FATE OF ELEMENTAL SULFUR IN A SULFIDIC CAVE AQUIFER MIXING ZONE

A Thesis in
Geosciences
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2017
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ABSTRACT

The Frasassi Cave system (Italy) is intersected by a perennially sulfidic deep aquifer that mixes with oxygenated meteoric water in the upper 1-10 meters. It therefore presents an ideal setting to study dark sulfur cycling under oxygen-limiting conditions. Conspicuous white biofilms are ubiquitous in the cave streams and pools within the oxic-anoxic mixing zone of the aquifer. Elemental sulfur concentrations in biofilms are 20 to 25% S(0) by mass, while underlying anoxic sediments contain less than 5% S(0). I analyzed paired biofilm-sediment samples using Illumina high-throughput sequencing with universal (bacterial and archaeal) 16S rRNA primers and found that the most common biofilm type is dominated by Gamma-, Beta-, Epsilon-, and Deltaproteobacteria. Underlying sediment microbial communities are nearly identical except for the lower abundance of Gammaproteobacteria related to freshwater Beggiatoa strains. My community composition data are consistent with Beggiatoa-dominated microbial mats in which cells orient along the oxic-anoxic interface at the sediment surface, as measured in microsensor profiles. Microsensor profiles indicate net acid production in the sediments but not in the biofilms, suggesting that sulfide is primarily oxidized to S(0) rather than sulfuric acid in the biofilms. Deltaproteobacteria populations in both biofilms and sediments are dominated by diverse relatives of Desulfocapsa thiozymogenes, an autotroph that carries out sulfur disproportionation in culture. Although the biochemistry and ecology of sulfur disproportionation are not yet well understood, my data suggest that S(0) disproportionation is an important sink for the large quantity of S(0) generated in the system.
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PREFACE

This thesis contains work contributed by multiple people. Sulfur concentration samples were collected by Daniel Jones in early field seasons. Microsensor profiles were completed by Lubos Polerecky and assembled by Daniel Jones. Samples for community composition analyses were collected by Daniel Jones, Amanda Labrado, and Jennifer Macalady. I personally completed fieldwork to obtain samples for iron analysis in summer 2016 with the help of Sandro Mariani, Maurizio Maniero, Jennifer Macalady, Pauline Henri, and Clara Chan.
ACKNOWLEDGEMENTS

I could not have completed the work presented in this thesis without the guidance and support of multiple individuals. I thank my thesis advisor, Jennifer Macalady, for fostering my passion for the cave environment and reviewing this thesis. I would also like to thank committee members Katherine Freeman and Chris House for helping me with research questions and guidance.

The Macalady lab members have been key in maintaining a stimulating working environment and always offered assistance. Muammar Mansor contributed time and effort with DNA extractions and was instrumental in carrying out iron analyses. Christy Grettenberger, Irene Schaperdoth, and Zena Cardman assisted with DNA extractions and laboratory training and experiments. Trinity Hamilton and Zena Cardman were incredibly helpful with high-throughput analysis and phylogenetic tree constructions. As well, I would like to thank my graduate cohort for lending a helping hand and ear when needed and for always providing encouragement.

Working on the project allowed me to visit and go caving multiple times in the amazing Frasassi caves of central Italy. I thank Alessandro Montanari at the Osservatoria Geologico di Coldigioco for coordinating logistics and introducing me to the geological setting. Sandro Mariani and Maurizio Maniero were extremely capable cavers, guides, and teachers.

Finally, I thank my family and friends for their encouragement throughout the process and for their love and support. Most importantly, I would like to thank my partner Erica Piteavage for help with geological and chemical concepts and for the many nights of engaging scientific discussion. As well, I would like to express my appreciation to The Pennsylvania State University Department of Geosciences for financial assistance. During my time at Penn State I received funding from the Pennsylvania Space Grant Consortium as a Graduate Student Research Fellow and the Krynine Travel Grant. Major funding came from the NSF FastLane Grant and the Little Salt Spring DDF Grant under P.I. Jennifer Macalady as well as one semester’s support from the Biogeochemistry Dual Title Program.
CHAPTER 1: INTRODUCTION

1.1 Geological context

The Frasassi caves in central Italy are an expansive, over 25 kilometers of mapped passages, hypogene cave system (Fig. 1.1). Located on the eastern side of the central Apennine Mountain chain, the cave system occupies the core of an anticline bisected by the Frasassi Gorge and Sentino River. The Frasassi anticline originated during the late Miocene during a tectonic compressive phase that lead to the uplift and emersion of the Apennine Mountains (Galdenzi and Maruoka 2003), and is still rising (Mariani et al. 2007). Marian et al. 2007 found that the Apennine area around the Frasassi caves has been rising for the last 8,000 years at an average rate of 0.6 mm per year. The cave system is located in the eastern limb of a northeast verging fold-and-thrust belt where faulting has likely enhanced the upward movement of sulfidic groundwater (Galdenzi and Maruoka 2003). The rocks hosting the cave system are underlain by the organic-rich Triassic Calcaria Raethavicula Formation (limestone) and anhydrites in the Triassic Burano Formation (Galdenzi et al. 2008). Microbial sulfate reduction in these uppermost Triassic formations is assumed to be the source of the sulfide and elevated dissolved solids in the aquifer that intersects the cave system. The caves are formed primarily in limestone of the 600 to 1000 meter thick Jurassic Calcare Massiccio Formation (Galdenzi and Maruoka 2003). The Calcare Massiccio Formation is composed of greater than 99% calcium carbonate and is overlain by the Bugarone Formation, which acts as an aquitard. Galdenzi et al. (2008) suggest that the Bugarone Formation channels meteoric water flow to the Calcare Massiccio Formation limestone exposed in the gorge, which then aids in
the formation of the cave system by creating a path of least resistance for water percolation to the water table (Fig. 1.2).

Elevations in the Frasassi region range from 200 meters above sea level in the valleys to as great as 1000 meters above sea level in the surrounding mountains (Galdenzi and Maruoka 2003). Cave entrances range from 200 to 500 meters above sea level with the majority of the Frasassi entrances varying between 200 and 360 meters (Mariani et al. 2007; Galdenzi and Maruoka 2003). The cave system itself is made up of four dominant levels with the older, non-sulfidic zones at higher elevations and actively forming, sulfidic zones at low elevation near the water table surface. The above-water passages form a ramiform, horizontal network of passages indicative of corrosion by sulfidic groundwater (Palmer 2001). Both active and relict meter-scale gypsum deposits are present (Mariani et al. 2007). Phreatic passages are a mix of anastomotic and network zones currently being explored by cave divers.

1.2 Hydrogeology

The Frasassi caves contain two chemically distinct water types: carbonate and sulfidic (Jones et al. 2015, Galdenzi and Maruoka, 2003; Tazioli et al., 1990; Cocchioni et al., 2003; Sighinolfi, 1990). Carbonate waters originate from meteoric water that percolates through voids in the Calcare Massiccio limestone formation before meeting the groundwater table. This vadose zone water is characterized by low salinity (~500 uS cm^{-1}), high dissolved oxygen (0.32 mM L^{-1}), and low sulfate concentrations (Galdenzi and Maruoka 2003). Using δ^{18}O, δD, and tritium isotopic analyses, Tazioli et al. 1990 found that the sulfidic groundwater likely has a meteoric origin and is recharged in an approximately 5 km² area at altitudes between 600 to 1000 meters above mean sea level.
However, the dissolved constituents of the two types of water are modified as the water passes along drip and flow paths in the vadose zone versus along groundwater flow paths in the underlying anhydritic Burano Formation. The sulfidic aquifer has higher salinity (up to 2 g L$^{-1}$), is enriched in sodium and chloride, is under saturated with respect to gypsum, is higher in sulfate (up to 2.5 mM L$^{-1}$), and has hydrogen sulfide concentrations up to ~ 600 mM L$^{-1}$ (Galdenzi and Maruoka 2003; Galdenzi et al. 2008; Macalady et al. 2008; Jones et al. 2015).

Sulfidic groundwater is diluted throughout the year with downward percolating carbonate water with seasonal variations from 30 to 60% (Galdenzi et al. 2008; Sarbu et al. 2000). Persistent spatial patterns in vadose water inputs result in Type 1 (less dilute) and Type 2 (more dilute) sulfidic waters. Sites PC (this study) and FS are characterized by Type 1 waters, with specific conductivity values between 2700-3400 uS cm$^{-1}$ and total dissolved sulfide concentrations between 325-600 uM. Sites GS and VC (this study) as well as CS and GB are characterized by Type 2 waters, with specific conductivity values between 1500-2300 uS cm$^{-1}$ and total dissolved sulfide concentrations <300 uM (Jones et al. 2015). Consistent with an important role for downward percolating vadose water in the cave system, stream levels and aquifer surface heights fluctuate seasonally by up to 50 cm. Oxygen concentrations in sulfidic waters range from below detection limits (<2 uM) to nearly saturated (>200 uM), but are most commonly <25 uM (Macalady et al. 2008). Redox disequilibrium between the oxidized carbonate water and reduced sulfidic water provides the necessary chemical energy for lithoautotrophic microorganisms to thrive.
1.3 Figures

**Figure 1.1:** Location map and plan view of the Frasassi cave system. Dark areas are mapped cave passages, dashed lines illustrate surface topography, and labels indicate sampling locations for this study. Base map from Mariani et al. 2007.
Figure 1.2: Schematic hydrogeologic cross-section of the Frasassi Gorge. The white area filled with perpendicular black lines represents the Calcare Massaccio Formation, a thickly bedded Jurassic platform limestone. The Calcare Massiccio Formation is overlain by the Bugarone Formation, which is a lower-permeability marly platform facies. Used with permission from Galdenzi and Maruoka, 2003.
1.4 References


Jones, Daniel S., Lubos Polerecky, Sandro Galdenzi, Brian A. Dempsey, and Jennifer L Macalady. 2015. Fate of Sulfide in the Frasassi Cave System and Implications for Sulfuric Acid Speleogenesis. *Chemical Geology* 410 (September). Elsevier B.V.


CHAPTER 2: FATE OF ELEMENTAL SULFUR IN A SULFIDIC CAVE AQUIFER MIXING ZONE

2.1 Abstract

The Frasassi Cave system (Italy) is intersected by a perennially sulfidic deep aquifer that mixes with oxygenated meteoric water in the upper 1-10 meters. It presents an ideal setting for studying dark sulfur cycling under oxygen-limiting conditions and is a potential window into subsurface processes that are widespread but inaccessible. Conspicuous white biofilms colonize the cave streams and pools in the oxic-anoxic mixing zone of the aquifer, and are dominated by freshwater Beggiatoa strains where water flow conditions allow for the accumulation of fine sediment. Sediment geochemistry and microbiology at Frasassi have not previously been investigated, but a recent microsensor study (Jones et al. 2015) suggested that sediment processes are important for elemental cycling and potentially for limestone dissolution. Microsensor profiles indicate net acid production in the sediments but not in the biofilms, suggesting that sulfide is primarily oxidized to S(0) rather than sulfuric acid in the biofilms. In order to gain further insight, I analyzed the community composition, S(0), and Fe concentrations in paired biofilm-sediment samples. Elemental sulfur concentrations in biofilms are 20 to 25% by mass, while underlying anoxic sediments contain less than 5%. Biofilms are dominated by representatives of 5 autotrophic, sulfur cycling Proteobacterial genera, plus multiple genera of anaerobic heterotrophs affiliated with the Bacterial phylum Bacteroidetes. Underlying sediment microbial communities are essentially identical to biofilms except for the much lower abundance of gliding filaments related to Beggiatoa and Desulfonema. Deltaproteobacteria populations in both biofilms and sediments are dominated by relatives of Desulfocapsa thiozymogenes, which carries out
S(0) disproportionation in culture. Although the biochemistry and ecology of sulfur disproportionation are not yet well understood, my data suggest that disproportionation is the primary sink for the large quantity of S(0) generated in the system.

2.2 Introduction

The majority of life remains hidden out of sight. Microorganisms inhabit every ecological niche on Earth while the preponderance of them survive in permanent darkness removed from the basking energy of the Sun. Caves offer a unique glimpse into one of the least understood dark biospheres on the planet and present an exciting setting to study biogeochemical cycles that generally escape observation. The research described in this study focuses on understanding the dynamic sulfur cycle within the perennially sulfidic Italian Frasassi Cave system with an emphasis on the largely neglected sulfur metabolism, sulfur disproportionation. Currently, relatively little is known about how microbes precipitate and utilize S(0) even though the majority of economic supergene sulfur deposits are thought to be biologically mediated.

Frasassi represents a small portion of caves (less than 10%) that is formed via hypogenetic groundwater processes in which hydrogen sulfide rich water from below reaches the groundwater table in actively forming cave passages (Palmer 2001). The cave aquifer is comprised of two sources: the sulfidic aquifer and oxygenated meteoric water percolating from the surface (Galdenzi et al. 2008). The mixing of these two water sources in morphologically different zones of the cave leads to two distinct water chemistries flowing through the system, type 1 waters with high hydrogen sulfide (H₂S) to oxygen (O₂) ratios and type 2 waters with lower H₂S to O₂ ratios (Jones et al. 2015).
Conspicuous white biofilms are found throughout the turbulent cave streams in the oxic-anoxic zone of the water–sediment/rock interface (Jones et al. 2015; Macalady et al. 2006; Macalady et al. 2008). These microbial communities are composed of sulfur-oxidizing *Epsilonproteobacteria*, *Gammaproteobacteria*, and *Betaproteobacteria* as well as sulfur-reducing *Deltaproteobacteria*. Numerous published studies from the Frasassi Cave system have characterized the sulfur-oxidizing bacteria (SOB) and their potential role in the formation of elemental sulfur (Jones et al. 2015; Macalady et al. 2006; Macalady et al. 2008; Hamilton et al. 2015). However, the sulfur-reducing bacteria (SRB) populations of these microbial mats have been largely overlooked. In addition, no study to date in the Frasassi Cave system has examined the microbial communities of sediments underlying stream biofilms and their function in the biogeochemical sulfur cycle. Therefore this study represents a missing link in understanding the microbial sulfur cycle of the Frasassi cave system (Fig. 2.1).

Interestingly, there is a large disparity between elemental sulfur S(0) concentrations in biofilms versus underlying cave sediments. Based on preliminary data from a previous study it seems that *Desulfocapsa spp.*, a sulfur disproportionating *Deltaproteobacteria*, are likely present in the system. If *Desulfocapsa spp.* represent a significant portion of the cave microbial population then it is expected that elemental sulfur disproportionation is likely an important sink of S(0) in the Frasassi stream sediments leading to the discrepancy in sulfur concentrations between the two niche spaces. Sulfur disproportionation is a cryptic sulfur metabolism that is only energetically favorable under low H₂S (<10mM) concentrations and an optimal pH range of 6.8-7.4 (Finster et al. 1998; Finster 2008; Finster et al. 2013; Janssen et al. 1996). These
conditions are present in the sediments below stream biofilms, based on micro-sensor profiles, and represent an ideal anoxic environment in which active sulfur disproportionation could lead to the removal of S(0) from the system.

2.3 Methods

2.3.1. Elemental analyses

Elemental analysis was carried out for total sulfur on 19 samples, 8 biofilm and 11 sediment, and total carbon, 4 biofilm and 6 sediment, at the Pennsylvania State University Agricultural Analytical Services Laboratory using EPA Method 3050B + 6010 using inductively coupled plasma atomic emission spectroscopy. Samples were collected between 2005 and 2011 from Ramo Sulfureo (RS), Grotta Sulfurea (GS), Pozzo di Cristalli (PC), Vecchio Condotto (VC), and Lago Verde (LV). It is important to note that Method 3050B is not considered a total acid digestion for most samples and is not able to breakdown silicate minerals. However, I was interested in biogenic sulfur and therefore could ignore sulfur that is locked up in silicate minerals.

2.3.2. Sample geochemistry

Microsensor profiles were created, in situ, of the biofilm-sediment interface in turbulent streams of the Frasassi cave system using the Caver Operated Microsensor System (COMS), as characterized in Jones et al. 2015, during a field expedition in 2010. The COMS are a smaller and more robust version of the Diver Operated Microsensor System described in Weber et al. 2007. Oxygen, pH, and total dissolved hydrogen sulfide (H₂S_T) concentrations were measured in biofilms and underlying stream sediments. Microsensor tips ranged from 20-30 µm in diameter and were packed with hygroscopic
beads and all contacts were sealed to reduce moisture and H₂S (g) vapors from interacting with COMS electronics and sensor tips during trips to sampling locations (Jones et al. 2015). Profiles were compiled from the average of three separate microsensor profiles collected within 1 cm² of microbial mats in contact with sediment surfaces (Jones et al. 2015).

2.3.3. High-throughput sequencing

Paired biofilm and underlying sediment samples were collected in Pozzo di Cristalli in 2006 and 2007, Grotta Sulfurea in 2010, and Vecchio Condotto in 2010. One paired biofilm-sediment sample from PC was collected in 2006 while another sediment sample was collected in 2007 to show annual similarities in microbial community structure. Three paired samples were collected in 2010, one in GS and two in VC. Samples were collected in sterile 50 ml Falcon tubes (Thermo Fisher Scientific Inc., Waltham, MA), RNALater was added (Sigma-Aldrich Corp., St. Louis, MO), and were frozen until processing at Pennsylvania State University.

DNA was extracted in replicates with blanks from samples in a clean fume hood using MoBio Power Lyzer Soil DNA isolation kit #12855-50 according to kit protocol (Mo Bio Laboratories Inc., Carlsbad, CA). The microbial community of each sample was analyzed by 16S tag sequencing (Illumina MiSeq platform) (Illumina Inc., San Diego, CA) using the 515FB and 806RB universal primers at the MrDNA Laboratory (Molecular Research LP, Shallowater, TX). The 16S rRNA primers were used in a 28 cycle PCR with the HotStarTaq Plus Master Mix Kit (Qiagen Inc., Hilden, Germany). The PCR cycle conditions were as follows: 94°C for 3 minutes, succeeded by 28 cycles
at 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute. Upon completion a final elongation step was carried out at 72°C for 5 minutes. Following sampled amplification, PCR products were assessed in 2% agarose gel to determine amplification success. Pooled samples were purified using calibrated Ampure XP beads. The purified PCR products were then used to compile the Illumina DNA library. Sequence data was processed using the MrDNA pipeline (Molecular Research LP, Shallowater, TX). Forward and reverse reads were joined, their barcodes were removed, sequences with less than 150 base pairs were removed, and ambiguous basecalls were removed. Operational Taxonomic Units (OTUs) were created by clustering at 97% similarity, chimeras were eliminated, and sequences were denoised. Final OTUs were classified according to taxonomy using BLASTn (SINA) from curated databases from NCBI (The National Center for Biotechnology Information, Bethesda, MD) and RDP (II).

2.3.4. *Desulfocapsa spp.* phylogenetic tree

A 16S phylogenetic tree was created from full-length 16S *Desulfocapsa spp.* sequences from the NCBI database. In total, 88 sequences were used to build the tree with two sequences representing a distant out-group, *Sulfurimonas autotrophica* and *Sulfurimonas denitrificans*. Of the remaining 86 sequences 72 were full-length *Desulfocapsa spp.* sequences of previous clone libraries from the Frasassi cave system. Sequences were aligned using ARB-SILVA’s online SINA aligner version 1.2.11. Pre-aligned sequences were then imported into ARB and final alignments were carried out before tree construction. The 16S phylogenetic tree was compiled via ARB’s Neighbor-Joining function using the bootstrap method (1000 bootstrap replications).
2.3.5. Fe in stream biofilms and sediments

Iron analyses were carried out on biofilm and sediment samples collected during July of 2016 and summer of 2014 from locations within PC. Sampling locations GS and VC were not sampled due to difficulties from a century flood years earlier. The VC sampling locations was removed all together due to the flood in the Sentino River Valley and due to hydrologic changes from the flood GS no longer supports a viable microbial community or flowing water. Samples were collected using sterile plastic pipettes, stored in 15 ml sterile tubes with no headspace, and placed on ice until transport to the laboratory at the Geological Observatory of Coldigioco. Samples were then placed into an anoxic chamber and 2 ml of sample was removed from each, capped with an N₂ headspace, and frozen until analysis at Pennsylvania State University.

Samples were then thawed at 4°C overnight in the Macalady Geobiology Laboratory and bulk-porewater was removed after being spun down at 8°C and 7,000 rpm for 15 minutes, samples were frozen overnight, and then freeze dried for 72 hours. Freeze dried samples were then powdered with a mortar and pestle and 100 mg of each was weighed into acid-cleaned 50 ml sample vials before carrying out acid dissolution steps. The iron extraction method used was based on Huerta-Diaz and Morse 1990 and Severmann et al. 2006 and adapted by Muammar Mansor.

Samples were leached with 6N HCl for 2 hours, shaking continuously, to remove reactive Fe, Fe-oxides, Fe-carbonates, and some Fe-silicates. Residual sample material was then centrifuged at 8000g for 10 minutes. Supernatant (SUP) was decanted into a clean 50 ml tube before being washed once with 10 ml of H₂O. After washing, the
sample material was centrifuged again at 8000g for 10 minutes and SUP was combined into previous tube, this represents the reactive Fe species in the HCl solution.

Remaining sample material was then leached with 10 ml 10N HF for 1 hour to remove Fe-silicates. Following the initial leaching the sample tube was centrifuged at 8000g for 10 minutes before the SUP was decanted into a clean 50 ml tube. Residual sample material was leached overnight a second time for greater than 16 hours before being centrifuged at 8000g for 10 minutes. SUP was then pipetted and combined with previous SUP tube. A final wash step of 10 ml H2O was carried out, followed by centrifuging at 8000g for 10 minutes, before the SUP was combined with the previous SUP tube. This SUP sample tube represents the fraction of Fe-silicates in the original samples.

Finally remaining sample material was leached with 5 ml of concentrated HNO3 for 2 hours, to ensure proper mixing tubes were inverted regularly. Following leaching, 10 ml of H2O was added to the sample before filtering through a 0.2 um filter into a clean 50 ml falcon tube. To ensure all material was collected the leaching tube was rinsed with 5 ml of H2O and filtered again through the previous filter into the new sample tube. This SUP consists of the pyrite fraction of the initial samples. All samples were then analyzed in triplicate using a HACH DR2700 Spectrophotometer (Hach Co., Loveland, CO). The Fe-HCl fraction was analyzed using the Ferrover Hach reagent for total Fe and the Ferrous Iron Hach reagent was used for Fe (II). Hach Ferrover reagent tests were also used to evaluate analytes in both the HF fraction and HNO3 fraction (Hach Co., Loveland, CO).
2.4 Results

2.4.1 Zero-valent sulfur concentrations

I observed abundant $S^0$ inclusions in all filaments in biofilm samples analyzed in this study via field microscopy, consistent with previous observations of *Beggiatoa* spp. morphology and ecophysiology at Frasassi (Macalady et al. 2006; Macalady et al. 2008; Rench 2015). Solid-phase $S^0$ concentrations were much higher in biofilms compared to underlying sediments (Fig. 2.2). In total, 8 biofilms and 11 sediments were analyzed over two field seasons. Biofilms contained much higher concentrations of $S^0$, with a maximum of 41.7% mass dry weight and a mean concentration of 18.9%. In contrast, concentrations of $S^0$ in underlying sediment samples ranged from 0.72% to 6.42% mass dry weight with a mean of 2.27%.

2.4.2 Total carbon concentrations

Total carbon concentrations were much higher in biofilms compared to underlying sediments (Fig. 2.3). In total, 4 biofilms and 6 sediments were analyzed over two field seasons. Biofilms contained much higher concentrations of carbon, with a maximum of 16.9% mass dry weight and a mean concentration of 14.1%. In contrast, concentrations of carbon in underlying sediment samples ranged from 5.3% to 8.4% mass dry weight with a mean of 6.5%.

2.4.3 Fe concentrations

Seasonal fluctuations in cave stream water levels periodically expose sediments to the cave air, resulting in Fe redox cycling that can be observed in the form of rust-colored staining on sediment surfaces during periods of low water (Fig. 2.4). Because iron and
sulfur cycling can be tightly linked in environments where strong redox fluctuations occur, I analyzed iron in paired biofilm-sediment samples. To my knowledge these data represent the first solid phase Fe analyses carried out in the Frasassi cave system to date.

The sample series PC16-24b, PC16-25b, and PC16-25c represent a biofilm sample (PC16-24b) and two directly underlying sediment samples. Sample 16-25c was the deepest sediment sample in the series and was black in color rather than grey like all other samples in this study. Samples PC14-11, PC14-13, and PC14-14 were sampled contemporaneously within 20 cm of each other in the same cave stream. Sample PC14-11 represents an *Epsilonproteobacteria* spp. dominated streamer attached to limestone rock surfaces and not directly in contact with sediments. Samples PC14-13 and PC14-14 are paired biofilm-sediment samples.

Total Fe concentrations (% mass dry weight) were higher in sediments compared to paired biofilms (Fig. 2.5A). Total Fe ranged from 0.90 - 2.03% with mean 1.51% in biofilms and from 1.85 – 3.33% with mean 2.55% in sediments. Iron in silicates represents the largest pool of iron measured in the system and followed the same trends as total Fe (Fig. 2.5B). Fe in silicates ranged between 0.58 – 1.14% in biofilms (mean 0.94%) and between 1.28 – 2.14% in underlying sediments (mean 1.59%). Iron in the pyrite pool was also higher in sediments and ranged between 0.09 – 0.23% in biofilms (mean 0.15) and 0.19 – 0.56% in sediments (mean 0.35%). Concentrations of reactive Fe (III) were similar in all samples (0.18 – 0.45% in biofilms with mean 0.30% vs. 0.34 – 0.73% in sediments with mean 0.50%). Concentrations of reactive Fe (II) made up the smallest pool and were also similar in all samples (0.04 – 0.24% in biofilms with mean 0.12% vs. 0.05 – 0.25% in sediments with mean 0.12%).
2.4.4 pH, O\textsubscript{2}, and H\textsubscript{2}S microsensor profiles

Vertical microsensor profiles through *Beggiatoa*-dominated Frasassi biofilms were measured in a previous study (Jones et al. 2015) a pattern with significant implications for understanding S\textsuperscript{0} cycling (Fig. 2.6). Oxygen and sulfide concentrations in the bulk water above the mats were 28 uM and 3 uM, respectively. Oxygen was completely consumed in the biofilm, resulting in an anoxic environment beginning at the base of the biofilm. H\textsubscript{2}S concentrations increased with depth from 3 uM to approximately 48 uM at the sediment-biofilm interface. Below the biofilm H\textsubscript{2}S concentrations increased with depth. The sediment zone yielded a range of 48-567 uM H\textsubscript{2}S concentrations to a depth of 3 mm below the biofilm-water interface. A significant pH decrease from 7.3 to 6.8 was observed below, but not within, the depth zone where H\textsubscript{2}S and oxygen overlap.

2.4.5 Microbial community composition

High-throughput sequencing using Illumina 515f/806r universal 16S rRNA gene primers revealed the microbial community composition in Frasassi stream sediments for the first time. Reads were affiliated primarily with Bacteria, with Archaea making up less than 7% of reads from any sample and generally less than 3% (Fig. 2.7). Eukaryote reads made up <1 to 6 % of the dataset and were affiliated almost exclusively with the protist genera *Stokesia*, ciliates common in a variety of environments, and *Paraclusilocola*, ciliates commonly found in the guts of oligochaete worms (Nana et al. 2014; Fokam et al. 2015). Bacterial reads made up 92- > 99% of the total reads in each sample. Four bacterial classes dominated in all samples independent of geochemical parameters and sample type. *Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria, and*
Epsilonproteobacteria collectively make up an average of 61% of bacterial reads (~70% in biofilms and ~55% in sediments) (Fig. 2.8).

Consistent with my expectation based on field observations, the biofilm samples all contained a significant proportion of reads affiliated with Beggiatoa (22-32%, Fig. 2.9, Fig. 2.10). Biofilm samples also contained a significant number of reads associated with the filamentous sulfate-reducing bacterial genus Desulfonema (1-9%, Fig. 2.9) (Janssen et al. 1996; K Finster, Liesack, and Thamdrup 1998; Kai Finster and Frederiksen 2003; Frederiksen and Finster 2004; K. W. Finster et al. 2013), which has previously been found at Frasassi in association with Beggiatoa (Macalady et al. 2006). When sequence reads affiliated with Beggiatoa and Desulfonema are removed from the dataset, biofilm samples and paired sediments have essentially the same community composition (Fig. 2.11). Neglecting differences in community composition due to Beggiatoa and Desulfonema, all samples are dominated by reads associated with 4 additional genera: Sulfurovum (Epsilonproteobacteria, Fig. 2.12), Desulfocapsa (Deltaproteobacteria, Fig. 2.13), Thiobacillus (Betaproteobacteria, Fig. 2.14), and Cytophaga (Bacteroidetes, Fig. 2.15). Reads associated with the genera Solitalea (Bacteroidetes), Anaerophaga (Bacteroidetes), Sphingobacterium (Bacteroidetes), Geobacter (Deltaproteobacteria), Bellilinea (Chloroflexi), Ignavibacterium, Verrucomicrobium, Paludibacter (Bacteroidetes), Spirochaeta, Lutibacter (Bacteroidetes), Longilinea (Chloroflexi), Flavobacterium (Gammaproteobacteria), Desulfobacterium (Deltaproteobacteria), Caldithrix, Syntrophus (Deltaproteobacteria), Arcobacter (Epsilonproteobacteria), Sulfuricurvum (Epsilonproteobacteria), and Bacteroides (Bacteroidetes) are also abundant across all samples. Combined, these 24 genera make up 75-90% of all Bacteria-
associated reads in each sample (Fig. 2.9).

*Sulfurovum* reads make up 5-26% of all reads (Fig. 2.8) and 60-98% of reads associated with Epsilonproteobacteria (Fig. 2.12). *Epsilonproteobacteria* reads associated with the genera *Arcobacter, Nitratiruptor, Sulfuricurvum,* and *Sulfurimonas* are also abundant across all samples.

Reads affiliated with *Desulfocapsa* make up the majority of *Deltaproteobacteria* reads in all samples except for biofilm sample PC07-20, in which *Desulfonema* reads are very abundant (Fig. 2.13). Other abundant *Deltaproteobacterial* reads were associated with the genera *Geobacter, Desulfobacterium, Syntrophus, Desulfobacca,* and *Desulfatibacillum* (Fig. 2.13). Reads representing more than 65 *Deltaproteobacterial* genera were retrieved in significant numbers. Bacteria affiliated with the Desulfobulbaceae family capable of long-distance electron transport (“cable bacteria”) were not detected in any of the samples.

Having identified *Desulfocapsa* spp. as potentially major players controlling the fate of zero-valent S in Frasassi streams, I extracted full-length 16S rRNA sequences related to *Desulfocapsa* spp. from the NCBI database and compared them to Frasassi 16S rRNA clones retrieved in previous studies. In total, 88 high quality and aligned sequences were used to compute the tree in Figure 2.16. Of the 88 sequences, strains *Desulfocapsa thiozymogenes* (X95181), *Desulfocapsa Cad626* (AJ511275), *Desulfocapsa sulfexigens* (CP003985), and *Desulfocapsa sulfexigens* (Y13672) represent cultivated isolates. All 71 strains of *Desulfocapsa* spp. retrieved from Frasassi cave streams branch most with the
species *Desulfocapsa thiozymogenes*, an autotrophic, freshwater, elemental sulfur-disproportionating bacterium.

*Thiobacillus* reads make up 3-17% of all reads (Fig. 2.7) and 81-94% of reads associated with *Betaproteobacteria* (Fig. 2.14). Closely related clones retrieved in other Frasassi *Beggiatoa* biofilms are most closely related to “*Candidatus Thiobacillus baregensis*” (Macalady et al. 2006; Jones et al. 2010).

Archaeal reads were primarily affiliated with Euryarchaeota (65-95%) and Thaumarchaeota (2-35%), with Thaumarchaeota consistently more abundant in sediments versus biofilms collected above them. Crenarchaeota reads were rare (<2%) (Fig. 2.17). The majority of samples were dominated by Euryarchaeota reads associated with either Methanomicrobia (genus *Methanosaeta*, 35-90 % of total Archaea) or Thermoplasmata (genera *Thermogymnomonas*, 3-45%, and *Thermoplamsa*) (Fig. 2.18, Fig. 2.19).

### 2.5 Discussion

2.5.1 Microbial sulfur cycling

Vertical oxygen, sulfide and pH microsensor profiles (Fig. 2.6) through Frasassi stream biofilms depict a setting in which oxygen is completely consumed at the sediment-water interface. Hydrogen sulfide concentrations increase with depth in the mat and continue to increase throughout the sediment profile with increasing depth. Opposing sulfide and oxygen gradients coincide with the vertical position of *Beggiatoa* filaments and suggest that *Beggiatoa* are oxidizing sulfide at the expense of oxygen (and possibly nitrate). Complete H$_2$S oxidation is accompanied by H$_2$SO$_4$ (sulfuric acid) production. However, pH in Frasassi microsensor profiles was constant through the zone of overlapping sulfide and oxygen gradients where sulfide oxidation and biofilm growth is
occurring, and decreased only in the sediment below the biofilm. This pattern is consistent with incomplete microbial $\text{H}_2\text{S}$ oxidation to $\text{S}(0)$ as suggested in a previous study (Jones et al. 2015). Sulfide profiles show that sulfide is not only present in the overlying bulk water, but is also being produced at depth within the sediment. Sulfate reduction (equation 1) consumes protons (Miao et al. 2012), whereas $\text{S}(0)$ disproportionation (equation 2) produces protons (Finster et al. 1998):

\begin{align*}
\text{Equation 1: } & \text{SO}_4^{2-} + 2\text{CH}_2\text{O} \rightarrow \text{H}_2\text{S} + 2\text{HCO}_3^- \\
\text{Equation 2: } & 4\text{S}^0 + 4\text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 3\text{HS}^- + 5\text{H}^+
\end{align*}

Therefore, $\text{S}(0)$ disproportionation rather than sulfate reduction may be the primary source of sulfide in the sediments. Together with the observation that $\text{S}(0)$ content in biofilms is much higher than in sediments (Fig. 2.2), the microsensor data inspired me to use a high-throughput 16S rRNA gene sequencing approach to investigate the community composition of the biofilms and underlying sediments.

Neglecting potential artifacts due to DNA extraction, primer biases, and pipeline protocol, Illumina amplicons revealed that biofilm and sediment communities are dominated by representatives of 5 bacterial genera: Beggiatoa, Sulfurovum, Thiobacillus, Desulfocapsa, and Cytophaga (Fig. 2.9). The cultivated Beggiatoa, Sulfurovum, and Thiobacillus strains most closely related to Frasassi strains oxidize reduced sulfur compounds such as sulfide, and are either autotrophs or mixotrophs (Campbell et al. 2006, Teske & Nelson 2006, Mori & Suzuki 2008).

\textit{Beggiatoa spp.} migrate vertically to take advantage of optimal geochemical parameters at the oxic-anoxic interface (Dunker et al. 2011; Berg et al. 2014; Schwedt et
Beggiatoa spp. are capable of incomplete sulfide oxidation to elemental sulfur and store elemental sulfur intracellularly. As well, Beggiatoa spp. are easily identified by their filament morphology under the microscope and due to their relatively large size (filaments up to 200 um in diameter) (Mills et al. 2004; Teske and Nelson 2006). The lack of cultivated strains of Beggiatoa spp. has lead to a limited knowledge of the phylogenetic diversity and metabolic abilities of marine and freshwater Beggiatoa. However, Beggiatoa spp. are prevalent in diverse environments where sulfide and oxygen overlap.

Studies of the few cultivated strains of marine Beggiatoa indicate that they are capable of fixing carbon by the Calvin cycle, however freshwater Beggiatoa are almost exclusively heterotrophic (Teske and Nelson 2006; Hagen and Nelson 1996). While Beggiatoa spp. are known sulfur oxidizers, using nitrate or oxygen as electron acceptors, they are also able to use pyruvate, ethanol, methanol, acetate, and succinate as electron donors via organotrophy (Hagen and Nelson 1997; Jewell, Huston, and Nelson 2008; Teske and Nelson 2006; Rench 2015). As well, all species of Beggiatoa are able to reduce intracellular sulfur in anaerobic environments using carbon (Grabovicz et al. 2001), hydrogen (Brock et al. 2011), and polyhydroxyalkanoates (Schmidt et al. 1987; Schwedt et al. 2011; Schwedt et al. 2012). Current knowledge of Beggiatoa spp. indicates that marine Beggiatoa are capable of utilizing nitrate as an electron accepter (Faheem and Khan 2009; Teske and Nelson 2006) however previous studies in the Frasassi cave system found that the freshwater species of Beggiatoa colonizing stream sediments is capable of nitrate reduction (Mills et al. 2004; Rench 2015). Additionally, the study by
Rench 2015 found genes encoding for RuBisCO, suggesting that freshwater *Beggiatoa* in Frasassi are most similar to known marine *Beggiatoa* strains.

Analysis of 8 metagenomes from Frasassi stream samples showed that Frasassi *Sulfurovum* strains are rTCA cycle autotrophs capable of oxidizing sulfide incompletely to $S^0$ with either oxygen or nitrate as a terminal electron acceptor (Hamilton et al. 2015). These conclusions are based on the analysis of 4 nearly complete genomic bins affiliated with *Sulfurovum* spp. In the same metagenomes, genomic bins affiliated with *Thiobacillus* spp. (*Gammaproteobacteria*) and *Sulfuricurvum* and *Arcobacter* spp. (*Epsilonproteobacteria*) contained complete SOX pathways and therefore have the genetic potential to oxidize sulfide completely to sulfate. Most of the *Epsilonproteobacteria* isolated to date can respire $S(0)$ with $H_2S$ as a waste product (Campbell et al. 2006). However, cultivated strains in the family Thiovulgaceae, including *Sulfurovum* and related genera like *Nitratifractor, Sulfurimonas, Sulfuricurvum,* and *Thiomicrospira,* seem to be the exceptions. Consistent with the findings of Hamilton et al. (2015), Thiovulgaceae strains including *S. lithoautotrophicum* typically grow by oxidizing sulfide and other reduced sulfur compounds using oxygen and/or nitrate as electron acceptors (Campbell et al. 2006).

Although *Thiobacillus* is a polyphyletic genus, all Frasassi *Thiobacillus* strains retrieved to date are related to cultivated members of the family Hydrogenophilae, which includes the genera *Halothiobacillus, Thiovirga, Thiofaba,* and others (Elshahed et al. 2003; Ito et al. 2005; Macalady et al. 2008; Jones et al. 2010) (Elshahed et al. 2003; Ito et al. 2005; Macalady et al. 2008; Jones et al. 2010; Tsao 2014). Although some representatives of all of these Hydrogenophilae genera have been retrieved from Frasassi
streams in prior studies, the most abundant strains (and the only strains retrieved from *Beggiatoa* biofilms) are most closely related to *Thiofaba tepidiphila*, an obligately lithoautotrophic sulfur-oxidizer, and even more closely related to a culture that has been lost, “*Candidatus* Thiobacillus baregensis” originally isolated from the sulfidic groundwater of a spring at Barège, France (Hédoin et al. 1996; Hédoin et al. 1996). Using a probe targeting close relatives of *Thiobacillus baregensis*, Tsao (2014) used Fluorescence In Situ Hybridization (FISH) to show that the relative population size of this species in Frasassi stream biofilms is not dependent on dissolved oxygen concentrations, dissolved sulfide concentrations, or on the ratio of their concentrations. Since other major populations of sulfur oxidizers in Frasassi streams are correlated with dissolved sulfide to oxygen ratios (Macalady et al. 2008), her result suggests that *T. baregensis* uses alternative or additional electron donors and acceptors, perhaps including S(0) and nitrate or nitrite. Consistent with the results obtained by Tsao (2014), a parallel study showed using metagenomic data that *T. baregensis* populations are RuBisCO autotrophs that can oxidize sulfur compounds completely to sulfuric acid using a complete SOX pathway and Sqr, Fcc, and SOR genes, and that can use nitrate as an electron acceptor (Hamilton et al., unpublished).

*Desulfocapsa spp.* are reverse CO-dehydrogenase autotrophs that are able to couple growth to the disproportionation of S(0) in addition to thiosulfate and sulfite (Janssen et al. 1996; K Finster, Liesack, and Thamdrup 1998; Kai Finster and Frederiksen 2003; Frederiksen and Finster 2004; Poser et al. 2013; K. W. Finster et al. 2013). Other genera in the same family Desulfobulbaceae (e.g. *Desulfobulbus, Desulfofustis*) have similar physiology but were not detected in my amplicon survey. *Desulfocapsa*
thiozymogenes, the freshwater strain most closely related to Frasassi strains (Fig. 2.16) is also able to grow as a sulfate reducer by oxidizing short chain alcohols to their corresponding fatty acids (Janssen et al. 1996).

Under standard conditions, elemental sulfur disproportionation is endergonic with a $\Delta G^0 = +41 \text{ kJ/rxn}$. In culture, D. thiozymogenes and D. sulfexigens are only able to grow via disproportionation in the presence of a sulfide scavenger such as Fe(III) (Thamdrup et al. 1993; Janssen et al. 1996; K Finster, Liesack, and Thamdrup 1998), equation 2.

$$4S^0 + 3Fe^{3+} + 4H_2O \rightarrow SO_4^{2-} + 3FeS + 8H^+$$

Equation 3: 4S$^0$ + 3Fe$^{3+}$ + 4H$_2$O $\rightarrow$ SO$_4^{2-}$ + 3FeS + 8H$^+$

In this reaction, Fe$^{2+}$ acts a sulfide scavenger keeping sulfide concentrations below 10 mM and allowing the reaction to be energetically favorable. However, under in situ conditions in Frasassi cave streams, the S(0) disproportionation reaction is exergonic without a sulfide scavenger and has a $\Delta G$ ranging from -89 kJ/rxn to -26 kJ/rxn. Hydrogen sulfide concentrations in Frasassi rarely exceed ~600 uM, and sulfate concentrations are roughly an order of magnitude lower than marine values. Nonetheless, I measured the concentrations of Fe species present in biofilms and sediments in order to calculate the energy available from S(0) disproportionation with sulfide scavenging (Fig. 2.5, Table 2.1). The presence of reactive Fe(III) and reduced Fe(II) indicate the potential for FeS products that would sequester sulfide and increase the energetic favorability of disproportionation. Based on equation 3 and in situ conditions in Frasassi streams, $\Delta G$ values for S(0) disproportionation range from -61 to -65 kJ/rxn in the presence of Fe sulfide scavengers. Therefore, in this system the presence of iron as a sulfide scavenger
does not affect the overall energetic favorability of the reaction. This is in direct contrast to modern marine environments in which sulfate values are greater and sediment sulfide fluxes can be much higher requiring the presence of a sulfide scavenger. The Frasassi system suggests this metabolism might have been important in the Earth’s past when ocean redox environments were anoxic to micro-oxic and sulfidic.

_Cytophaga_ affiliated reads (phylum Bacteroidetes) made up more than 5% of total reads in all samples, and are joined by 5 other genera in the Bacteroidetes phylum that are among the most abundant 24 genera in my amplicon libraries. Along with non-phototrophic members of the phylum Chloroflexi (e.g. _Bellilinea_ spp., _Longilinea_ spp.), Bacteroidetes populations likely act as the dominant anaerobic and aerobic complex organic matter degraders in the system (Kirchman 2002; Mayrberger 2011).

Other prominent _Deltaproteobacteria_ retrieved in the 16S rRNA amplicon survey include _Desulfonema_ spp., _Desulfatibacillum_ spp., _Desulfo bacca_ spp., _Desulfo bacterium_ spp., _Geobacter_ spp., and _Syntrophus_ spp. _Syntrophus_ spp. carry out an essential role in the anaerobic recycling of organic matter to CO₂ and CH₄ (McInerney et al. 2007). Both _Desulfonema_ spp. and _Desulfo bacterium_ spp. are dissimilatory sulfate reducing bacteria (SRB) that completely oxidize acetate. _Desulfonema_ spp. are filamentous SRB with gliding motility, and are frequently found in association with filamentous sulfur oxidizing bacteria such as _Beggiatoa_ spp. (Macalady et al. 2006). In my dataset, they are one of the rare taxa that are always more abundant in biofilms than in underlying (paired) sediment samples (Fig. 2.13).
*Geobacter* reads are abundant in all samples irrespective of sample type. *Geobacter* strains are able to respire S\(^0\) at any pH (Flynn et al. 2014), and are known for their capacity to oxidize organic compounds and reduce metals through the use of nanowires and pili in sedimentary environments (Childers et al. 2002). Direct interspecies electron transfer (DIET) is carried out by *Geobacter* spp. in a syntrophic relationship with methanogenic archaea in the genus *Methanosaeta*. In this recently described consortium, *Geobacter* cells shuttle electrons directly to *Methanosaeta* cells, resulting in the production of methane (Snider et al. 2012; Rotaru et al. 2014; Zhao et al. 2015; Holmes et al. 2017). Interestingly, *Methanosaeta* reads are the most prevalent archaea in all samples (mean of 50% archaeal sequences, Fig. 2.19) and are found in similar relative abundance as *Geobacter* reads (data not shown). Members of the family containing *Geobacter* strains (Desulfuromonadales) have recently been implicated in marine sediment S(0) reduction (Pjevac et al. 2014).

Based on this data and what is known about elemental cycling and microbial ecology in Frasassi streams from prior work, I can hypothesize about the most likely fate of abundant S(0) generated at the expense of oxygen and nitrate by sulfur oxidizers related to *Beggiatoa* and *Sulfurovum*. Potential fates include:

1. physical transport (export from the cave system in flowing water)
2. microbial oxidation
3. microbial reduction
4. microbial disproportionation (Equations 2 and 3)
I currently have no good estimate of S(0) export from the cave system, but I have observed S(0) in spring water exiting the cave system into the Sentino River. Given the presence of abundant sulfide oxidizing microbial biofilms in the spring mouths, and the lack of sulfide depletion in the spring waters, I suggest that transport is not the only important sink for S(0). Abundant populations of *Sulfovum* present in both biofilms and sediments appear to lack the necessary genes for complete sulfide oxidation to sulfate. Numerous organisms with the genetic potential to oxidize S(0) completely to sulfate are present in Frasassi streams, including *Beggiatoa*. However, it is clear from microsensor profiles that *Beggiatoa* are not producing sulfuric acid *in situ*, and microscopic observations confirm they are storing large amounts of S(0) in intracellular inclusions that are inaccessible to other potential S(0) oxidizing populations. *Thiobacillus baregensis* relatives are the most likely S(0) oxidizers in the biofilms and sediments I studied, although acid production resulting from their potential activity is effectively neutralized. More importantly, it is not clear how *T. baregensis* populations could remain active in anoxic sediments below the biofilms during normal hydrologic flow where oxygen and nitrate are unavailable.

In contrast to S(0) oxidation, both reduction and disproportionation of S(0) can proceed in the absence of oxidants and are therefore more likely sinks for S(0) produced at the sediment-water interface. Both S(0) reduction and S(0) disproportionation are consistent with microsensor data showing that sulfide is produced below the biofilms. Although sulfate reducers are present, their activity should consume protons and therefore raise the pH. The extraordinarily high abundance of 16S rRNA amplicons affiliated with *Desulforcapsa thiozymogenes*, a S(0) disproportionation specialist, strongly
suggests that this process is important, and also helps to explain the drop in pH with increasing depth in sediments below the biofilms (Equation 2). In addition, the known S(0) reduction capability of *Geobacter* populations may be minimized in this environment by direct electron transfer to *Methanosaeta* cells. The abundance of *Geobacter-Methanosaeta* consortia could be evaluated in future work using FISH.

Microbial consumption of zero-valent sulfur has recently been studied in marine sediments (Pjevac et al. 2014). In sulfidic marine environments including tidal flats and deep-sea hydrothermal vents, the authors demonstrated using a combination of *in situ* incubations, activity measurements using microsensors, clone libraries, and FISH observations that organisms capable of S(0) disproportionation, reduction and oxidation (*Sulfurimonas* spp.) are all present, and suggest that S(0) oxidation by *Sulfurimonas* spp. is the primary sink for marine sedimentary S(0). However, they found *Desulfocapsa* spp. colonizing sulfur granules in anoxic sediments, which suggested disproportionation was a key player in the anoxic consumption of S(0) at the seafloor (Pjevac et al. 2014).

Frasassi differs strongly from modern marine sedimentary environments, which have non-sulfidic, oxygen-saturated bulk water at one extreme of spatial redox gradients. At Frasassi, the bulk water is micro-oxic and sulfidic, indicating that the geochemical niche space available for complete sulfur oxidizers is narrow or non-existent. Combined with the results obtained by Pjevac et al. (2014), my study implies that the fate of marine sedimentary S(0) has likely varied in Earth history as the redox chemistry of ocean water evolved, with strong implications for S isotope fractionation preserved in the geologic record (Zerkle et al. 2016).
2.5.2 Conclusions and future work

Since *Desulfocapsa* spp. are both widespread and abundant in Frasassi stream sediments, its metabolism potentially has a significant effect on sulfur cycling in the environment. Based on the work presented in this study it is likely that *Desulfocapsa*, positioned in sediments underlying *Beggiatoa* dominated biofilms, are actively carrying out elemental sulfur disproportionation leading to the large disparity in sulfur concentrations between biofilms and sediments. The other dominant genera are made up of sulfide oxidizers and fermentators and likely are not actively using S(0) energetically in the anoxic sediments. This study represents the first investigation into the role of *Desulfocapsa* spp. and sulfur disproportionation in the geochemical cycle of sulfidic cave systems.

Future work should focus on in situ experiments similar to (Pjevac et al. 2014) in which sulfur granules are placed in sediments and their colonizing microbial populations analyzed via high-throughput 16S rRNA sequencing. As well, samples should be collected for stable isotope analysis to determine if large depletions in $^{34}$S, characteristic of sulfur disproportionation, are present. Samples could also be collected for metatranscriptomics, however the enzymatic pathway for sulfur disproportionation is not yet well understood and therefore it may prove difficult to determine if disproportionation is occurring from gene transcripts. Future research should also investigate the potential role of *Desulfocapsa* spp. in active cave formation due to the production of acid associated with elemental sulfur disproportionation.
2.6 Acknowledgements

The research presented in this chapter would not have been possible without the help and support of members in the Macalady lab. I would like to thank Jennifer Macalady for sample collection, photo contribution, and for multiple field expeditions, Muammar Mansor, Zena Cardman, Irene Schaperdoth, and Christy Grettenberger for lab assistance, and Trinity Hamilton and Zena Cardman for help with 16S sequences. As well, I would like to thank Perry Oddo for help with R and creating figures. Support for this work came from NSF S Cycling 424-19 65MR to J.L.M..
2.7 Tables and Figures

Figure 2.1: Simplified sulfur cycle in the Frasassi cave system with major sulfur metabolisms represented. Modified from Fike et al. 2015.
Figure 2.2: Analysis of elemental sulfur by percent mass of dry weight for biofilm and sediment samples from Frasassi cave streams. Mean sulfur concentrations are 18.9% for biofilms vs. 2.27% for underlying sediments.
Figure 2.3: Analysis of carbon by percent mass of dry weight for biofilm and sediment samples from Frasassi cave streams. Mean carbon concentrations are 14.1% for biofilms vs. 6.5% for underlying sediments.
Figure 2.4: Fe staining during high and low water conditions in Pozzo di Cristalli in the Frasassi cave system. Image on the left is during high water conditions with no Fe staining visible. Figure on the right is during low water conditions, red arrow indicates prevalent Fe staining in oxidized sediments.
Figure 2.5: (A) Solid phase total Fe, and Fe in silicates, Pyrite, reactive Fe(III), and reactive Fe(II) (B) for Beggiatoa biofilms (o) and underlying sediments (Δ). X for comparison, indicates the Epsilonproteobacterial streamer biofilm described in the text and collected at the same time and location as PC-14-14.
Figure 2.6: Vertical H$_2$S, O$_2$, and pH micro-sensor profiles in *Beggiatoa*-dominated Frasassi biofilms. Empty circles represent O$_2$ values (µM), solid squares represent H$_2$S values (µM), and X’s represent pH values. The oxic water column is indicated in white, biofilms are shown in dark gray shading, and the underlying sediment is shown in light gray.

Modified from Jones et al., 2015.
Figure 2.7: 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped Kingdoms. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.8: Bacteria-associated 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped by taxonomic class. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.9: Bacteria-associated 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped by taxonomic genera. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.10: Gammaproteobacterial 16S rRNA reads from paired biofilm and sediment samples from 3 Frasassi cave streams. Reads are grouped by genus. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.11: Bacteria-associated 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped by taxonomic genera excluding Beggiatoa spp. and Desulfonema spp. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.12: Epsilonproteobacterial 16S rRNA reads from paired biofilm and sediment samples from 3 Frasassi cave streams. Reads are grouped by genus. Sample locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.13: Deltaproteobacterial 16S rRNA reads from paired biofilm and sediment samples from 3 Frasassi cave streams. Reads are grouped by genus. Sample locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.14: Betaproteobacterial 16S rRNA reads from paired biofilm and sediment samples from 3 Frasassi cave streams. Reads are grouped by genus. Sample locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.15: Bacteroidetes phylum 16S rRNA reads from paired biofilm and sediment samples from 3 Frasassi cave streams. Reads are grouped by genus. Sample locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.16: Phylogenetic tree of full-length 16S rRNA sequences affiliated with *Desulfocapsa*, n=88, from the NCBI database. Sequence names in bold are isolates. Frasassi strains have not been isolated to date.
Figure 2.17: Archaea-associated 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped by taxonomic phylum. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.18: Archaea-associated 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped by taxonomic class. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.19: Archaea-associated 16S rRNA Illumina amplicons from paired biofilm and sediment samples in 3 Frasassi cave streams. Reads are grouped by taxonomic genera. Cave locations are Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Circles represent biofilm samples while triangles represent underlying sediments. PC06 and PC07 sediments were collected from the same location in different years (2006 and 2007).
Figure 2.20: Paired biofilm-sediment sample high-throughput bacterial sequence data from the Frasassi cave system. Samples are from three cave locations; Grotta Sulfurea (GS), Vecchio Condotto (VC), and Pozzo dei Cristalli (PC). Genera indicated represent 5% or greater of the overall bacterial community. Dominant metabolisms represented are:

- Sulfide Oxidation: \(2\text{HS}^- + \text{O}_2 \rightarrow 2\text{S}^0 + 2\text{OH}^-\) (Incomplete),
- Organic Matter Degradation: \(\text{CH}_2\text{O} + \text{O}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O}\),
- Sulfur Disproportionation: \(4\text{S}^0 + 4\text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 3\text{HS}^- + 5\text{H}^+\), \(3\text{S}^0 + 2\text{FeOOH} \rightarrow \text{SO}_4^{2-} + 2\text{FeS} + 2\text{H}^+\),
- Sulfate Reduction: \(\text{SO}_4^{2-} + \text{H}_2 \rightarrow \text{H}_2\text{S} + \text{H}_2\text{O}\), and
- Sulfur Reduction: \(\text{S}^0 + \text{H}_2 \rightarrow \text{H}_2\text{S}\). Sulfate and sulfur reduction are grouped together.
2.8 References


APPENDIX: IRON ANALYSIS IN PERCENT DRY WEIGHT

Iron Analysis

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Total Fe (Weight %)</th>
<th>Total Fe in HF Fraction (Weight %)</th>
<th>Total Fe in HNO3 Fraction (Weight %)</th>
<th>Reactive Fe(III) in HCl (Weight %)</th>
<th>Reactive Fe(II) in HCl (Weight %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC16-24b</td>
<td>1.898</td>
<td>0.582</td>
<td>0.101</td>
<td>0.179</td>
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<tr>
<td>PC16-25b</td>
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<td>0.216</td>
<td>0.335</td>
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<tr>
<td>PC16-25c</td>
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<td>1.714</td>
<td>0.558</td>
<td>0.423</td>
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<tr>
<td>PC16-27b</td>
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<td>1.129</td>
<td>0.212</td>
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<tr>
<td>PC16-28b</td>
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<td>2.137</td>
<td>0.364</td>
<td>0.726</td>
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<tr>
<td>PC16-30a</td>
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<td>1.158</td>
<td>0.117</td>
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<td>PC16-30b</td>
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<td>0.053</td>
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</tbody>
</table>

Table 3.1: Total Fe, Fe-silicates, Pyrite, Fe(III), and Fe(II) values for Frasassi stream biofilms and underlying sediments in percent dry weight. Samples in grey indicate sediment samples while samples in white represent *Beggiatoa spp.* dominated biofilms. Samples in series are paired with each other (i.e. PC16-27b is a biofilm and PC16-28b is it’s underlying sediments).