Received: 30 March 2023 | Revised: 24 July 2023 | Accepted: 29 August 2023

DOI: 10.1111/gbi.12574

gebiology WILEY

ORIGINAL ARTICLE

Sulfur disproportionating microbial communities in a dynamic, microoxic-sulfidic karst system

Heidi S. Aronson[1] | Christian E. Clark[2] | Douglas E. LaRowe[3] | Jan P. Amend[1,3] | Lubos Polerecky[4] | Jennifer L. Macalady[2]

[1]Department of Biological Sciences, University of Southern California, Los Angeles, California, USA

[2]Department of Geosciences, Pennsylvania State University, University Park, Pennsylvania, USA

[3]Department of Earth Sciences, University of Southern California, Los Angeles, California, USA

[4]Department of Earth Sciences, Utrecht University, Utrecht, The Netherlands

Correspondence
Jennifer L. Macalady, 210 Deike Building, University Park, PA 16802, USA.
Email: jlm80@psu.edu

Funding information
Cave Conservancy Foundation; Josephine De Karman Fellowship Trust; Lewis and Clark Fund for Exploration and Field Research in Astrobiology; National Aeronautics and Space Administration; National Cave and Karst Research Institute; National Science Foundation; University of Southern California Dornsife College of Letters, Arts, and Sciences

Abstract

Biogeochemical sulfur cycling in sulfidic karst systems is largely driven by abiotic and biological sulfide oxidation, but the fate of elemental sulfur (S^0) that accumulates in these systems is not well understood. The Frasassi Cave system (Italy) is intersected by a sulfidic aquifer that mixes with small quantities of oxygen-rich meteoric water, creating Proterozoic-like conditions and supporting a prolific ecosystem driven by sulfur-based chemolithoautotrophy. To better understand the cycling of S^0 in this environment, we examined the geochemistry and microbiology of sediments underlying widespread sulfide-oxidizing mats dominated by *Beggiatoa*. Sediment populations were dominated by uncultivated relatives of sulfur cycling chemolithoautotrophs related to *Sulfurovum*, *Halothiobacillus*, *Thiofaba*, *Thiovirga*, *Thiobacillus*, and *Desulfocapsa*, as well as diverse uncultivated anaerobic heterotrophs affiliated with *Bacteroidota*, Anaerolineaceae, Lentimicrobiaceae, and Prolixibacteraceae. *Desulfocapsa* and *Sulfurovum* populations accounted for 12%–26% of sediment 16S rRNA amplicon sequences and were closely related to isolates which carry out autotrophic S^0 disproportionation in pure culture. Gibbs energy (ΔG_r) calculations revealed that S^0 disproportionation under in situ conditions is energy yielding. Microsensor profiles through the mat-sediment interface showed that *Beggiatoa* mats consume dissolved sulfide and oxygen, but a net increase in acidity was only observed in the sediments below. Together, these findings suggest that disproportionation is an important sink for S^0 generated by microbial sulfide oxidation in this oxygen-limited system and may contribute to the weathering of carbonate rocks and sediments in sulfur-rich environments.

KEYWORDS
karst, sediment, sulfide oxidation, sulfur disproportionation

Heidi S. Aronson and Christian E. Clark contributed equally to the study.

1 | INTRODUCTION

Actively forming sulfidic caves are valuable natural laboratories for studying microbial ecosystems that are supported entirely by in situ chemolithotrophic sulfur cycling and chemolithoautotrophic carbon fixation. These restricted environments provide constrained geochemical contexts in which to investigate the role of microorganisms in mediating the geochemistry of subsurface environments and their contributions to sulfur cycling and cave formation. The karst at Frasassi (Italy) is a rare example of a cave system actively forming by sulfuric acid speleogenesis (cave formation, SAS; Table 1, Rxn 1) (Galdenzi et al., 1999; Macalady et al., 2019). The Frasassi cave system is intersected by a perennially sulfidic (up to 600 μM total dissolved sulfide) deep aquifer that is anoxic due to insufficient contact with the atmosphere or oxygenated meteoric water. Sulfide that degasses from the water table reacts with oxygen that is either in the downward percolating meteoric water or in the cave air, to form sulfuric acid, which corrodes subaerial carbonate host rock to form gypsum and carbonic acid (Rxn 1).

In the water table, the mixing of oxygenated meteoric water with the sulfidic aquifer forms anoxic, microoxic, and oxygenated zones (Galdenzi et al., 2008; Lyons et al., 2009; Macalady et al., 2008; Meyer & Kump, 2008). White microbial mats form at the interface between the microoxic stream waters and underlying sediments. These mats are dominated by chemolithotrophic sulfur-cycling taxa, including freshwater *Beggiatoa* strains, Campylobacterota (f.k.a. Epsilonproteobacteria), Burkholderiales (f.k.a. Betaproteobacteria), and Desulfobacterota. The Frasassi stream microbial mats have been well described (Hamilton et al., 2015; Jones et al., 2015; Macalady et al., 2006, 2008). The subaqueous *Beggiatoa* mats primarily oxidize sulfide, and abundant visible sulfur inclusions within their cells suggest that sulfide is oxidized incompletely to form elemental sulfur (S^0, Table 1, Rxn 2) (Hamilton et al., 2015; Macalady et al., 2006, 2008). Sulfur speciation measurements on Frasassi microbial mats using K-edge X-ray absorption near-edge structure spectroscopy confirmed that the mats contained S^0, primarily in the form of S_8 (Engel et al., 2007). Additionally, microsensor measurements through the mats did not detect a decrease in pH, indicating that incomplete oxidation of sulfide, rather than complete oxidation to sulfuric acid, was occurring (Table 1, Rxn 3; Figure 1; Jones et al., 2015).

In contrast to the mats, net acid and sulfide production was detected in the sediments, suggesting that S^0 is being used as an electron donor and/or acceptor (Figure 1, Jones et al., 2015). There are four potential fates of S^0 in the Frasassi sediments, including (1) physical transport (export from the cave system by flowing water), (2) microbial oxidation, (3) microbial reduction, and (4) microbial disproportionation. There is currently no good estimate of S^0 export, but S^0 can be observed exiting the cave system into the Sentino River. Here, we explored possible microbial roles in S^0 consumption by analyzing the microbial communities within the sediments and calculating the energetic yield of reactions involving S^0 using in situ water chemistry. This study represents the first investigation of the microbial communities in the sediments at Frasassi and their involvement in the cave biogeochemical sulfur cycle. Our findings are important for understanding acidic weathering of carbonates in other sulfur-rich environments and for understanding Proterozoic sulfur cycling that may have occurred under similar geochemical conditions.

2 | MATERIALS AND METHODS

2.1 | Site description, geochemistry, and sample collection

The stream waters at Frasassi are circumneutral (pH 6.9–7.4) and have a nearly constant temperature of 13–14°C, total sulfide concentrations up to 600 μM, and conductivity between 1000 and 3500 μS/cm, with an ionic strength equivalent to a salinity of approximately 3.5–4.5 ppt (Galdenzi et al., 2008; Macalady et al., 2008). The stream waters have consistently low levels of dissolved oxygen (<30 μM), nitrate concentrations below detection limits (~7 nM), and ammonium concentrations between 30 and 175 μM (Macalady et al., 2006, 2008).

Beggiatoa mat and sediment samples for geochemical and microbial community analysis were collected in 2006, 2007, and 2010 from sulfidic streams in the Frasassi cave system, specifically from Ramo Sulfureo (RS), Grotta Sulfurea (GS), Pozzo dei Cristalli (PC), and Vecchio Condotto (VC) (Table 2; for details on sample locations see Hamilton et al., 2015; Macalady et al., 2006, 2007, 2008). Paired mat-sediment samples were collected using a sterile transfer pipette to remove the white mat into a sterile 50 mL centrifuge tube. The underlying sediment was then collected with a new transfer pipette and placed into a sterile 50 mL centrifuge tube. All samples were preserved by adding 3:1 RNAlater:sample volume (Sigma-Aldrich Corp.), and stored at −20°C until processing at Pennsylvania State University in 2015.

2.2 | Reaction energetics

The Gibbs energy yields (ΔG_r) for various sulfur redox reactions (Table 1) were calculated with

$$\Delta G_r = \Delta G_r^{\,0} + RT\ln Q_r \quad (1)$$

where $\Delta G_r^{\,0}$ is the standard state Gibbs energy, R is the universal gas constant, T is the temperature in Kelvin, and Q_r refers to the reaction activity quotient. Values of $\Delta G_r^{\,0}$ were calculated at 13.4°C and 1 bar with the revised Helgeson-Kirkham-Flowers (HKF) equations of state (Helgeson et al., 1981; Shock et al., 1992; Tanger & Helgeson, 1988) using the "subcrt" command from the R software package CHNOSZ v1.4.1 (Dick, 2019). Thermodynamic data in CHNOSZ are derived from the OrganoBioGeoTherm database, which come from a number of sources (https://chnosz.net/download/refs.html). The sources of these data are provided in the CHNOSZ package documentation. Values of Q_r were calculated with

TABLE 1 Selected chemical and catabolic reactions and their average Gibbs energy yields, ΔG_r, under in situ conditions along the microsensor depth profile through Frasassi *Beggiatoa* mats and sediments shown in Figure 1.

Reaction number	Reaction name	Reaction	ΔG_r (kJ/Mol S consumed)	ΔG_r (kJ/Mol O_2)	ΔG_r (kJ/Mol H_2)
1	Sulfuric acid speleogenesis	$H_2SO_4 + CaCO_3 + 2H_2O \rightleftharpoons CaSO_4{\cdot}2H_2O + H_2CO_3$			
2	Incomplete aerobic sulfide oxidation	$2HS^- + O_2 + 2H^+ \rightleftharpoons 2S^0 + 2H_2O$	−283.1	−411.2	
3	Complete aerobic sulfide oxidation	$HS^- + 2O_2 \rightleftharpoons SO_4^{2-} + H^+$	−761.0	−381.6	
4	Aerobic S^0 oxidation	$2S^0 + 3O_2 + 2H_2O \rightleftharpoons 2SO_4^{2-} + 4H^+$	−583.3	−383.3	
5	Incomplete sulfide oxidation coupled to DNRA	$4HS^- + NO_3^- + 6H^+ \rightleftharpoons 4S^0 + NH_4^+ + 3H_2O$	−89.0		
6	Complete sulfide oxidation coupled to DNRA	$HS^- + NO_3^- + H_2O + H^+ \rightleftharpoons SO_4^{2-} + NH_4^+$	−606.2		
7	S^0 oxidation coupled to DNRA	$4S^0 + 3NO_3^- + 7H_2O \rightleftharpoons 4SO_4^{2-} + 3NH_4^+ + 2H^+$	−331.8		
8	Incomplete sulfide oxidation coupled to denitrification	$5HS^- + 2NO_3^- + 7H^+ \rightleftharpoons 5S^0 + N_2 + 6H_2O$	−174.0		
9	Complete sulfide oxidation coupled to denitrification	$5HS^- + 8NO_3^- + 3H^+ \rightleftharpoons 5SO_4^{2-} + 4N_2 + 4H_2O$	−735.5		
10	S^0 oxidation coupled to denitrification	$5S^0 + 6NO_3^- + 2H_2O \rightleftharpoons 5SO_4^{2-} + 3N_2 + 4H^+$	−583.2		
11	S^0 disproportionation	$4S^0 + 4H_2O \rightleftharpoons 3HS^- + SO_4^{2-} + 5H^+$	−16.2		
12	S^0 reduction	$S^0 + H_2 \rightleftharpoons HS^- + H^+$	−25.6		−25.6
13	Sulfate reduction	$SO_4^{2-} + 4H_2 + H^+ \rightleftharpoons HS^- + 4H_2O$	−37.3		−9.3
14	S_2^{2-} disproportionation	$4S_2^{2-} + 4H_2O \rightleftharpoons 7HS^- + SO_4^{2-} + H^+$	−49.9		
15	S_3^{2-} disproportionation	$2S_3^{2-} + 4H_2O \rightleftharpoons 5HS + SO_4^{2-} + 3H^+$	−18.4		
16	S_4^{2-} disproportionation	$4S_4^{2-} + 12H_2O \rightleftharpoons 13HS^- + 3SO_4^{2-} + 11H^+$	−16.7		
17	Ferric iron reduction	$HS^- + 2FeOOH + 5H^+ \rightleftharpoons 2Fe^{2+} + S^0 + 4H_2O$			
18	Iron sulfide formation	$HS^- + Fe^{2+} \rightleftharpoons FeS + H^+$			
19	Sulfur disproportionation with ferric iron as a sulfide scavenger	$3S^0 + 2FeOOH \rightleftharpoons 2FeS + SO_4^{2-} + 2H^+$			

Note: Chemical concentration data that were used to calculate activities can be found in Appendix S1.

Abbreviation: DNRA, dissimilatory nitrate reduction to ammonium.

$$Q_r = \Pi a_i^{\nu_{i,r}} \tag{2}$$

where a_i represents the activity of the ith species raised to its stoichiometric reaction coefficient $\nu_{i,r}$, in the rth reaction, which is positive for products and negative for reactants.

Activities were calculated with the relation

$$a_i = m_i \gamma_i \tag{3}$$

where m_i and γ_i are the molality and activity coefficients of the ith species. Concentrations were sourced from median values from Pozzo dei Cristalli stream water data collected from 2002 to 2011 and were converted to molality by assuming stream water density of 1 kg/L (Appendix S1). Concentrations of sulfide, oxygen, and protons were sourced from microsensor data from Jones et al. (2015; Figure 1; Appendix S1). Oxygen concentrations below the detection limit (≤2 μM) for depths below 0.5 mm were set between 10^{-6} molal and 10^{-8} molal to illustrate the effects of extremely low oxygen concentration on Gibbs energy yields (Appendix S1). H_2 concentrations were assumed to range between 10^{-10} and 10^{-8} molal (Hoehler et al., 1998), and polysulfide concentrations were assumed to be 10^{-5} molal. Activities for aqueous species were determined using the aqueous speciation package AqEquil v0.9.1 (Boyer et al., 2021), which is based on EQ3/6 (Wolery, 1979). The activities of elemental sulfur and water were assumed to be unity (a_i=1). Values of ΔG_r were calculated for various redox reactions involving sulfur in various oxidation states (see Table 1) along the depth profile shown in Figure 1. The Gibbs energy for S^0 disproportionation was calculated for sulfate and sulfide concentrations ranging from 1×10^{-9} molal to 3×10^{-3} or 1×10^{-3} molal, respectively, with pH set at 7.26 and a temperature of 13.4°C (median pH and temperature of Frasassi waters).

2.3 | 16S rRNA gene amplicon sequencing and analysis

Replicate DNA extractions from samples and blanks were performed in 2015 in a clean fume hood using MoBio Power Lyzer Soil DNA isolation kit #12855-50 according to kit protocol (Mo Bio Laboratories Inc.). The microbial community of each sample was analyzed by 16S rRNA gene amplicon sequencing of the V4-V5 hypervariable region (Illumina MiSeq platform) (Illumina Inc.) using 515F and 806R universal primers at the MrDNA Laboratory (Molecular Research LP). The primers were used in a 28 cycle PCR with the HotStarTaq Plus Master Mix Kit (Qiagen Inc.). The PCR cycle conditions were as follows: 94°C for 3 min, succeeded by 28 cycles at 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min. A final elongation step was carried out at 72°C for 5 min. PCR products were assessed in 2% agarose gel to determine amplification success. Pooled samples were purified using calibrated Ampure XP beads. Purified PCR products were then used to compile the Illumina DNA library. Sequence data were processed using the MrDNA pipeline (Molecular Research LP). Forward and reverse reads were joined, their barcodes were removed, sequences with less than 150 base pairs were removed, and ambiguous base calls were removed.

16S rRNA amplicon sequence data were processed using QIIME2 v2019.7.0 (Bolyen et al., 2019). Fastq data were imported into QIIME2 using the fastq manifest format. Sequences were demultiplexed and denoised into amplicon sequence variants (ASVs) using the QIIME2 DADA2 "denoised-paired" plugin (Callahan et al., 2016). Taxonomy of each ASV was assigned using the "feature-classifier classify-sklearn" plugin. The classifier was trained on the SILVA138 database clustered at 99% and trimmed to the amplified region (Quast et al., 2012). Mitochondrial sequences were removed using "taxa filter-table" plugin. The R package phyloseq was used to further analyze and visualize data from the filtered OTU table and taxonomy (McMurdie & Holmes, 2013). Sequences that were unassigned at the domain level were removed and sequence counts were transformed to relative abundance. Plots were produced in R using the package ggplot2 (R Core Team, 2019; Wickham, 2016). Interpretations of microbial metabolic potential were inferred from metabolisms of the closest cultured relatives within the same genus and from previous metagenomic studies of Frasassi biofilms (Hamilton et al., 2015; Labrado, 2017; McCauley Rench, 2015; Pavia et al., 2018; Tsao, 2014).

Phylogenetic trees were constructed using *Desulfocapsa*, *Sulfurovum*, and Halothiobacillaceae 16S rRNA gene sequences from this study and from close relatives. *Desulfocapsa*, *Sulfurovum*, and Halothiobacillaceae sequences from this study were blasted against all 16S rRNA sequences from previous Frasassi studies (Hamilton et al., 2015; Jones et al., 2008, 2010, 2012, 2014, 2016; Macalady et al., 2006, 2007, 2008), against the NCBI 16S rRNA gene sequence reference database, and against the NCBI nt sequence database. The Halothiobacillaceae tree also contains additional *Thiobacillus* sequences from this study. Frasassi sequences ≥97% similar to sequences from this study, reference sequences ≥90% similar to sequences from this study, and nt sequences 100% similar to sequences from this study were included in the trees. The trees were pruned by selecting representative sequences with the dist.seqs, cluster (furthest neighbor at most 2% distant), and get.oturep commands in mothur (Schloss et al., 2009). Sequences were aligned using MUSCLE (Edgar, 2004) and trimmed with trimal (Capella-Gutiérrez et al., 2009). Phylogenetic trees were constructed using IQ-TREE with a maximum-likelihood method and the generalized time-reversible model of nucleotide evolution with 1000 bootstrap replications (Nguyen et al., 2015).

3 | RESULTS

3.1 | Reaction energetics

Gibbs energies of the reactions listed in Table 1 are shown in Table 1, Figure 2, and Figure S4. For S^0 disproportionation and reduction, sulfate reduction, and polysulfide disproportionation (Table 1, Rxns. 11–16), values of ΔG_r along the microsensor depth profile were negative (exergonic) (Figure 2). The Gibbs energy yields for these reactions became less negative with greater depth because of increasing sulfide concentration and reached local negative maxima at 0.1 mm depth because of the minimum sulfide concentration (1 μM). Values of ΔG_r along the depth profile different substantially among the different sulfur species that were disproportionated or reduced, with S_2^{2-} disproportionation yielding the most energy at up to −55 kJ/mol S and S^0 disproportionation yielding the least energy at as low as −13 kJ/mol S (Figure 2). Values of ΔG_r for S^0 disproportionation were between −8 and −25 kJ/mol S for the range of sulfate and sulfide activities found at Frasassi (Figure S4). The oxidation reactions with oxygen or nitrate yield significantly more energy (−91 to −763 kJ/mol S) even with activities equivalent to oxygen concentrations 10^{-8} molal (Table 1, Rxns. 1–10, Appendix S1).

3.2 | Microbial community composition

Analysis of the 16S rRNA gene V4-V5 hypervariable region revealed similar community compositions for each sample type across locations and sampling times (Figure 3). Taxonomy of each ASV was identified at the genus level, and interpretations of potential metabolic functions were inferred from the metabolisms of the closest cultured relatives and from previous metagenomic studies (Hamilton et al., 2015; Labrado, 2017; McCauley Rench, 2015; Pavia et al., 2018; Tsao, 2014). Sequences were affiliated primarily with Bacteria; Archaea represented fewer than 3% of reads from any sample and typically fewer than 1% (Supplementary information).

All mat samples were dominated by the sulfur-oxidizing Gammaproteobacterial genus *Beggiatoa* (20%–30%), consistent with field and microscopic observations of gliding filaments coiled at the sediment surface. *Beggiatoa* sequences were significantly lower in relative sequence abundance (<3%) in anoxic sediment immediately

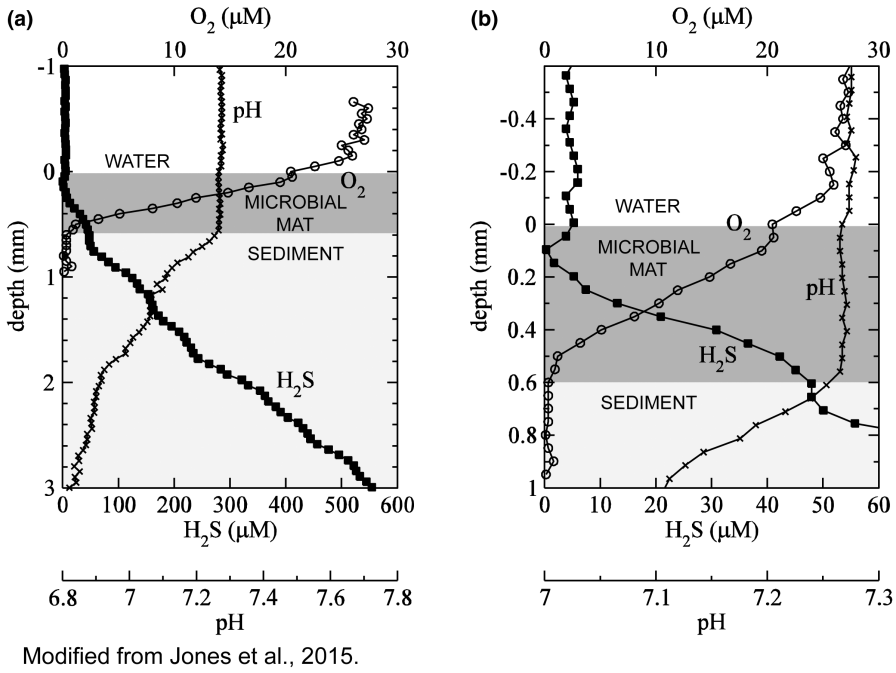

FIGURE 1 Representative in situ concentration profiles of H_2S, O_2, and pH as a function of depth through a Frasassi Cave *Beggiatoa* mat and sediment layer at Pozzo dei Cristalli. Panel (b) is a magnified version of panel (a).

TABLE 2 Samples collected for 16S rRNA gene sequencing.

Location	Date	Samples collected	Sample name
PC	2006	Sediments ($n=1$)	PC06-113-sed
PC	2007	Paired microbial mats and sediments ($n=1$)	PC07-20-mat, PC07-22-sed
GS	2010	Paired microbial mats and sediments ($n=1$)	GS10-5-mat, GS10-5-sed
VC	2010	Paired microbial mats and sediments ($n=2$)	VC10-2-mat, VC10-3-sed; VC10-7-mat, VC10-8-sed

Note: Location names refer to Pozzo dei Cristalli (PC), Grotta Sulfurea (GS), and Vecchio Condotto (VC).

below the mat surface. The remaining populations in all samples were dominated by *Sulfurovum*, (Campylobacterota, f.k.a. Epsilonproteobacteria, 5%–15%), uncultured strains of Halothiobacillaceae (Gammaproteobacteria, 2%–9%), *Desulfocapsa* (Desulfobacterota, 5%–11%), and Bacteroidetes BD2-2 (4%–7%). Other anaerobic chemoorganotrophs, including Bacteroidetes vadinHA17, uncultured Anaerolineaceae, uncultured Lentimicrobiaceae, uncultured Prolixibacteraceae, and Pedosphaeraceae ADurb.Bin063-1, were consistently higher in abundance in the sediments than in the mats.

There were six clades of Frasassi *Sulfurovum* sequences that were most closely related to sulfide-oxidizing taxa *Nitratifractor salsuginis*, *Sulfurovum aggregans*, *Sulfurovum lithotrophicum*, and *Sulfurovum riftiae*, with the most abundant sequences in clade 4 (Figure S5). Reads affiliated with uncultured Halothiobacillaceae made up 2%–10% of the sediment and mat communities (Figure 3). There were three clades of sequences associated with the Halothiobacillaceae (Figure S6). One clade clustered with *Halothiobacillus neapolitanus*, one clustered with *Thiovirga* sequences, and the third, which included the most abundant sequences, clustered with *Thiofaba tepidiphila*. There were two clades of *Thiobacillus* from Frasassi sequences, which both clustered with *Thiobacillus thioparus*, *Thiobacillus sanjanensis*, *Thiobacillus denitrificans*, *and Thiobacillus thiophilus* (Figure S6). *Desulfocapsa* sequences made up 5%–11% of total reads (Figure 3). The majority ($n=19$) of *Desulfocapsa* strains from this study were most closely related to *Desulfocapsa thiozymogenes* (Figure S7). Other abundant (>1%) populations were associated with other sulfur-oxidizing taxa (*Thiobacillus*, *Sulfuricurvum*, and *Arcobacteraceae*), sulfate-reducing taxa (*Desulfobacca*, *Desulfonema*, and *Desulfatirhabdium*), and anaerobic chemoorganotrophs (uncultured Anaerolinaceae, Lentimicrobiaceae, and Prolixibacteraceae) (Figure 3).

4 | DISCUSSION

4.1 | S^0 is produced by dominant members of the microbial mat community

The microoxic, sulfidic streams in the Frasassi karst host perennially abundant white sulfide-oxidizing microbial mats that develop at the water–sediment interface (Macalady et al., 2006, 2008).

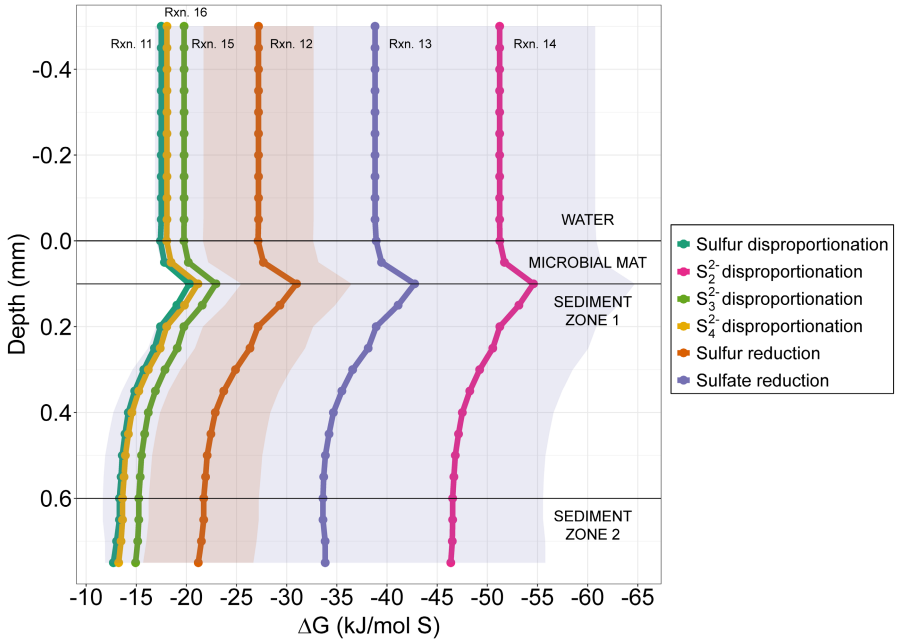

FIGURE 2 Gibbs energies, ΔG_r, of S^0 disproportionation, S^0 reduction, sulfate reduction, and polysulfide disproportionation (reactions 11–16 in Table 1) as a function of depth into Frasassi cave sediment at T = 13.5°C and pH = 7.26 expressed in units of kJ/mol S. Activities of sulfide, oxygen, and protons were derived from microsensor data shown in Figure 1b. Dots correspond to ΔG_r at each point on the microsensor data from Figure 1b, solid lines designate guiding fit lines, and the colored ribbons around these lines indicate the energetic yield when hydrogen concentrations were set at one order of magnitude higher and lower than the values used for the plotted lines (1 nM) for S^0 reduction (orange) and sulfate reduction (purple). Reaction numbers on the plot refer to reactions listed in Table 1.

The schematic shown in Figure 4 provides an overview of our current understanding of the biogeochemistry and microbial ecology governing sulfur cycling in the Frasassi mats and sediments. The *Beggiatoa*-dominated mats consume sulfide and oxygen without a concomitant decrease in pH (Figure 1, Jones et al., 2015), which is consistent with incomplete microbial sulfide oxidation to S^0 within the mat (Table 1, Rxn 2). A decrease in pH would be expected if complete sulfide oxidation (Table 1, Rxn 3) was occurring (Jørgensen & Revsbech, 1983; Schwedt et al., 2012). Dominant members of the mat community, including *Beggiatoa*, *Sulfurovum*, and Halothiobacillaceae, show the genetic potential to perform incomplete and complete sulfide oxidation, suggesting that the end product of sulfide oxidation may be determined by both environmental and physiological factors as discussed in the following (Hamilton et al., 2015; McCauley Rench, 2015; Pavia et al., 2018; Tsao, 2014).

Beggiatoa spp., which dominated the mat community at 20%–30% relative sequence abundances, have the genetic potential to oxidize reduced sulfur compounds (sulfide, S^0, thiosulfate). *Beggiatoa* MAGs from Frasassi contain genes encoding enzymes involved in reduced sulfur oxidation, including *soxYZ*, *rDSR*, *fccAB*, and heterodisulfide reductases (McCauley Rench, 2015). While the presence of *rDSR* suggests that *Beggiatoa* spp. at Frasassi have the capacity to oxidize sulfide completely to sulfate (Mußmann et al., 2007), the availability of both sulfide and oxygen may determine the final product of sulfide oxidation (Klatt et al., 2016; Klatt & Polerecky, 2015). In marine and freshwater environments, *Beggiatoa* spp. have been shown to migrate vertically to take advantage of optimal geochemical parameters at oxygen and sulfide diffusion gradient zones. Under low sulfide flux conditions for a given O_2 supply, *Beggiatoa* oxidize sulfide directly to sulfate, while under high sulfide flux conditions, *Beggiatoa* are only able to oxidize ~50% of sulfide to sulfate, leading to intracellular S^0 deposition (Berg et al., 2014). Eventually, *Beggiatoa* may accumulate so much S^0 that they burst (Berg et al., 2014). *Beggiatoa* may save themselves from this fate by migrating into anoxic/sulfidic regions where they can respire excess S^0 using polyhydroxyalkanoates as electron donors (Schwedt et al., 2012). At Frasassi, *Beggiatoa* populations were not dependent on sulfide/oxygen supply ratios but were observed in areas with less turbulent flow where they could accumulate on fine sediments (Macalady et al., 2008). *Beggiatoa* MAGs from Frasassi also contained genes encoding cbb3-type cytochrome c oxidases and bd-type quinol oxidases, which are aerobic terminal oxidases that function under low oxygen conditions (Buschmann et al., 2010; McCauley Rench, 2015; Pitcher & Watmough, 2004; Preisig et al., 1996; Rich et al., 1996). In *E. coli*, cytochrome bd oxidases have been shown to promote sulfide-resistant respiration and growth and are expressed under microoxic conditions (Borisov et al., 2011, 2021; Forte et al., 2016). At Frasassi, *Beggiatoa* likely oxidize sulfide incompletely to S^0 under the microoxic, sulfidic conditions of the streams, which is supported by the perennial observation of visible S^0 inclusions within their cells and high concentration of dry weight sulfur in the cells (Figure S1).

Sulfurovum spp., which were present at 5%–15% relative sequence abundances across all samples, were most closely related to

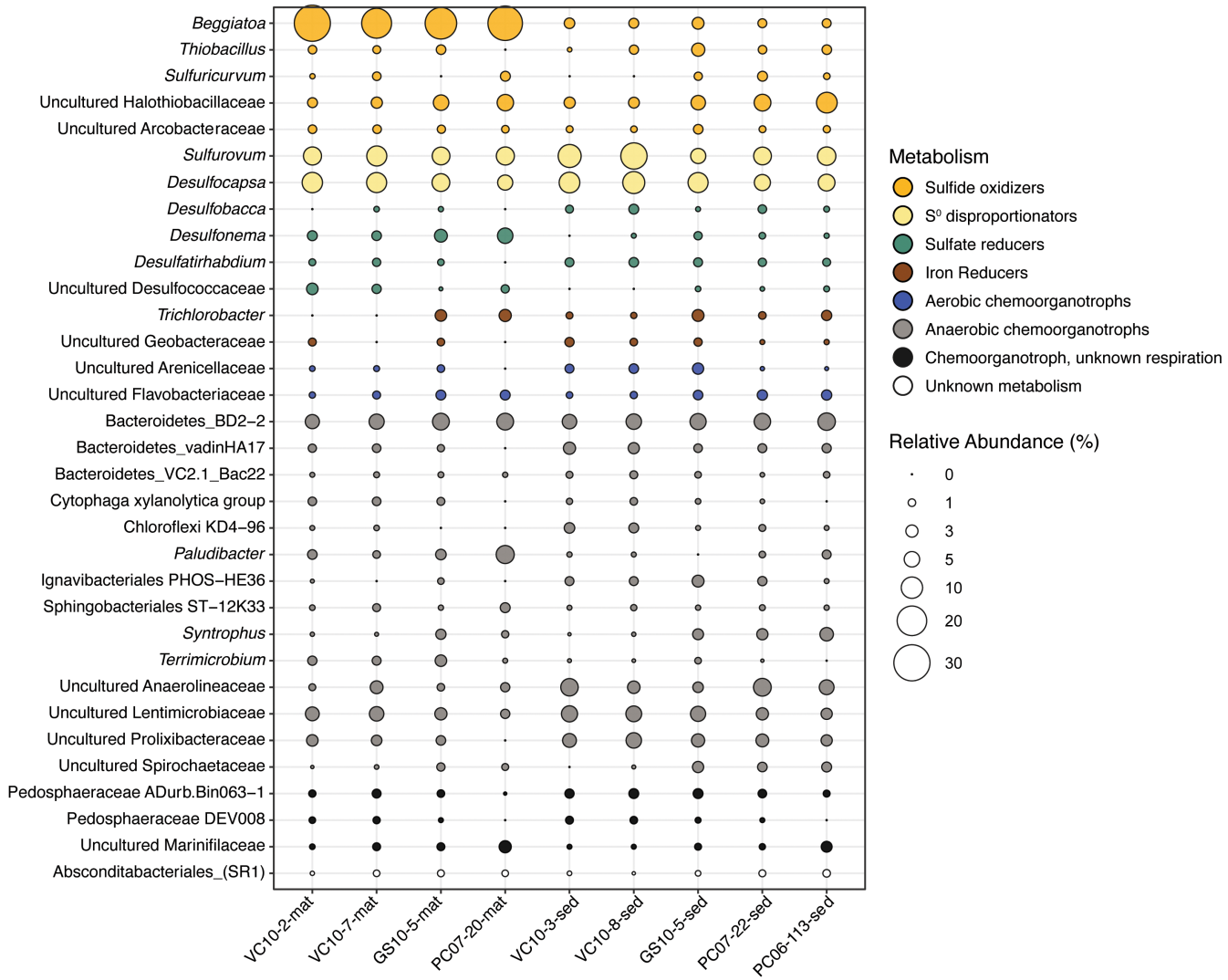

FIGURE 3 Abundance and inferred catabolisms of microbial taxa from paired Frasassi biofilm and sediment samples. Catabolisms were assigned based on published accounts of closely related species and data from the current study. Only taxonomic affiliations of Illumina V4 amplicons that comprised >0.5% of genera were included. Some strains related to the sulfide oxidizers and disproportionators shown can also reduce S^0 using H_2, e.g. *Sulfurovum*, *Sulfuricurvum*, and *Geobacter*.

Sulfurovum aggregans, *Sulfurovum lithotrophicum*, *Sulfurovum riftiae*, and *Nitratifractor salsuginis* (Figure S5). These isolates are incapable of utilizing sulfide as an electron donor, and instead obligately oxidize H_2 with S^0, thiosulfate, or nitrate (*N. salsuginis* and *S. aggregans*; Mino et al., 2014; Nakagawa et al., 2005) or oxidize S^0 or thiosulfate with oxygen or nitrate (*S. lithotrophicum* and *S. riftiae*; Giovannelli et al., 2016; Inagaki et al., 2004). However, prior analysis of four nearly complete *Sulfurovum* MAGs from Frasassi showed that these populations are capable of oxidizing sulfide and thiosulfate to S^0 (*sqr* and *fcc*) using oxygen (cbb3-type cytochrome c oxidases and bd-type quinol oxidases) or nitrate (denitrification pathways; Hamilton et al., 2015), suggesting that certain *Sulfurovum* spp. may contribute to S^0 deposition within the microbial mats. It is also possible that different *Sulfurovum* populations in the sediment, which may be more closely related to cultured representatives, consume S^0. Further cultivation and/or transcriptomic studies are necessary to determine the S^0 consumption or production activity of *Sulfurovum* spp. in both the mats and sediments.

Uncultured strains related to the family Halothiobacillaceae were present at 2%–10% across all samples and formed three clades with cultured relatives from the genera *Halothiobacillus*, *Thiovirga*, and *Thiofaba* (Figure 3, Figure S6). *Halothiobacillus neapolitanus*, *Halothiobacillus kellyi*, *Thiovirga sulfuroxydans*, and *Thiofaba tepidiphila*, the only cultured isolates from these genera, are autotrophs capable of complete aerobic sulfide, thiosulfate, and S^0 oxidation to sulfate (Ito et al., 2005; Mori & Suzuki, 2008; Parker & Prisk, 1953; Sievert et al., 2000). Halothiobacillaceae from Frasassi mats have been shown to express the *sox* complex and *fccAB*, suggesting that these populations can oxidize sulfide and deposit S^0, but also expressed *soxCD*, indicating that they completely oxidize intracellular S^0 to sulfate (Pavia et al., 2018). In the mats, where oxygen is limiting and is rapidly scavenged to below detection limits (≤2 μM), some populations may oxidize sulfide or S^0 using nitrate as an alternative electron acceptor. Such reactions yield −89 to −735 kJ/mol S (Table 1, Rxns 5–10) and could feasibly serve as alternative metabolisms when oxygen

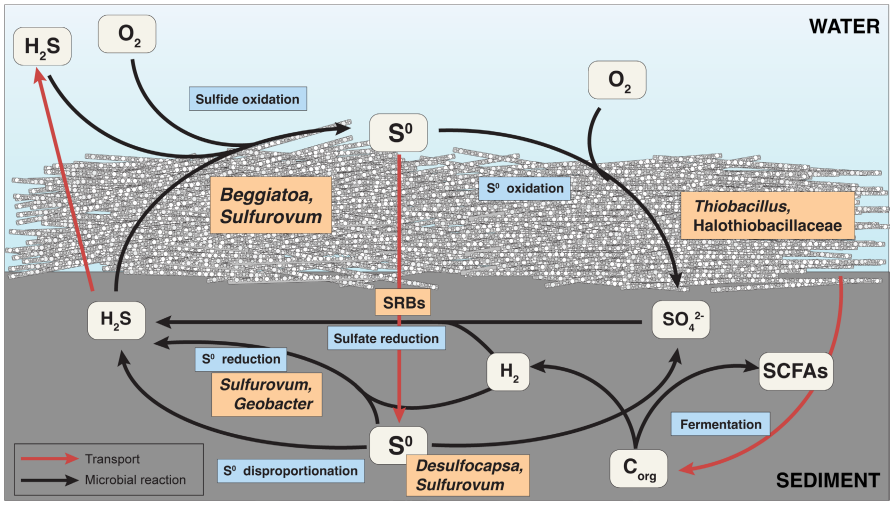

FIGURE 4 Suggested conceptual model of sulfur cycling in Frasassi biofilms and sediments. Blue boxes denote microbially catalyzed processes and orange boxes indicate the taxa responsible for them. In the *Beggiatoa* mats, incomplete sulfide oxidation to S^0 is a dominant process, with possible minor contributions of sulfide or S^0 oxidation with oxygen or nitrate. In the anoxic sediment, sulfide production may occur via S^0 reduction or disproportionation, or sulfate reduction. SCFA stands for short chain fatty acids produced through fermentation reactions. The functions of microbes represented in the model were inferred from their closest cultured relatives and from previously analyzed metagenome assembled genomes.

is limiting. Numerous taxa present in the mats, including *Beggiatoa* (Kamp et al., 2006; McCauley Rench, 2015), *Sulfurovum* (Giovannelli et al., 2016; Hamilton et al., 2015; Inagaki et al., 2004; Pavia et al., 2018), *Thiobacillus* (Schedel & Trüper, 1980), *Sulfuricurvum* (Kodama & Watanabe, 2004), and uncultured Halothiobacillaceae sequences closely related to '*Candidatus* Thiobacillus baregensis' (Hédoin, 1997; Hédoin et al., 1996; Tsao, 2014) have close cultured relatives capable of sulfide, thiosulfate, and S^0 oxidation with nitrate as an electron acceptor or have the genetic potential to perform this metabolism. The use of nitrate as an alternative electron acceptor could explain why nitrate levels at Frasassi are consistently below detection. However, the constant pH profile with depth through the microbial mat suggests that complete sulfide oxidation or S^0 oxidation, either with oxygen or nitrate, is not a predominant sink for S^0.

4.2 | S^0 reduction and disproportionation in anoxic sediments

With increasing depth into the anoxic sediment (Figure 1), a significant pH decrease (from 7.3 to 6.8) was observed and sulfide concentrations reached almost 600 μM at a depth of 3 mm below the mat-water interface (Jones et al., 2015). Upward diffusion of sulfide from the anoxic sediments can explain the increasing sulfide concentration within the microbial mat (Figure 1), contributing to the dynamic cycling of sulfide that occurs at Frasassi. Microbial metabolisms that generate sulfide include sulfate and S^0 reduction or disproportionation of intermediate oxidation-state sulfur species (sulfite, polythionates, thiosulfate, S^0, or polysulfides) (Table 1, Rxns 11–16). Sulfur disproportionation, or the inorganic fermentation of sulfur, is the simultaneous oxidation and reduction of an intermediate oxidation-state sulfur compound to form sulfate and sulfide. Under sulfidic conditions, polysulfides form through the reaction of S^0 and sulfide, and concentrations of polysulfides increase proportionally with sulfide concentration (Milucka et al., 2012; Schauder & Müller, 1993; Schwarzenbach & Fischer, 1960). Under in situ conditions (pH ~7, ~500 μM sulfide), 47 μM of sulfide should be present as polysulfides, with $S_n = 2$, 4, 5, and 6 as the most abundant forms (Milucka et al., 2012), so it is possible that both polysulfides and S^0 are disproportionated by microorganisms in Frasassi sediments.

Based on the microsensor data, sulfate reduction with H_2 is more exergonic than S^0 disproportionation or reduction ($\Delta G_{\text{sulfate reduction}} = -34$ to -42 kJ/mol S, Figure 2). However, when calculated per mole of H_2, which is likely limiting in the sediments compared to S^0, sulfate reduction yields only −8 to −10 kJ/mol H_2 while sulfur reduction yields −19 to −29 kJ/mol H_2 (Table 1). Although sulfate-reducing taxa including *Desulfonema*, *Desulfobacca*, and *Desulfatirhabdium* were detected in the amplicon dataset in low abundance (Figure 3), sulfate reduction consumes rather than produces protons (Table 1, Reaction 5). Thus, while sulfate reduction was likely occurring in the sediments and could explain some of the increase in sulfide concentration, it was not occurring at rates high enough to counteract competing processes that produce protons. The decrease in pH with increasing depth in the anoxic sediments may also be partially attributed to short-chain fatty acid production through fermentation by anaerobic chemoheterotrophs (Figures 3 and 4).

We therefore evaluated which sulfide-producing processes would also produce significant acidity. S^0 reduction and disproportionation of S^0 and polysulfides generate sulfide and protons, and in contrast to S^0 oxidation, can proceed in the absence of oxidants and are therefore more likely to be sinks for S^0 produced at the sediment–water interface. Taxa closely related to isolates that can reduce S^0

in culture (*Sulfurovum* (Campbell et al., 2006; Mino et al., 2014), *Sulfuricurvum* (Campbell et al., 2006; Kodama & Watanabe, 2004), uncultured Arcobacteraceae (Campbell et al., 2006), and *Geobacter* (Caccavo et al., 1994)) were abundant in the sediments. While S^0 reduction with hydrogen yields more free energy per mole of sulfur than S^0 disproportionation, S^0 reducers are likely in competition for H_2 with sulfate reducers. This could allow S^0 and polysulfide disproportionating taxa to reach significant populations in the sediment because they are not in direct competition with sulfate reducing bacteria. Cultivated species of the genus *Desulfocapsa* are strictly anaerobic and couple autotrophic growth to the disproportionation of intermediate oxidation-state sulfur species (S^0, sulfite, and thiosulfate) to form sulfide and sulfate (Finster et al., 1998, 2013; Frederiksen & Finster, 2003, 2004; Janssen et al., 1996; Poser et al., 2013). Recently, it was shown that several species of *Sulfurovum* from diverse natural environments carry out S^0 disproportionation in pure culture (Wang et al., 2023). It is therefore possible that *Sulfurovum* populations are also carrying out S^0 disproportionation at Frasassi, although their S^0-disproportionating activity should be confirmed with laboratory studies. *Desulfocapsa* spp. and *Sulfurovum* spp. were found both in *Beggiatoa* mats and in sediments at 5%–10% and 5%–12% relative abundance, respectively, making up a total of 12%–26% relative abundance in all samples (Figure 3). Additionally, a novel family, genus, and species of S^0-disproportionating bacteria was recently isolated from the Frasassi sediments (Aronson et al., 2022). Sequences associated with this strain were present at 0.11% relative abundance in the GS10-5-mat sample, suggesting that both rare and abundant members of the sediment and mat communities are potentially capable of S^0 disproportionation. The presence of diverse sulfur disproportionating taxa and the geochemical trends in the sediments indicates that disproportionation is an important sink for S^0 at Frasassi.

There are few studies that attribute polysulfide disproportionation to microbial processes (Findlay, 2016; Milucka et al., 2012; Poser et al., 2013). Based on our calculations, disproportionation of S_2^{2-} is more exergonic than S^0 disproportionation, S^0 reduction, and sulfate reduction at Frasassi and could also be a significant contributor to sulfide and proton production in the sediments. Since S^0 is a solid that cannot pass through cell membranes and polysulfides readily form by reaction of S^0 and sulfide, it is likely that some S^0 disproportionation occurs via a polysulfide intermediate produced either abiotically or biologically. While the biochemistry of S^0 disproportionation is still poorly understood, environments with sufficient S^0 and sulfide to produce abundant polysulfides may be especially important habitats for organisms carrying out disproportionation. *Sulfurovum* and *Sulfuricurvum* MAGs contained genes encoding polysulfide reductase (*psr*) and may be important contributors to polysulfide cycling in the Frasassi sediments (Hamilton et al., 2015).

In contrast to sulfur cycling in marine sediments, which is primarily driven by dissimilatory sulfate reduction to sulfide, S^0 and polysulfide disproportionation are likely the predominant sulfur reactions in the anoxic Frasassi sediments. Despite numerous claims to the contrary (Bak & Cypionka, 1987; Finster, 2008; Finster et al., 2013; Lovley & Phillips, 1994; Morrison & Mojzsis, 2021; Poser et al., 2013; Thamdrup et al., 1993), S^0 disproportionation is an energetically favorable process under a range of natural conditions (Alain et al., 2022). The argument that S^0 disproportionation is an energetically unfavorable process is based on the misconception that the sign and value of the standard Gibbs energy ($\Delta G_r°$) define the energy yield of a reaction. $\Delta G_r°$ is only a part of the total Gibbs energy yield of a reaction (ΔG_r) and is dependent on temperature and pressure of the system, and thus cannot be used to determine the favorability of a reaction (Amend & LaRowe, 2019). Thus, although the free energy yields of S^0 disproportionation under in situ conditions are low, our evidence indicates that they provide enough energy to sustain significant and diverse populations of S^0 disproportionating microorganisms in the Frasassi sediments.

Certain laboratory cultures of disproportionators show increased growth in the presence of a sulfide scavenger such as Fe(III) (Finster et al., 1998; Janssen et al., 1996; Thamdrup et al., 1993). In these reactions, sulfide produced by sulfur disproportionation reduces FeOOH to form Fe^{2+} and S^0 (Table 1, Rxn. 17). Sulfide reacts with Fe^{2+} to form FeS (Rxn. 18), giving Rxn. 19 as the overall reaction stoichiometry. Rusty-red iron crusts can be observed near Frasassi streams (Figure S2), and both ferrous and ferric iron were measured in microbial mats and sediments (Figure S3), indicating the potential for FeS products that could sequester sulfide and perhaps increase growth of sulfur disproportionating organisms in the sediment. However, the increase in sulfide concentration with depth in the sediment indicates that iron is limiting in the sediment, as the fast reaction of sulfide with FeOOH would prevent sulfide from accumulating in the sediment. Future studies are needed to investigate the dynamics of iron cycling in the Frasassi caves and their potential impact on sulfur cycling microbial communities.

In addition to serving as a sink for S^0 at Frasassi, S^0 or polysulfide disproportionation may also contribute to the weathering of carbonate rocks and sediments in sulfur-rich environments. Subaerial sulfuric acid speleogenesis via the abiotic and biological oxidation of sulfide in sulfidic karst has been studied intensively (Jones et al., 2014; Macalady et al., 2007; Stern et al., 2002). At Frasassi, subaqueous sulfuric acid speleogenesis is thought to have a minor contribution to cave formation because sulfide degassing rates exceed the rates of microbial sulfide oxidation, and because S^0 rather than sulfuric acid is the major end product of subaqueous microbial sulfide oxidation (Jones et al., 2015). This is likely due to O_2:H_2S availability in the oxygen-rich cave air, where sulfide oxidation can proceed completely to sulfuric acid, while it is more limited by O_2 availability in the microoxic streams. S^0 disproportionation, which is an acid-producing process, may prove to be a significant contributor to anoxic, subaqueous sulfuric acid speleogenesis at Frasassi and in other sulfidic karst environments. This could be tested by incubating limestone tablets in the sediment, stream water, and cave air and measuring average mass loss over time (Galdenzi, 2012; Jones et al., 2015; Peterson, 1966), or by comparing rates of S^0 disproportionation using radiolabeled compounds in field incubations.

4.3 | Frasassi sulfidic sediments as a Proterozoic analog environment

Microbial consumption of S^0 has been studied in modern marine sediments (Pjevac et al., 2014). In sulfidic marine environments, including tidal flats and deep-sea hydrothermal vent systems, oxidation in surface sediments by *Sulfurimonas* and *Sulfurovum* was the main sink for S^0. In anoxic sediments, *Desulfocapsa* colonized and consumed S^0, indicating that disproportionation could be a sink for S^0 in the absence of oxygen in marine sediments (Pjevac et al., 2014).

Sulfur cycling at Frasassi, which occurs under oxygen-limited conditions with significantly lower salinity and sulfate concentrations than modern oceans, may have significant similarities to Proterozoic sulfur cycling in fresh to brackish sediments, when euxinic marine ecosystems encountered the initial rise of oxygen (Lyons et al., 2009; Meyer & Kump, 2008). Proterozoic waters with high sulfide concentrations and oxygen-limited conditions may have hosted microbial communities that produced S^0 through incomplete sulfide oxidation and subsequently disproportionated the S^0, analogously to the microbial communities found at Frasassi (Canfield & Teske, 1996). The fate of marine sedimentary S^0 has likely varied in Earth history as the redox chemistry of the ocean evolved, and sulfur isotope fractionations preserved in the geologic record may reveal shifts in the predominance of competing sulfur metabolisms. In particular, the S^0 disproportionation is thought to be an ancient catabolism based on phylogenetic and isotopic evidence (Canfield & Raiswell, 1999). Sulfur disproportionation produces sulfide depleted in ^{34}S and sulfate enriched in ^{34}S, and repeated cycles of sulfide oxidation to S^0 followed by disproportionation are thought to produce sulfides that are strongly isotopically depleted by over 50‰ relative to seawater sulfate (Canfield et al., 1998; Canfield & Thamdrup, 1994; Johnston et al., 2005). The closely linked ecology of sulfide oxidizers, sulfate reducers, S^0 reducers, and S^0 disproportionators at Frasassi may inform on how microbial metabolisms shaped the biogeochemical sulfur cycle in Proterozoic sedimentary ecosystems. At Frasassi, the $\delta^{34}S$ fractionations observed between sedimentary sulfate and sulfides were (<40‰) smaller than what is expected in systems driven by reductive or disproportionative sulfur cycling (Zerkle et al., 2016). Further studies are necessary to interpret the effects of chemolithotrophic sulfur oxidation on sulfur isotopic fractionation observed at Frasassi.

5 | CONCLUSIONS

The Frasassi Cave system hosts a biogeochemical sulfur cycle that is largely driven by microbial oxidation of sulfide. In the Proterozoic-like oxygen-limited sulfidic stream waters, sulfide is incompletely oxidized by members of the stream microbial mat to form S^0. The fate of this S^0 is not well understood, and we hypothesized that it could serve as an electron donor and/or acceptor for microbial communities inhabiting the stream sediment. Here, we investigated the sediment microbial communities for the first time and found that almost 30% of the community was composed of taxa closely related to isolates shown to carry out S^0 disproportionation, including *Desulfocapsa* and *Sulfurovum*. Microsensor profiles through the mat-sediment interface showed that dissolved sulfide and oxygen are consumed, but a net increase in acidity was only observed in the sediments below. Calculations of the Gibbs energy using the microsensor data revealed that S^0 disproportionation is energy yielding under in situ conditions and could explain the acid production in the sediment. Our results suggest that S^0 disproportionation could serve as an important sink for microbially produced S^0 in this Proterozoic-like system and could be a significant contributor to the weathering of carbonate rocks and sediments in other sulfur-rich environments.

AUTHOR CONTRIBUTIONS

HSA analyzed the data, interpreted results, designed the figures, and drafted the manuscript. CEC collected field samples, analyzed the data, interpreted results, designed the figures, and drafted the manuscript. DEL and JPA assisted with thermodynamic calculations and revised the manuscript. LP assisted with microsensor data collection and revised the manuscript. JLM designed the study and revised the manuscript. All authors approved the final version of the manuscript. The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank A. Montanari for providing logistical support and the use of facilities and laboratory space at the Osservatorio Geologico di Coldigioco, Apiro, Italy; S. Mariani, S. Cerioni, M. Mainiero, F. Baldoni, S. Carnevali, and members of the Gruppo Speleologico C.A.I. di Fabriano for technical assistance during field campaigns, and to S. Dattagupta, R. McCauley Rench, KS Dawson, and C. Chan for assistance in the field. We thank DS Jones for helpful discussions on Frasassi sediment biogeochemistry. This work was supported by National Science Foundation grants EAR-0525503 and EAR-1124411 (JM), the NASA Habitable Worlds program under grant 80NSSC20K0228 (DEL) and the USC Dornsife College of Letters, Arts and Sciences (DEL). HSA was supported by an NSF Graduate Research Fellowship grant DGE-1842487, the Lewis and Clark Fund for Exploration and Field Research in Astrobiology, the National Cave and Karst Research Institute NCKRI Scholar Fellowship, the Josephine de Karman Fellowship, and the Cave Conservancy Foundation Graduate Fellowship in Karst Studies.

DATA AVAILABILITY STATEMENT

16S rRNA amplicon reads are available in GenBank under BioProject accession number PRJNA871755. Chemical concentration data used for thermodynamic calculations are available in Appendix S1.

ORCID

Heidi S. Aronson https://orcid.org/0000-0002-6777-0304

REFERENCES

Alain, K., Aronson, H. S., Allioux, M., Yvenou, S., & Amend, J. P. (2022). Sulfur disproportionation is exergonic in the vicinity of marine hydrothermal vents. *Environmental Microbiology*, *24*, 2210–2219.

Amend, J. P., & LaRowe, D. E. (2019). Minireview: Demystifying microbial reaction energetics. *Environmental Microbiology*, *21*, 3539–3547.

Aronson, H. S., Thomas, C., Bhattacharyya, M. K., Eckstein, S. R., Jensen, S. R., Barco, R. A., Macalady, J. L., & Amend, J. P. (2022). *Thiovibrio frasassiensis* gen. Nov., sp. nov., an autotrophic, elemental sulfur disproportionating bacterium isolated from sulfidic karst sediment, and proposal of *Thiovibrionaceae* fam. Nov. *bioRxiv* 2022.10.27.514068.

Bak, F., & Cypionka, H. (1987). A novel type of energy metabolism involving fermentation of inorganic sulphur compounds. *Nature*, *326*, 891–892.

Berg, J. S., Schwedt, A., Kreutzmann, A.-C., Kuypers, M. M. M., & Milucka, J. (2014). Polysulfides as intermediates in the oxidation of sulfide to sulfate by *Beggiatoa* spp. *Applied and Environmental Microbiology*, *80*, 629–636.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*, 852–857.

Borisov, V. B., Gennis, R. B., Hemp, J., & Verkhovsky, M. I. (2011). The cytochrome bd respiratory oxygen reductases. *Biochimica et Biophysica Acta*, *1807*, 1398–1413.

Borisov, V. B., Siletsky, S. A., Paiardini, A., Hoogewijs, D., Forte, E., Giuffrè, A., & Poole, R. K. (2021). Bacterial oxidases of the cytochrome bd family: Redox enzymes of unique structure, function, and utility as drug targets. *Antioxidants & Redox Signaling*, *34*, 1280–1318.

Boyer, G., Ely, T., & Shock, E. (2021). *AqEquil v0.9.1*.

Buschmann, S., Warkentin, E., Xie, H., Langer, J. D., Ermler, U., & Michel, H. (2010). The structure of cbb3 cytochrome oxidase provides insights into proton pumping. *Science*, *329*, 327–330.

Caccavo, F., Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F., & Mcinerneyl, M. J. (1994). *Geobacter sulfurreducens* sp. nov., a hydrogen-and acetate-oxidizing dissimilatory metal-reducing microorganism. *Applied and Environmental Microbiology*, *60*, 3752–3759.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*, 581–583.

Campbell, B. J., Engel, A. S., Porter, M. L., & Takai, K. (2006). The versatile ε-proteobacteria: Key players in sulphidic habitats. *Nature Reviews Microbiology*, *4*, 458–468.

Canfield, D. E., & Raiswell, R. (1999). The evolution of the sulfur cycle. *American Journal of Science*, *299*, 697–723.

Canfield, D. E., & Teske, A. (1996). Late proterozoic rise in atmospheric oxygen concentration inferred from phylogenetic and Sulphur-isotope studies. *Nature*, *382*, 127–132.

Canfield, D. E., & Thamdrup, B. (1994). The production of 34S-depleted sulfide during bacterial disproportionation of elemental sulfur. *Science*, *266*, 1973–1975.

Canfield, D. E., Thamdrup', B., & Fleischer, S. (1998). Isotope fractionation and sulfur metabolism by pure and enrichment cultures of elemental sulfur-disproportionating bacteria. *Limnology and Oceanography*, *43*, 253–264.

Capella-Gutiérrez, S., Silla-Martínez, J. M., & Gabaldón, T. (2009). trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*, *25*, 1972–1973.

Dick, J. M. (2019). CHNOSZ: Thermodynamic calculations and diagrams for geochemistry. *Frontiers in Earth Science*, *7*, 180.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

Engel, A. S., Lichtenberg, H., Prange, A., & Hormes, J. (2007). Speciation of sulfur from filamentous microbial mats from sulfidic cave springs using X-ray absorption near-edge spectroscopy. *FEMS Microbiology Letters*, *269*, 54–62.

Findlay, A. J. (2016). Microbial impact on polysulfide dynamics in the environment. *FEMS Microbiology Letters*, *363*, fnw103.

Finster, K. (2008). Microbiological disproportionation of inorganic sulfur compounds. *Journal of Sulfur Chemistry*, *29*, 281–292.

Finster, K., Liesack, W., & Thamdrup, B. (1998). Elemental sulfur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. *Applied and Environmental Microbiology*, *64*, 119–125.

Finster, K. W., Kjeldsen, K. U., Kube, M., Reinhardt, R., Mussmann, M., Amann, R., & Schreiber, L. (2013). Complete genome sequence of *Desulfocapsa sulfexigens*, a marine deltaproteobacterium specialized in disproportionating inorganic sulfur compounds. *Standards in Genomic Sciences*, *8*, 58–68.

Forte, E., Borisov, V. B., Falabella, M., Colaço, H. G., Tinajero-Trejo, M., Poole, R. K., Vicente, J. B., Sarti, P., & Giuffre, A. (2016). The terminal oxidase cytochrome bd promotes sulfide-resistant bacterial respiration and growth. *Scientific Reports*, *6*, 1–8.

Frederiksen, T. M., & Finster, K. (2003). Sulfite-oxido-reductase is involved in the oxidation of sulfite in *Desulfocapsa sulfoexigens* during disproportionation of thiosulfate and elemental sulfur. *Biodegradation*, *14*, 189–198.

Frederiksen, T. M., & Finster, K. (2004). The transformation of inorganic sulfur compounds and the assimilation of organic and inorganic carbon by the sulfur disproportionating bacterium *Desulfocapsa sulfoexigens*. *Antonie Van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *85*, 141–149.

Galdenzi, S. (2012). Corrosion of limestone tablets in sulfidic groundwater: Measurements and speleogenetic implications. *International Journal of Speleology*, *41*, 149–159.

Galdenzi, S., Amici, V., Cocchioni, M., Morichetti, L., & Scuri, S. (2008). Sulfidic ground water chemistry in the Frasassi cave, Italy. *Journal of Cave and Karst Studies*, *70*, 94–107.

Galdenzi, S., Menichetti, M., Sarbu, S., & Rossi, A. (1999). Frasassi caves: A biogenic hypogean karst system? In: *European Conference Karst '99*. pp. 101–106.

Giovannelli, D., Chung, M., Staley, J., Starovoytov, V., Le Bris, N., & Vetriani, C. (2016). *Sulfurovum riftiae* sp. nov., a mesophilic, thiosulfate-oxidizing, nitrate-reducing chemolithoautotrophic epsilonproteobacterium isolated from the tube of the deep-sea hydrothermal vent polychaete *Riftia pachyptila*. *International Journal of Systematic and Evolutionary Microbiology*, *66*, 2697–2701.

Hamilton, T. L., Jones, D. S., Schaperdoth, I., & Macalady, J. L. (2015). Metagenomic insights into S(0) precipitation in a terrestrial subsurface lithoautotrophic ecosystem. *Frontiers in Microbiology*, *5*, 1–16.

Hédoin, H. (1997). Eaux thermales de Barège: Microbiologie de l'eau. *L'Eurobiologiste*, *31*, 43–51.

Hédoin, H., Couté, A., Kaiser, P., & Laugier, R. (1996). Nature and occurrence of sulfoxidizing bacteria in Barégine developing in sulfurated thermal waters at Barèges (France). *Hydrobiologia*, *323*, 75–81.

Helgeson, H. C., Kirkham, D. H., & Flowers, G. C. (1981). Theoretical prediction of the thermodynamic behavior of aqueous electrolytes by high pressures and temperatures; IV, calculation of activity coefficients, osmotic coefficients, and apparent molal and standard and relative partial molal properties to 600 degrees C and 5kb. *American Journal of Science*, *281*, 1249–1516.

Hoehler, T. M., Alperin, M. J., Albert, D. B., & Martens, C. S. (1998). Thermodynamic control on hydrogen concentrations in anoxic sediments. *Geochimica et Cosmochimica Acta*, *62*, 1745–1756.

Inagaki, F., Takai, K., Nealson, K. H., & Horikoshi, K. (2004). *Sulfurovum lithotrophicum* gen. Nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the E-Proteobacteria isolated from Okinawa trough hydrothermal sediments. *International Journal of Systematic and Evolutionary Microbiology*, *54*, 1477–1482.

Ito, T., Sugita, K., Yumoto, I., Nodasaka, Y., & Okabe, S. (2005). *Thiovirga sulfuroxydans* gen. Nov., sp. nov., a chemolithoautotrophic sulfur-oxidizing bacterium isolated from a microaerobic waste-water biofilm. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 1059–1064.

Jørgensen, B. B., & Revsbech, N. P. (1983). Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovulum* spp., in 02 and H2S microgradients. *Applied and Environmental Microbiology*, *45*, 1261–1270.

Janssen, P. H., Schuhmann, A., Bak, F., & Liesack, W. (1996). Disproportionation of inorganic sulfur compounds by the sulfate-reducing bacterium *Desulfocapsa thiozymogenes* gen, nov., sp. nov. *Archives of Microbiology*, *166*, 184–192.

Johnston, D. T., Wing, B. A., Farquhar, J., Kaufman, A. J., Strauss, H., Lyons, T. W., Kah, L. C., & Canfield, D. E. (2005). Active microbial sulfur disproportionation in the Mesoproterozoic. *Science*, *310*, 1477–1479.

Jones, D. S., Albrecht, H. L., Dawson, K. S., Schaperdoth, I., Freeman, K. H., Pi, Y., Pearson, A., & MacAlady, J. L. (2012). Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm. *ISME Journal*, *6*, 158–170.

Jones, D. S., Lyon, E. H., & Macalady, J. L. (2008). Geomicrobiology of biovermiculations from the Frasassi cave system, Italy. *Journal of Cave and Karst Studies*, *70*, 78–93.

Jones, D. S., Polerecky, L., Galdenzi, S., Dempsey, B. A., & Macalady, J. L. (2015). Fate of sulfide in the Frasassi cave system and implications for sulfuric acid speleogenesis. *Chemical Geology*, *410*, 21–27.

Jones, D. S., Schaperdoth, I., & Macalady, J. L. (2014). Metagenomic evidence for sulfide oxidation in extremely acidic cave biofilms. *Geomicrobiology Journal*, *31*, 194–204.

Jones, D. S., Schaperdoth, I., & Macalady, J. L. (2016). Biogeography of sulfur-oxidizing *Acidithiobacillus* populations in extremely acidic cave biofilms. *ISME Journal*, *10*, 2879–2891.

Jones, D. S., Tobler, D. J., Schaperdoth, I., Mainiero, M., & Macalady, J. L. (2010). Community structure of subsurface biofilms in the thermal sulfidic caves of Acquasanta Terme, Italy. *Applied and Environmental Microbiology*, *76*, 5902–5910.

Kamp, A., Stief, P., & Schulz-Vogt, H. N. (2006). Anaerobic sulfide oxidation with nitrate by a freshwater *Beggiatoa* enrichment culture. *Applied and Environmental Microbiology*, *72*, 4755–4760.

Klatt, J. M., Meyer, S., Häusler, S., Macalady, J. L., De Beer, D., & Polerecky, L. (2016). Structure and function of natural sulphide-oxidizing microbial mats under dynamic input of light and chemical energy. *ISME Journal*, *10*, 921–933.

Klatt, J. M., & Polerecky, L. (2015). Assessment of the stoichiometry and efficiency of CO_2 fixation coupled to reduced sulfur oxidation. *Frontiers in Microbiology*, *6*, 484.

Kodama, Y., & Watanabe, K. (2004). *Sulfuricurvum kujiense* gen. Nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity. *International Journal of Systematic and Evolutionary Microbiology*, *54*, 2297–2300.

Labrado, A. L. (2017). *Evidence for dissimilatory nitrate reduction to ammonia by aqueous biofilms in the sulfidic caves of Frasassi, Italy.*

Lovley, D. R., & Phillips, E. J. P. (1994). Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. *Applied and Environmental Microbiology*, *60*, 2394–2399.

Lyons, T. W., Anbar, A. D., Severmann, S., Scott, C., & Gill, B. C. (2009). Tracking Euxinia in the Ancient Ocean: A multiproxy perspective and Proterozoic case study. *Annual Review of Earth and Planetary Sciences*, *37*, 507–534.

Macalady, J. L., Dattagupta, S., Schaperdoth, I., Jones, D. S., Druschel, G. K., & Eastman, D. (2008). Niche differentiation among sulfur-oxidizing bacterial populations in cave waters. *ISME Journal*, *2*, 590–601.

Macalady, J. L., Jones, D. S., & Lyon, E. H. (2007). Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. *Environmental Microbiology*, *9*, 1402–1414.

Macalady, J. L., Lyon, E. H., Koffman, B., Albertson, L. K., Meyer, K., Galdenzi, S., & Mariani, S. (2006). Dominant microbial populations in limestone-corroding stream biofilms, Frasassi cave system, Italy. *Applied and Environmental Microbiology*, *72*, 5596–5609.

Macalady, J. L., Mainiero, M., & Galassi, D. M. P. (2019). The Frasassi caves, Italy. In W. B. White, D. C. Culver, & T. Pipan (Eds.), *Encyclopedia of caves* (pp. 435–443). Elsevier.

McCauley Rench, R. (2015). *Metabolic potential and diversity of the microbial communities in a sulfidic cave system* (PhD).

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*, e61217.

Meyer, K. M., & Kump, L. R. (2008). Oceanic Euxinia in earth history: Causes and consequences. *Annual Review of Earth and Planetary Sciences*, *36*, 251–288.

Milucka, J., Ferdelman, T. G., Polerecky, L., Franzke, D., Wegener, G., Schmid, M., Lieberwirth, I., Wagner, M., Widdel, F., & Kuypers, M. M. M. (2012). Zero-valent sulphur is a key intermediate in marine methane oxidation. *Nature*, *491*, 541–546.

Mino, S., Kudo, H., Arai, T., Sawabe, T., Takai, K., & Nakagawa, S. (2014). *Sulfurovum aggregans* sp. nov., a hydrogen-oxidizing, thiosulfate-reducing chemolithoautotroph within the Epsilonproteobacteria isolated from a deep-sea hydrothermal vent chimney, and an emended description of the genus *Sulfurovum*. *International Journal of Systematic and Evolutionary Microbiology*, *64*, 3195–3201.

Mori, K., & Suzuki, K.-i. (2008). *Thiofaba tepidiphila* gen. Nov., sp. nov., a novel obligately chemolithoautotrophic, sulfur-oxidizing bacterium of the Gammaproteobacteria isolated from a hot spring. *International Journal of Systematic and Evolutionary Microbiology*, *58*, 1885–1891.

Morrison, P. R., & Mojzsis, S. J. (2021). Tracing the early emergence of microbial sulfur metabolisms. *Geomicrobiology Journal*, *38*, 66–86.

Mußmann, M., Hu, F. Z., Richter, M., de Beer, D., Preisler, A., Jørgensen, B. B., Huntemann, M., Glöckner, F. O., Amann, R., Koopman, W. J. H., Lasken, R. S., Janto, B., Hogg, J., Stoodley, P., Boissy, R., & Ehrlich, G. D. (2007). Insights into the genome of large sulfur bacteria revealed by analysis of single filaments. *PLoS Biology*, *5*, e230.

Nakagawa, S., Takai, K., Inagaki, F., Horikoshi, K., & Sako, Y. (2005). *Nitratiruptor tergarcus* gen. Nov., sp. nov. and *Nitratifractor salsuginis* gen. Nov., sp. nov., nitrate-reducing chemolithoautotrophs of the ε-Proteobacteria isolated from a deep-sea hydrothermal system in the Mid-Okinawa Trough. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 925–933.

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, *32*, 268–274.

Parker, C. U., & Prisk, J. (1953). The oxidation of inorganic compounds of sulphur by various sulphur bacteria. *Journal of General Microbiology*, *8*, 344–364.

Pavia, M. J., Fidelma Boyd, E., Watson, G. H., & Doren, D. J. (2018). *Colonization and S(0) mineralization of sulfur oxidizing biofilms in the Frasassi cave system.*

Peterson, M. N. A. (1966). Calcite: Rates of dissolution in a vertical profile in the Central Pacific. *Science*, *154*, 1542–1544.

Pitcher, R. S., & Watmough, N. J. (2004). The bacterial cytochrome cbb3 oxidases. *Biochimica et Biophysica Acta (BBA) – Bioenergetics*, *1655*, 388–399.

Pjevac, P., Kamyshny, A., Dyksma, S., & Mussmann, M. (2014). Microbial consumption of zero-valence sulfur in marine benthic habitats. *Environmental Microbiology*, *16*, 3416–3430.

Poser, A., Lohmayer, R., Vogt, C., Knoeller, K., Planer-Friedrich, B., Sorokin, D., Richnow, H. H., & Finster, K. (2013). Disproportionation of elemental sulfur by haloalkaliphilic bacteria from soda lakes. *Extremophiles*, *17*, 1003–1012.

Preisig, O., Zufferey, R., Thöny-Meyer, L., Appleby, C. A., & Hennecke, H. (1996). A high-affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *Journal of Bacteriology*, *178*, 1532–1538.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*, D590–D596.

R Core Team. (2019) *R: A language and environment for statistical computing*. The R Foundation.

Rich, P. R., Meunier, B., Mitchell, R., & John Moody, A. (1996). Coupling of charge and proton movement in cytochrome c oxidase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1275*, 91–95.

Schauder, R., & Müller, E. (1993). Polysulfide as a possible substrate for sulfur-reducing bacteria. *Archives of Microbiology*, *160*, 377–382.

Schedel, M., & Trüper, H. G. (1980). Anaerobic oxidation of thiosulfate and elemental sulfur in *Thiobacillus denitrificans*. *Archives of Microbiology*, *124*, 205–210.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*, 7537–7541.

Schwarzenbach, G. V., & Fischer, A. (1960). Die Acidität der Sulfane Und Die Zusammensetzung wässeriger Polysulfidlösungen. *Helvetica*, *43*, 1365–1390.

Schwedt, A., Kreutzmann, A.-C., Polerecky, L., & Schulz-Vogt, H. N. (2012). Sulfur respiration in a marine chemolithoautotrophic *Beggiatoa* strain. *Frontiers in Microbiology*, *2*, 276.

Shock, E. L., Oelkers, E. H., Johnson, J. W., Sverjensky, D. A., & Helgeson, H. C. (1992). Calculation of the thermodynamic properties of aqueous species at high pressures and temperatures. Effective electrostatic radii, dissociation constants and standard partial molal properties to 1000°C and 5 kbar. *Journal of the Chemical Society, Faraday Transactions*, *88*, 803–826.

Sievert, S. M., Heidorn, T., & Kuever, J. (2000). *Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and emended description of the genus. *International Journal of Systematic and Evolutionary Microbiology*, *50*, 1229–1237.

Stern, L., Engel, A. S., Bennett, P. C., & Porter, M. L. (2002). Subaqueous and subaerial speleogenesis in a sulfidic cave. *Karst Waters Institute*, *7*, 89–91. Special Publication.

Tanger, J. C., & Helgeson, H. C. (1988). Calculation of the thermodynamic and transport properties of aqueous species at high pressures and temperatures; revised equations of state for the standard partial molal properties of ions and electrolytes. *American Journal of Science*, *288*, 19–98.

Thamdrup, B., Finster, K., Hansen, J. W., & Bak, F. (1993). Bacterial disproportionation of elemental sulfur coupled to chemical reduction of iron or manganese. *Applied and Environmental Microbiology*, *59*, 101–108.

Tsao, L. E. (2014). *Culture-dependent and independent studies of sulfur oxidizing bacteria from the Frasassi Caves*.

Wang, S., Jiang, L., Xie, S., Alain, K., Wang, Z., Wang, J., Liu, D., & Shao, Z. (2023). Disproportionation of inorganic sulfur compounds by mesophilic chemolithoautotrophic campylobacterota. *mSystems*, *8*(1), e00954-22.

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag.

Wolery, T. J. (1979). *Calculation of chemical equilibrium between aqueous solution and minerals: The EQ3/6 software package*. California Univ.

Zerkle, A. L., Jones, D. S., Farquhar, J., & Macalady, J. L. (2016). Sulfur isotope values in the sulfidic Frasassi cave system, Central Italy: A case study of a chemolithotrophic S-based ecosystem. *Geochimica et Cosmochimica Acta*, *173*, 373–386.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Aronson, H. S., Clark, C. E., LaRowe, D. E., Amend, J. P., Polerecky, L., & Macalady, J. L. (2023). Sulfur disproportionating microbial communities in a dynamic, microoxic-sulfidic karst system. *Geobiology*, *21*, 791–803. https://doi.org/10.1111/gbi.12574