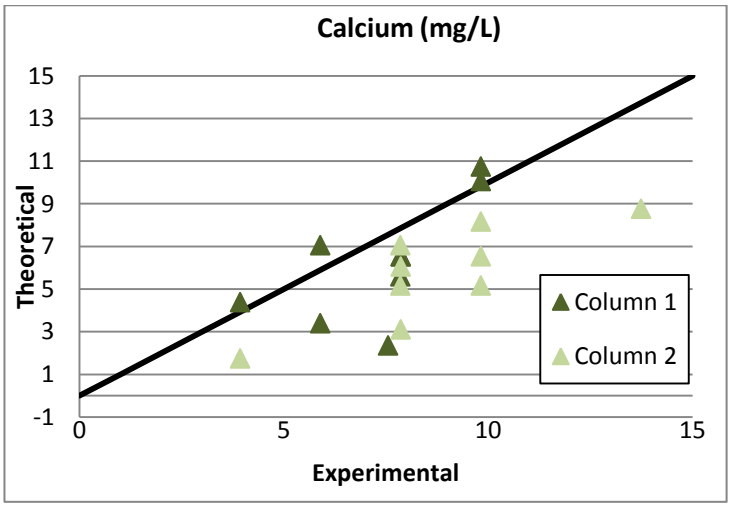


Figure 7a: Comparison between column results (experimental) and modeling results (theoretical) for each set SI at each sampling period. Time is not plotted as no trends with respect to time emerged.
pH: pH showed relative agreement between the theoretical and experimental results for both column systems.



7b. Alkalinity: Alkalinity showed a deviation between the theoretical and experimental results, trending towards higher alkalinity for the modeling results.



7c. Calcium: Calcium also showed a deviation between the theoretical and experimental results, but unlike alkalinity, the experimental results were consistently higher than the theoretical results.

concentrations based on the PHREEQCI model. For each parameter, pH, alkalinity, calcium concentrations, the experimental results were plotted against the theoretical results (Figure 7a-c).

For both column and model systems, there was general agreement between the experimental and theoretical results, especially with respect to pH. However, there was slight deviation for both alkalinity and calcium. Alkalinity was consistently lower for the experimental results when compared to the theoretical results. Since the experimental saturation index (Eqn. 4) for the experimental column results was used in the modeling, the lower experimental alkalinity measurements when compared to the theoretical results generated consistently higher calcium concentrations for the theoretical when compared to the experimental results.

b. Modeling the Abitoic cave environment

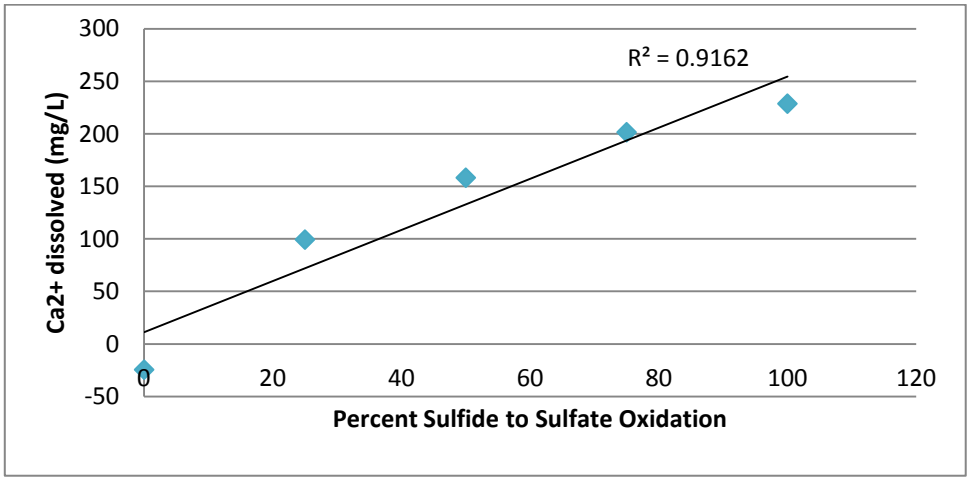


Figure 8: The relationship between percent of biologic oxidation from sulfur to sulfate and its effect on calcite dissolution. The amount of dissolution was modeled for 4 different scenarios, resulting in a positive-linear relationship with an acceptable R^2 value of 0.9162.

The abiotic cave modeling scenario indicated no dissolution would occur at the limestone wall surface due to abiotic sulfide oxidation, even when the mixing ratio between the groundwater and the cave water was 1:1. However, the model does indicate some dissolution within the pore space due to abiotic sulfide oxidation occurring as the sulfide oxidizes over long periods of time in the small oxygen concentrations of the groundwater. With respect to the biotic modeling, it can be assumed that all dissolution at the groundwater, cave interface will be due to biologic as opposed to chemical, sulfide oxidation.

c. Modeling the Biotic Cave Environment

Four different scenarios were modeled to determine the percent of biological sulfur oxidation versus the amount of calcite dissolution (Figure 8). It was assumed that all sulfide was oxidized to S^o and therefore, only the amount of S^o oxidized to sulfate varied among the scenarios. The difference in calcium concentration between the starting solution and the resultant solution was used as a proxy for calcite dissolution. Since calcite is the only variable in the defined modeling system capable of producing calcium ions, this assumption is considered a valid approximation.

A linear regression line was fit to the data and can be used to approximate the amount of calcite dissolution due to biological sulfide oxidation based on the percent of sulfur to sulfate oxidation. The data showed a positive-linear relationship between sulfide oxidation and calcite dissolution.

DISCUSSION

Batch Reactors

The results obtained from the two columns displayed reproducibility such that a similar arrangement could be used to detect differences due to microbiological activity when microbes are present in the system. There was a reasonable amount of variability and experimental noise in the two columns, indicating that a high amount of biologic sulfide oxidation would be necessary in order to see an effect on calcite dissolution; however, the modeling results strongly suggest that the amount of dissolution due to microbial sulfide oxidation should be large enough to overcome the background noise, even at only 25% oxidation from S^o to sulfate which results in about 99.4 mg/L calcite dissolution per 1 mL of groundwater.

Reaction-Path-Model

a. Abiotic Column Modeling

The abiotic column modeling scenario was mainly conducted to determine if the assumptions made in the modeling could accurately reflect the composition of a physical experimental solution, in this case the two replicate columns. Throughout the sampling period, the modeling results accurately reflected the results obtained in the *in vitro* columns. Calcium and alkalinity, despite showing similar patterns due to the relationship between the two in the saturation index calculation, did exhibit some variability between the experimental and

theoretical results. Calcium concentrations tended to be higher for the columns than the modeling, while alkalinity tended to be lower for the columns and higher in the modeling.

For the modeling results, out-gassing of CO_2 (g) was limited, resulting in higher bicarbonate concentrations in the theoretical solution when compared to the experimental solution. The higher alkalinity values in the modeling would lead to lower theoretical calcium concentrations based on the set saturation index, ultimately leading to the inverse relationship seen between the theoretical and experimental results. The tendency towards the modeling of a closed system is not as accurate when the experimental system is open, such as the columns *in vitro*, but when modeling another closed system, such as the cave environment, the results produced by the model should more accurately reflect the solution composition.

b. Modeling the Abiotic Cave Environment

Based on the relatively good agreement between the experimental column results and the theoretical modeling results, the assumptions used in the modeling are assumed to be accurate and could then be used to model the cave environment *in situ*. The abiotic cave modeling results indicate dissolution in the pore space, potentially leading to increased porosity within the limestone bedrock, but indicates no dissolution on the limestone cave-wall surface. The oxygen concentrations in the circulating cave water may be too low to support a large enough scale for abiotic sulfide oxidation to promote additional dissolution. In addition, the dilution factor between the incoming groundwater and the already circulating cave-water may be too high for the high sulfide and therefore, high sulfide-oxidizing potential groundwater to make much of an impact on the calcite-dissolving capacity of the circulating cave water. A better estimate for abiotic oxidation could be obtained if the flux of groundwater through the entire cave-wall system and the total amount of circulating cave-water was known for the cave environment;

however, the modeling, despite significantly increasing the ratio between groundwater and circulating cave water, indicates the amount of calcite dissolution due to abiotic sulfide oxidation along the cave-wall surface will be small.

c. Modeling the Biotic Cave Environment

Based on the results obtained for the abiotic cave modeling, it is assumed that all oxidation on the cave wall-surface for the biotic cave modeling scenario was due to biologic sulfide oxidation. In addition, because no sulfide has been detected in neither No-Mount cave nor the abiotic columns, it can be assumed that all the sulfide present in either the natural or artificial groundwater is completely oxidized to S^o or sulfate by the bacteria in the microbial mats.

Significant dissolution due to sulfide oxidation was obtained when the bacteria were modeled to oxidize all sulfide to sulfate. The high amount of dissolution is expected based on the production of two protons in the complete oxidation of sulfide (Eqns. 2 and 3). However, this assumption does not model the biotic system accurately. From field observations, sulfide-oxidizing bacteria deposit sulfur granules intercellularly; the microorganisms typically only oxidize the sulfur grains to sulfate when sulfide concentrations are low (Ehrlich and Newman, 2009). This indicates that not all the sulfide is fully oxidized to sulfate, but instead are, at least to some extent, deposited intercellulary as S^o granules.

At the other extreme, the microorganisms could only oxidize the sulfide to S^o and never produce sulfate and the subsequent proton's needed to effect calcite dissolution. During this scenario, no additional calcite is dissolved as a result of biological activity and the model suggested instead that calcite would precipitate from the solution. This extreme, as was the case with sulfate generation, is not expected based on observations from the field. The pH of water in

the micro-environments surrounding the biofilms has been measured in some subaerial caves to be as low as 0 (Hose et al, 2000, Sarbu et al, 1996). The low pH readings are attributed to the generation of acid during the biotic oxidation of sulfide at least partially to sulfate, but were measured before the generated acid was diluted and carried away into the larger pH neutral cave stream system.

These two observations suggest that the amount of sulfate produced by sulfide-oxidizing bacteria must lie somewhere in the middle of the two extremes. For modeling purposes, the assumption that all sulfide is oxidized to S^o and only half of the sulfur is oxidized to sulfate is a decent approximation and yields a dissolution of 158.3 mg/L of calcite per 1 mL of groundwater. This estimate assumes the oxidized water is in contact with calcite long enough to reach equilibrium.

In this specific modeling scenario, the mixing ratio was assumed to be 1:1 for the groundwater and circulating cave water, however, this is not a valid assumption. More information is needed to the groundwater flux into the cave and the amount of circulating cave water to more accurately predict the mixing ratio. The changing mixing ratio could result in more dissolution if the groundwater flux is greater than the amount of circulating cave water or there is the potential for less dissolution if the cave water is present in large enough quantities to sufficiently dilute the acidic groundwater.

In addition, the model assumes the acidic groundwater is in contact with the limestone wall long enough for calcite to reach equilibrium (SI = 0). This may also be a false assumption, especially if the circulating cave water is moving at a rapid enough velocity to quickly and will sufficiently mix the groundwater with the circulating cave water.

Although, it is possible that the high dissolution rates obtained from the model are accurate for the cave environment, especially in microenvironments and pore spaces. The filamentous bacteria that make up the large biofilms are often rooted in previously formed pore spaces along the limestone wall. As the filaments obtain energy from sulfide-oxidation, the resulting acid generate is trapped in the pore space, allowing ample time for the solution to approach equilibrium with respect to calcite. This type of microenvironment would allow for the high amounts of calcite dissolved that were reflected in the modeling results. If each pore space in the limestone wall contained filaments of sulfide-oxidizing bacteria, a large amount of cave biospeleogenesis could occur due to biologically mediated sulfide oxidation.

In addition, the hydraulics of the biofilms could allow increased contact time between the acidic water produced by the sulfide oxidizing bacteria and the limestone cave wall. Typically, filamentous microbial mats are found in areas of turbidity (Macalady et al, 2008). Due to the high turbidity and the filamentous nature of biofilms, there is a large amount of friction between the biofilm strands and the circulating cave water (Stoodley et al, 1997). Because of this friction, water at the biofilm interface, including the acidic water produced due to biologically mediated sulfide oxidation, will have a significantly slower velocity when compared to rest of the cave conduit water. This slower velocity could allow enough time along the cave-wall and biofilm interface for the acidic solution to come into equilibrium with the calcite wall, resulting in high amounts of dissolution.

More information on the physical cave-environment and the structure of biofilms is needed to determine the effect of pore spaces and filamentous biofilms have on the residence time of the acidic water with the cave-wall. However, these factors do indicate that the

assumptions made in the modeling scenario and the resulting high rate of calcite dissolution could be valid in the *in situ* cave environment.

CONCLUSIONS

Despite a lack of bacterial growth, the results of this study indicate a strong potential for increased calcite dissolution, and ultimately cave enlargement due to sulfide-oxidizing bacteria. Based on the PHREEQCI modeling results, up to 228.8 mg/L of calcium could be dissolved per square centimeter, however, the more conservative estimate of 158.3 mg/L per square centimeter of dissolved calcium is expected. Under either scenario, the presence of sulfide-oxidizing bacteria in a limestone aquifer would indicate accelerated calcite dissolution. The study also sets groundwork for future research in the actual rate and stochiometry for biotic sulfide oxidation, subsequent rate of calcite dissolution, and rate of cave enlargement by demonstrating the use of a two column comparison system between abiotic and biotic sulfide-oxidation and subsequent calcite dissolution in a simulated environment.

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APPENDIX

Appendix A: Bacteria Growth

A series of growth media was used in an attempt to grow sulfide-oxidizing bacteria, such as *Beggiatoa* and *Thiothrix*, from three different microbial mat samples obtained from No-Mount cave. The first sample was taken about three years ago and had been refrigerated since sampling. The second sample was taken from the cave system by SCUBA divers during the Summer of 2011 and refrigerated since sampling. The third sample was a sediment sample taken from the aphotic zone in No-Mount cave during October, 2011 and refrigerated upon sampling and was thought to have contained bacterial cells. Ideally, a recent biofilm sample would have been obtained and used for growth and subsequent column inoculation; however, due to the isolation and degree of difficulty in obtaining samples, no new samples were obtained within the constrained time period.

The first media was composed of artificial cave water (Table 2) with added sodium sulfide. The cultures were continuously mixed on a shake place to ensure an oxygenated environment. Each of the three samples from the cave system were introduced into three separate flasks of growth medium. This procedure resulted in no additional sulfur granule formation in any of the samples, despite what appeared to be filamentous growth.

The second media was adopted from Williams and Unz, 1989 and Eikelboom, 1975. The same three samples were used. No new sulfur granule deposition was monitored but rapid growth of an unidentified bacterial contamination was evident both with the naked eye and under a microscope.

Filamentous, microbial mats mainly composed of *Beggiatoa* were grown from samples collected in a salt-water marsh along the Eastern Shore of Virginia (samples courtesy L. Blum). Both *Beggiatoa* from the cave environment and *Beggiatoa* found in salt water marshes use

sulfide-oxidation as an energy source, indicating that the samples from the marsh would be analogous to samples from No-Mount cave (Howarth, 1984). The bacteria were grown in a direct simulation of the marsh environment.

To accurately represent the marsh environment, sodium sulfide was laid below a mud layer containing organic plant material and bacterial biofilm. The entire solution was immersed in the artificial cave-water with additional NaCl. The buried sulfide represents the slow diffusion of hydrogen sulfide through the anoxic marsh sediments to the aerobic boundary near the sediment surface. This allows the sulfide-oxidizing bacteria to colonize the aerobic - anaerobic interface where high sulfide concentrations co-exist with low oxygen concentrations, making the ideal environment for biologically mediate sulfide oxidation (Jorgensen & Revsbech, 1983).

Appendix B: Data Tables

Table A1: Raw Data for both columns at each sampling time. Note: n.d. indicates no samples were taken.

Circulation Time	pH	HCO_3^- (mg/L)	Ca^{2+} (mg/L)	SI	HS^- (mg/L)	SO_4^{2-} (mg/L)	Cl^- (mg/L)	NO_3^- (mg/L)
Day 1								
Column 1	5.9	3.02	7.55	-4.1	0	17.20	5.030	0
Column 2	5.9	6.04	3.93	-4.4	0	17.20	5.030	0
Day 4								
Column 1	6.47	9.06	5.89	-3.7	0.008	29.9	5.47	0.90
Column 2	6.25	12.08	7.86	-3.8	0	31.11	5.44	0.92
Day 8								
Column 1	6.42	9.06	3.93	-3.4	0	26.14	5.62	0.71
Column 2	6.14	12.08	9.82	-3.2	0	31.56	5.63	0.89
Day 10								
Column 1	6.40	15.10	7.86	-2.9	0	22.78	5.87	0.59
Column 2	6.37	15.10	9.82	-2.9	0	28.55	6.05	0.48
Day 15								
Column 1	6.63	9.06	7.86	-2.9	0	23.34	5.87	0.31
Column 2	6.45	12.08	7.86	-3.0	0	28.50	6.25	0.45
Day 18								
Column 1	6.41	24.16	5.89	-2.8	0	22.24	5.37	0
Column 2	6.24	12.08	7.85	-3.2	0	27.88	6.27	0
Day 22								

Column 1	6.61	6.04	7.85	-3.1	0	19.92	5.45	0
Column 2	6.40	21.14	7.85	-2.8	0	24.89	5.90	0
Day 23								
Column 1	6.95	18.12	9.82	-2.2	0	n.d.	n.d.	n.d.
Column 2	6.05	n.a.	13.72	-2.5	0	n.d.	n.d.	n.d.
Day 25								
Column 1	7.03	12.08	9.82	-2.3	0	19.67	5.19	0
Column 2	6.52	18.12	9.82	-2.6	0	26.27	6.45	0