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Cryogenic minerals in Hawaiian lava tubes: A geochemical and microbiological exploration
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Abstract
Mauna Loa volcano, on the Island of Hawaii, has numerous young lava tube caves. Among them, two at high altitude are known to contain ice year-round: Mauna Loa Icecave and Arsia Cave. These unusual caves harbor cold, humid, dark, and biologically restricted environments. Secondary minerals and ice were sampled from both caves to explore their geochemical and microbiological characteristics. The minerals sampled from the deep parts of the caves, where near freezing temperatures prevail, are all multi-phase and consist mainly of secondary amorphous silica SiO₂, cryptocrystalline calcite CaCO₃, and gypsum CaSO₄·2H₂O. Based on carbon and oxygen stable isotope ratios, all sampled calcite is cryogenic. The isotopic composition of Arsia Cave pond water falls on the global meteoric line, indicating that little evaporation has occurred. The microbial diversity of a silica and calcite deposit in Mauna Loa Icecave and from ice pond water in Arsia Cave was explored by analysis of ~50,000 SSU rRNA gene fragments via amplicon sequencing. Analyses reveal that the Hawaii ice caves harbor unique microbial diversity distinct from other environments, including cave environments, on Hawaii and worldwide. Actinobacteria and Proteobacteria were the most abundant microbial phyla detected, which is largely consistent with studies
of other oligotrophic cave environments. The cold, isolated, oligotrophic basaltic lava cave environment on Hawaii provides a unique opportunity to understand microbial biogeography not only on Earth, but also on other planets.
Introduction

Context and Motivation

Mauna Loa (19°N 156°W; 4,169m above sea level), on the Island of Hawaii, is an active shield volcano with countless lava tubes (Halliday, 2004). At the Mauna Loa Meteorological Observatory (MLO, 3,397m asl), which is in the vicinity of our study sites, the average annual precipitation is only 452mm (Giambelluca et al., 2013), in the form of rain and snow. At this altitude the annual mean temperature is still well above freezing (+7°C), but in rare cases, perennial ice is found in lava tubes, partly due to cold air descending into the caves (Geiger, 1965). Two such ice caves (or ice-filled lava tubes) are known on the north flank of Mauna Loa volcano: Arsia Cave and Mauna Loa Icecave. Here, “Icecave” is spelled as one word, as it was on the original map (Kempe, 1979). These high-altitude lava tubes are the world’s most geographically isolated ice caves, farthest from any ice sheets and glaciers (Pflitsch et al., 2016). Situated in a barren stone desert, there is no vegetation in the vicinity of the study sites, only a few lichens hidden in pockets, and hence very little organic carbon is available to cave microorganisms that have to rely on other energy sources. Here, these rare and unusual caves are characterized in terms of their mineralogical and microbiological content, as well as their particular geochemical environment. We investigate the geochemistry of its water, identify secondary minerals, and search for microbial nucleic acid gene markers in the deepest and coldest parts of the lava tubes. The findings help to understand their environment and inspire insight into what may be expected in Martian lava tubes (Banfield et al., 2001; Leveille & Datta, 2010).

Lava tubes can contain a variety of secondary minerals formed by seepage waters that extract ions from the overlying basalt (Forti, 2005; Onac & Forti, 2011). White (2010) analyzed many specimens of secondary minerals from a selection of caves on Hawaii, including “Sample Cave” at 2,900m asl on Mauna Loa, where gypsum CaSO₄·2H₂O, thenardite Na₂SO₄, and mirabilite Na₂SO₄·10H₂O were identified. Our study sites contain perennial ice and are at elevations above 3,200m. Lava tubes are oligotrophic environments characterized by little to no light, and thus potentially contain chemoheterotrophic microorganisms that obtain energy from weathering the cave surfaces (Tomczyk-Zak & Zielenkiewicz, 2016) and oxidation of manganese and iron from rocks (Northup & Lavoie, 2001). As the conditions on Mars are further understood, the microbial life of many potential Earth-based analogues have been characterized, for example, Antarctic Dry Valleys (e.g. Wierzchos et al., 2005), sulfur-rich habitats (e.g. Sarbu et al., 1996; Boston et al., 2001), and hot springs (e.g. Preston et al., 2008). However, Fe-rich subsurface environments, like basaltic lava caves are most appealing to understand Mars microbial life because of the similar lithology (i.e. basaltic rock), offered protection from damaging solar
radiation, and possibility for liquid water retention (Northup et al., 2011; Popa et al., 2012; Northup and Lavoie, 2015). Thus, the perennial geographically-isolated ice caves of Mauna Loa offer a unique habitat to explore a refuge for life as it might exist on other planets.

*Site Descriptions*

Mauna Loa Icecave (MLIC) was briefly described by Kempe (1979), Kempe & Ketz-Kempe (1979a,b), and recently by Pflitsch et al. (2016). The lava tube is situated in geologic unit “k3”, 750--1,500 years old, and extends 235m from the entrance pit to the ice that blocks the remainder of the lava tube (Fig. 1). A survey from summer 1978 documented a massive ice floor (“Skating Rink”) that has disappeared sometime between then and our first visit in 2011; a whitish secondary mineral is left where the ice floor used to be. An ice plug still blocks the lava tube at its lower end.

Arsia Cave was discovered in 2009 by S.M. Smith and is briefly described here for the first time. Its name is based on the Arsia Mons region of Mars, where numerous putative lava tube entrances have been identified (Cushing, 2012). It is located in a lava flow dated 200--750 years old (geologic unit “k4”; U.S. Geologic Survey, 1996). Two side passages within this lava tube have frozen lakes at the distal end (Fig. 2). One of the branches (Branch A or “Freezeway”) descends for ~300m, although with several skylights, and ends in a large chamber with an ice pond ~30m long and ~7m wide; the beginning of the pond is marked by a perennial ice stalagmite (Fig. 2B). The other branch (B or “Icebat Hall”) descends for ~100m from the last skylight to the beginning of an ice lake ~30m long and on average ~4m wide (Fig. 2C,D). Water occasionally drips from the ceiling and freezes in the lakes that contain cm-sized air bubbles. Large air bubbles in clear ice are expected for slow freezing from above (Carte, 1961; Bari & Hallett, 1974).

*Sample Descriptions*

Table 1 lists the samples. The mineral deposits collected consist of veneer (basalt with white coating on the surface) and bulk samples (all powder) that occur only over a small area (decimeters). We refer to the latter type as “localized deposits”. Figure 3 shows a selection of photos of the specimens and their context.

Sampling in MLIC took place on April 12, 2015. The map in Figure 1 includes the sampling locations. Mineral sample M1 is located about 1.5m above the perennial ice deposit and the 4-year average temperature (Nov 2011 to Nov 2015, with a small gap) is +0.1°C (min/max: -1°C/+3°C). The relative humidity (RH) fluctuates strongly and has an average value of 81% (min 21%, max 100%) immediately above the ice. M1a and M1b are subsamples of the same deposit, collected for chemical and microbiological analysis, respectively. The three mineral samples M2-1, M2-3, and M2-5 were taken
along a transect at the lower end of Party Hall, with M2-1 and M2-3 at comparable height but opposite sides of the lava tube, whereas M2-5 is a sample from the cave floor (Fig. 1). The air temperature logger very near to these sample locations recorded a 4-year average of +1.0°C (min/max: -1°/+6°C). Water samples M4-1 and M4-2 were clear (fresh) icicles along the same transect. Samples M3-1 (veneer) and M4-3 (icicle) are from the upper end of Party Hall. Snow was sampled at the entrance pit on the same day.

In Arsia Cave, water was sampled from Icebat Hall (Branch B) on April 11, 2015. Unexpectedly, the ice pond in branch B was partially melted on the day of our visit, but covered by a thin layer of ice. Hence, it was easy to sample the previously frozen pond, yet the water was isolated from the atmosphere during the visit. A syringe was used to break through the ice and sample 10mL water each for the physical chemistry (A7a) and the microbiology analysis (A7b). Blank water samples (A5a & A5b) were apportioned as described below. Icicle A1 is from the area immediately in front of the ice pond. Two mineral samples were also collected from this area in Arsia Cave. Rock sample A6, from a ledge close to the ice pond, has a mineral coating (Fig. 3). The record of a nearby data logger indicates an environment that is near freezing but very humid (average RH 98%). A pile of breakdown material, about 3m high, blocks almost the entire lava tube just before the beginning of the ice lake. Sample A8 is a localized deposit of large soft grains on the cave floor in front of this rock barrier (on the outward side).

Methods

Sampling procedure

Decontamination procedures for inorganic constituents were based on Wilde (2004). Before the field trip, sample containers and instruments (metal spatula, saw, tweezers, and chisel) were repeatedly washed in distilled water, air dried, and stored in plastic bags. In the caves, icicles were handled with disposable powder-free vinyl gloves and, when necessary, sawed off and placed on aluminum foil for further cutting before being stored in leakproof plastic containers. Mineral samples were handled with clean instruments and gloves. As a control, two vials were filled with distilled water inside the cave, and otherwise treated like the water samples. All containers were promptly labeled on site and stored in leakproof aLOKSAK bags.

Two mineral samples (M1 and A8) were collected aseptically, using 70% isopropyl alcohol and flaming of the spatula immediately before collection. Hands were covered with gloves during the sampling. The sterile 1mL plastic vials were then sealed with Parafilm. A sterile syringe was used to collect 10mL of water (sample A7).
Samples from Arsia Cave were transported in a PolarBear cooler with dry ice to the base station at MLO. The goal was to transfer the mineral samples to the laboratory without dehydration and to impede microbial growth in the biological samples. Water samples were kept from freezing to avoid precipitation. We tried to keep the samples either continuously cooled or continuously frozen at MLO, during transport from MLO to Hilo airport, and on the flight from Hilo to Honolulu, until they reached the laboratories at the University of Hawaii at Manoa (UHM). The mineral samples were immediately subjected to a first round of Micro-Raman spectroscopy, and the biological samples were stored at -80°C at the Center for Microbial Oceanography until further processing. All samples not intended for biological analysis were ultimately allowed to warm up to room temperature, stored in a desiccator, and subjected to additional analyses.

**Analytical methods**

Ion analysis of the water samples was carried out at the UHM Water Resources Research Center Analytical Chemistry Laboratory with a Dionex ICS-1100s ion chromatograph (>0.1% precision), using 7 Anion-II and 6 Cation-II standards. For water isotope analysis, 2mL of each sample were submitted to the Center for Isotope Geochemistry at the University of California at Berkeley. A Picarro L2140-i analyzer was used; the in-house water standards for H and O isotopes had standard deviations of 0.73‰ and 0.13‰, respectively.

Each mineral sample was put through a 785nm excitation wavelength micro-Raman spectrometer at the UHM (Kaiser RXN1 Microprobe). Several spots on each sample were analyzed, with the veneer-coated side being the exterior face of the rock when it was collected, and the other side of the sample representing the interior face that was broken off the cave wall. The experimental spectra were plotted together with literature Raman spectra (Buzgar et al., 2009) using Essential FTIR software. Additional mineralogical analyses on selected samples were performed at the University of South Florida using a Bruker Analytical X-Ray System, Inc. D8 Endeavor XRD. Samples were scanned from 5 to 75° 20 with a step increment of 0.02° and a scan speed of 0.5 sec/step (analytical conditions: 50 kV, 40mA, CuK radiation, line source filtered with a Ni foil).

Secondary electron (SE) and backscattered electron (BSE) images of sample fragments and surfaces were acquired on a field-emission gun JEOL JXA-8500F electron probe microanalyzer at the Department of Geology and Geophysics at the UHM. Elemental analysis and element distribution maps were obtained using a Thermo-Noran System-six energy-dispersive spectrometry (EDS) system with a 10 mm² SDD detector. Accuracy is estimated to be better than 10% relative, based on the direct comparison between wavelength-dispersive analysis of the same polished certified silicate and carbonate standard.
materials. Operating conditions were 15 keV acceleration potential, a low probe current (3-5nA) to minimize sample damage, and a focused electron beam (~50nm). Samples were carbon coated prior to analyses to allow for a conductive surface. Carbon-k-alpha peak height in carbonates was at least a factor three higher than carbon-coated carbonate-free silicate.

The isotopic ratios $^{13}{\text{C}}/^{12}{\text{C}}$ ($\delta^{13}{\text{C}}$) and $^{18}{\text{O}}/^{16}{\text{O}}$ ($\delta^{18}{\text{O}}$) of the calcium carbonate samples were measured using a Thermo Delta V isotope ratio mass spectrometer at the Stable Isotope Laboratory in the School of Geosciences at the University of South Florida. Samples with white veneer were scraped using needles and forceps to collect at least 300 µg of secondary mineral. The carbonates were reacted for 24 hours with H₂PO₄ at 25°C to generate CO₂. The results are expressed in the δ-units in part per thousand (‰) relative to PDB; $\delta = (R1/ R2 – 1)·1000$, where R1 and R2 are the $^{13}{\text{C}}/^{12}{\text{C}}$ and $^{18}{\text{O}}/^{16}{\text{O}}$ ratios in the sample and the standard, respectively. For accuracy control, NBS-18 and NBS-19 were used as internal laboratory standards. Reproducibility was better than ±0.12‰.

HOBO data loggers (model no. U23-002, U23-003, and U12-008) recorded the meteorological conditions in the cave (T and RH) in half hour intervals over multiple years.

**DNA extraction, SSU rRNA gene PCR amplification and Illumina sequencing, and sequence read processing**

Fluid samples intended for the extraction of environmental DNA were thawed on ice; for each 10ml were manually filtered through a 0.1µm pore-sized polyethersulfone membrane filter (Pall Corporation, Port Washington, NY), and the filters stored for ~1.5 weeks at -80°C inside sterile tubes. Prior to nucleic acid extraction, the membrane filters and mineral samples were thawed to room temperature. Environmental DNA was extracted from the filters and ~1 cm³ of mineral sample using the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer’s protocol. A final elution step used 20µl of water, which was performed to concentrate the expected low amounts of environmental DNA recovered.

An Illumina sequencing approach (Caporaso et al., 2011) was used to characterize the ice cave samples. Briefly, the polymerase chain reaction (PCR) was used to amplify the V4 and V5 regions of the small subunit ribosomal RNA (SSU rRNA) gene using oligonucleotide primers 515FY (5’-GTGCCAGCMGCGCCGTAA-3’) and 926R (5’-AAACTYAAAAKRAATTGRCGG-3’) specific to Bacteria and Archaea (Parada et al., 2016), which were modified to include the Illumina flowcell adapter sequences (Bates et al., 2011). Forward and reverse primers contained an additional 8-bp barcode to assign individual sequences to samples (Kozich et al., 2013). Each 25µl PCR reaction was prepared in 5Prime HotMasterMix (Eppendorf-5Prime Inc., Gaithersburg, MD) and contained 0.5 U Taq DNA
polymerase, 45mM KCl, 2.5mM Mg$^{2+}$, 200μM of each of the four deoxynucleoside triphosphates (dNTPs), 200nM of both forward and reverse primer, and 14μl of genomic DNA template. PCR cycling conditions consisted of an initial denaturation step at 94°C for 3min, followed by 35 cycles of 94°C denaturation for 45 sec, 50°C annealing for 1min, 72°C extension for 1.5min, and a final extension step at 72°C for 10min. No amplification product was visible on an agarose gel so a second PCR was attempted using identical reactions and PCR cycling conditions, except that the template was 2μl of PCR product from the first PCR reaction. In all instances, negative control PCR reactions consisting of water and HotMasterMix were run in parallel. Amplification products were subsequently quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA), visualized on an agarose gel, pooled at equal concentrations, and purified with the MO BIO UltraClean PCR Clean-Up Kit (MO Bio Laboratories). Purified amplicons were sequenced (2 x 250 bp) on an Illumina (San Diego, CA, USA) MiSeq sequencer (Instrument ID M02308; run number 66) at the Hawaii Institute of Marine Biology.

Demultiplexing of the forward sequence reads was performed using the QIIME pipeline (version 1.9.1; Caporaso et al., 2010a) split_libraries_fastq.py script with a maximum of 1 error in the barcode, and the following quality-filtering parameters: r = 3, p = 0.75, q = 3, and n = 0. Because of the sequencing scheme used, primer regions within the amplicons were not present in the sequencing output and did not require removal. In preparation for clustering and subsequent diversity analyses, all sequences were aligned in QIIME (Caporaso et al., 2010a) using PyNAST (Caporaso et al., 2010b) and trimmed to the 250 bases corresponding to the amplicons generated here. Most sequences from other studies were retained in the same manner, with the exception of Cockell et al. (2009) where many non-overlapping sequences were present. Clusters were generated in mothur (Schloss et al., 2009; version 1.32.1) using the average neighbor method and with sequence clustering thresholds of 97% and 99% similarity. Beta diversity analysis was performed in QIIME using the binary_jaccard distance metric. Principal coordinates analysis was performed in QIIME and visualized in R using the ggplot2 library package. The venn diagram was generated using mothur.

Classification of all forward reads was performed with the SILVA rRNA gene database project NGS analysis pipeline (SILVAngs 1.2; Quast et al., 2013) using a classification similarity of 85% and default settings. For the phylogenetic analyses, the ten most abundant sequences from both samples A7 and M1b were identified using the USEARCH (Edgar, 2010; version 8.0.1623) ‘derep_fulllength’ command and similar sequences were identified within the NCBI non-redundant database via blastn with default parameters. The top three hits from the 20 total most abundant reads revealed 52 unique database sequences. To generate detailed gene phylogenies, SSU rRNA gene sequences from this and other studies
were aligned using the online SINA tool version 1.2.11 (Pruesse et al., 2012) before importing into the ARB software package (Ludwig et al., 2004; version 6.0.6). Phylogenetic analyses were performed with the RAxML maximum-likelihood method (version 7.2.2) using the GTR model of nucleotide substitution under the gamma- and invariable- models of rate heterogeneity (Stamatakis et al., 2006). Bootstrap analysis of the sequence alignments were determined by RAxML using the rapid bootstrap analysis algorithm (2000 bootstraps) implemented within ARB.

Sample metadata and the SSU rRNA sequence files used in this study were submitted to the NCBI BioSample and Sequence Read Archive databases and are accessible via BioProject identifier PRJNA340160.

Results

Geochemistry of waters

Ion analysis identified a variety of dissolved ions in the cave waters (Table 2). The two most abundant cations in all ice samples (by moles) are Ca$^{2+}$ and Na$^+$, followed by Mg$^{2+}$. This chemistry is consistent with waters that interact with and leach basalt, although absolute concentrations are low (Nesbitt and Wilson, 1992).

There is a greater abundance of ions in the icicles than in the snow and the rainwater (Erikson, 1957). As the water percolates through the young, porous basalt, it picks up ions. M4-1 and M4-2 were clear icicles, whereas icicle M4-3 had a grey tip suggesting it had begun to melt; this may explain its higher ion concentrations. The average ionic concentrations of the two clear sampled icicles best represent the source water, and their molar cation abundances are Ca$^{2+}$>Na$^+$>Mg$^{2+}$>K$^+$. The most abundant anion detected is generally SO$_4^{2-}$. The ion analyzer cannot detect Si, Al, and carbon-containing polyatomic ions such as bicarbonate (HCO$_3^-$) or carbonate (CO$_3^{2-}$). The pond is enriched in almost all the ion species relative to the source concentrations (Table 2).

The $\delta^{18}$O values of the samples vary from -43 to -94‰, whereas $\delta^2$H varies between -6.8 and -12.8‰. Figure 4 shows $\delta^2$H versus $\delta^{18}$O in the water samples plotted in comparison with the global meteoric water line (GMWL), which was defined by Craig (1961) and later refined by Rozanski et al. (1993) as $\delta^2$H = 8.17·$\delta^{18}$O + 11.27 (‰ VSMOW). Bodies of water that have experienced significant evaporation fall below the GMWL. All our sample results are consistent with the GMWL.

Secondary minerals

Laboratory Raman analysis identified calcite, gypsum, or both in all samples (Table 3). For samples identified as calcite, the characteristic fingerprint peaks are roughly 1086, 713, and 284 cm$^{-1}$ (Fig. 5). For gypsum-containing samples, the peaks are roughly 1142, 1008, 621, 496, and 417 cm$^{-1}$. Figure 5 shows
both calcite and gypsum diagnostic peaks at the same spot of a sample. The exterior of all four MLIC veneer samples exhibits Raman signatures for both minerals, but primarily calcite. These two minerals are even found on the interior side of the samples, but gypsum is the more common constituent.

The $\delta^{18}O$ values for four carbonate samples range from -4 to -10.5‰, whereas $\delta^{13}C$ varies between 5.7 and 8.4‰. Figure 6 shows the stable isotope $\delta^{13}C$ and $\delta^{18}O$ data for each sample, compared against calcium carbonate from various other ice caves (Zak et al., 2008). Samples from the current study fall outside the range of normal calcium carbonate speleothems and follow a trend characteristic of cryogenic cave carbonate (CCC) powder formed by rapid freezing. There are two known types of CCC: micron-sized crystals that formed during fast freezing and large pearls that experienced slow freezing and restricted CO$_2$ escape (Zak et al., 2008). Based on the isotopic signature, the CCC in MLIC belongs to the former category (Fig. 6). The two mineral samples from Arsia Cave did not reveal any evidence for carbonates in the Raman analysis, so no carbon isotope analysis was attempted.

Based on semi-quantitative EDS analysis, elements of high abundance include Si, Ca, Mg, and C (Table 3). The table entries are sorted based on how many of the sampled EDS points exhibited the respective elemental peak, and only elements identified in more than half of the sampled points are listed. These results confirm that there is indeed carbon in the mineral samples (beyond the C-coating that has been applied), consistent with the presence of calcium carbonate. A common phase also detected by EDS is silicon dioxide, SiO$_2$. Amorphous SiO$_2$ does not produce well-defined Raman peaks. SiO$_2$ is present in both bulk powder as well as secondary coating on the host basalt. It is found in many other lava tubes as well, as silica is derived from weathering of igneous rock (Hill & Forti, 1997). Chemtob & Rossman (2014) have found coatings of amorphous silica on freshly erupted Hawaiian lavas, and interpreted these to have formed by dissolution-reprecipitation reactions aided by acidification due to volcanic vapors. Mg$^{2+}$ and/or Na$^+$ ions are found on the samples in areas with SiO$_2$, whereas Mg$^{2+}$ ions occur in calcium carbonate-containing mineral samples. Other detected trace ions are iron, aluminum, and chromium.

Sample M1 consists primarily of SiO$_2$, and a smaller fraction is calcite (Fig. 7). Only integrated areas, as opposed to any single point on the sample registered stronger C signals along with Ca signals, suggesting that only a minor part of M1 is calcite. Additionally, Raman spectra indicate that the sample has peaks characteristic of calcite.

The white veneer in MLIC, left over from the disappearance of the Skating Rink (Fig. 1), is distributed very heterogeneously over the rough rock surface, with the highest concentration in pores (sample M2-1 in Fig. 2). This veneer contains SiO$_2$. SEM also shows gypsum crystals overlain by tiny
(cryptocrystalline) calcite crystals (Fig. 8). This is the grain size expected for the cryogenic calcite samples confirmed by isotopic analysis (Fig. 6).

The veneer sample from Arsia Cave (A6) consists primarily of gypsum and SiO$_2$, and, unlike the veneer in MLIC, only a small fraction may be calcite. SEM imaging reveals again gypsum crystals overlain by tiny (cryptocrystalline) calcite crystals, but also gypsum blades (not shown).

The localized floor deposit A8 has a soft, clay-like texture. In addition to a small amount of gypsum, it contains a large fraction of Al-rich silicate. SEM images reveal a heterogeneous sample, with most grains showing an Al-rich silicate composition, whereas other grains are smoother and contain gypsum. Based on the XRD pattern, sample A8 is not kaolinite, but may be a different clay mineral. A portion of this sample (1mL) was set aside for DNA extraction, but none was detected.

All mineral samples are multi-phase, containing SiO$_2$, and calcite, and/or gypsum (Table 2). All the calcite is cryogenic. The constituents are either cryptocrystalline or amorphous; none developed large crystals.

**Microbiology**

The caves are far from any vegetation, and hence the amount of organic carbon (the food source of heterotrophs) that enters these caves is likely low. Hence, microorganisms in the caves may be expected to include chemolithoautotrophs that survive via mineralization of inorganic substrates within the cave. To characterize microorganisms associated with high-altitude ice cave cryogenic minerals, we performed a survey of SSU rRNA genes from samples collected in the two caves. From MLIC (Fig. 1), loose white-colored powder identified as SiO$_2$ and calcite (M1, Fig. 2) was sampled from the upper tunnel parallel to the lower ice-filled lava tube at the terminal end. From Arsia Cave, water was sampled from a partially melted ice pond (A7). Mineral sample A8 did not yield any amplification product. Using the mineral and water samples, respectively, a total of 43,015 and 7,950 SSU rRNA reads of 250 base pairs (bp) were sequenced in the forward direction.

The microbial communities recovered from ice cave samples A7 and M1b were mostly similar to other microbial communities sampled from around the globe than to only samples collected in Hawaii (Fig. 9A). Samples that contained SSU rRNA gene sequences most closely matching the most abundant sequence reads from ice caves samples A7 and M1b include: Hawaii volcanic soil deposits (Gomez-Alvarez et al., 2007; Weber & King, 2010), Hawaii and Azores lava cave microbial mats (Hathaway et al., 2014), Icelandic volcanic glass (Cockell et al., 2009; Kelly et al., 2010), Antarctica ice caves (Tebo et al., 2015) and Dry Valleys (de la Torre et al., 2003), the cacti microbiome (Fonseca-Garcia et al., 2016), Chinese wetland soil (Feng, unpublished), Atacama desert soil (Lynch et al., 2012), Canary Island
volcanic rocks (Portillo and Gonzalez, 2007), and Alaskan volcanic soil (Zeglin et al., 2016). However, when compared to samples collected only in Hawaii, the microbial communities from samples A7 and M1b are more similar to each other than to other Hawaii samples (Fig. 9B). Samples from within Hawaii that contain microbial communities with the highest similarity to our Hawaii ice cave samples originate from volcanic soil deposits and previously uninvestigated lava cave microbial mats (Fig. 9B). An analysis of sequence clusters shared between the ice caves and other Hawaii samples further reveal the uniqueness of the Hawaii ice cave microbiome (Fig. 9C). While a small number of sequence clusters are common to both Hawaii ice cave samples, a majority are unique to individual samples (Fig. 9C). At a 97% sequence identity threshold, a single sequence cluster is common to all Hawaii samples, while a single sequence cluster of Actinobacteria is common to all of the samples analyzed here (Fig. 9C).

From a taxonomic perspective, the microbial communities inhabiting Hawaii ice caves are dominated by Bacteria. Thirty-two different bacterial phyla were identified in the Hawaii ice caves with a majority of the different phyla found in the ice cave melt water sample (Fig. 10). Actinobacteria dominated mineral sample M1b (86% of total), and Proteobacteria were the most abundant bacterial phylum within ice cave melt water sample A7 (39% of total) (Fig. 10). Other abundant phyla in mineral sample M1b include Deinococcus-Thermus (8%) and Bacteroidetes (3%). Other abundant phyla in ice cave melt water sample A7 include Bacteroidetes (18%), Actinobacteria (10%), Verrucomicrobia (8%), and Candidate Division OD1 (7%). Euryarchaeota lineages Marine Group II and Marine Benthic Group D (DHVEG-1) were both identified in low abundance (<1%) within the ice cave melt water (Fig. 10). A putative eukaryotic protist of genus Heteromita was identified in mineral sample M1b.

In a phylogenetic analysis that includes the ten most abundant SSU rRNA gene sequences from each Hawaii ice cave sample, eight from mineral sample M1b belong to the bacterial phylum Actinobacteria (Fig. 11). One of these comprises 11% of the M1b microbial community and is related to sequences previously recovered from soil (Fig. 11). The second most abundant sequence from sample M1b is a member of the bacterial phylum Deinococcus-Thermus, and makes up 3% of the community. A lineage within bacterial phylum Bacteroidetes related to sequences previously recovered from soil is the most abundant sequence within ice cave melt sample A7 and comprised 4% of the total microbial diversity (Fig. 11). Other abundant sequences from ice melt water sample A7 belong to the phylum Acidobacteria, Verrucomicrobia, TM6, Proteobacteria, Bacteroidetes, and Chloroflexi (Fig. 11).

A phylogenetic analysis that includes Hawaii ice cave Actinobacteria sequences and cultivated representatives of the phylum indicate both cultured and uncultured lineages are abundant in the ice cave
mineral sample. Five of the most abundant lineages, including the sequence representing 11% of the total microbial diversity in sample M1b, are in the suborder Frankineae and distantly related to the isolated strain *Acidothermus cellulolyticus* 11B (Fig. 12). Three of the most abundant lineages of Actinobacteria from mineral sample M1b are in the suborder Propinibacterineae (Fig. 12). Two lineages are distantly related to cultivated representatives, but a single lineage representing 2% of the total diversity in the sample was 99.6% similar to isolated strain *Nocardiodides dokdonensis* FR1436 (Fig. 12).

**Discussion**

**Chemical pathways**

Chemical weathering of basalt releases water-soluble cations such as Ca$^{2+}$, Na$^+$, and Mg$^{2+}$ (Table 1). Precipitation may contribute Cl and Na (Erikson, 1957; Kempe et al., 2016). The clear icicles (M4-1 and M4-2) are likely the most representative of water that has reached the cave ceiling. Based on values listed in Table 1, charge balances imply that a major additional ion species must be present. The missing negative charge could be made up of either 481 μM HCO$_3^-$ or 241 μM CO$_3^{2-}$. As this far exceeds the SO$_4^{2-}$ concentration in the same samples, (bi)carbonate is likely the most abundant anion.

Based on the identified ions in the water (Table 1), the secondary minerals are expected to contain Ca, Mg, and Na. Raman analysis indeed indicates calcite and gypsum, but no magnesium- or sodium-containing secondary minerals. EDS data show that Mg and Na are also present in the samples, associated with SiO$_2$ or calcite. Many of the highly soluble Na ions could also have been carried to the bottom of the debris covered cave floor and might not be represented in our sparse sample collection.

An additional source for SiO$_2$ and secondary silicates may be dust. Dust derived from Asia contributes significantly to the Hawaiian soils (e.g., Kurtz et al., 2001). Considering that eolian quartz is the most abundant mineral of this dust, its incorporation and subsequent dissolution and reprecipitation inside the ice may be one of the sources for the amorphous SiO$_2$ veneers.

During our most recent visit in November 2015 parts of the veneer in Party Hall had disappeared. This suggests that the amount of water that entered the chamber between April and November 2015 was the highest in decades, and this may be the beginning of the end of the life span of the veneer.

**Potential for paleoclimate studies**

The stable isotope composition of waters is affected by a variety of factors that include temperature, altitude, latitude, amount and origin of precipitation, and ocean temperature (Clark & Fritz, 1997). Similar to ice cores aboveground, the $\delta^{18}O$ and $\delta^2H$ in cave ice can in principle be used as proxy for paleo-temperature and source of moisture reconstructions (Yonge & MacDonald, 1999; Luetscher, 2005; Perșoiu et al., 2007). However, the interpretation of these data is more complex, because fractionation and
mixing can occur between the surface and the cave ice (Perșoiu et al., 2011). The fact that the Arsia pond water (temporarily melted perennial ice) still has the isotopic composition of meteoric water eliminates fractionation processes as a concern, although mixing of multi-year melt water could be a problem. Hence, an ice core from Arsia Cave, if its layers can be dated, has potential for paleoclimate reconstruction, particularly useful in a geographic area where no other such records are available at comparable resolution and historical climate records are short.

Relevance in cave ecology

Lava caves are suited for examining questions about microbial biogeography and controls on species diversity (Northup & Lavoie, 2015). Both Hawaii ice caves sampled in this study are away from vegetation and relatively low-trafficked; these traits make them ideal environments to understand the role of microorganisms in biomineralization and basaltic rock rock-weathering processes. Arsia Cave is more remote than MLIC and less visited and, therefore, more pristine. Despite the isolated nature of both caves, drip water enters through the cave surface and wind may flow through the tunnels of MLIC (Pflitsch et al., 2016). Further, dead moths and freeze-dried bats are found in both lava tubes. Altogether, a combination of heterotrophic and autotrophic microbial lineages are expected given that the ice caves represent an isolated environment with multiple limited routes of transport for microbes, particulates, and nutrients into and out of the lava caves.

The microbial communities recovered from the two ice caves reveal that they are more similar to each other than to other cave or environmental samples studied previously. Further, an analysis of only samples associated with the island of Hawaii revealed the ice caves to be distinct from previously described studies of lava cave microbial mats (Hathaway et al., 2014). Interestingly, the microbial communities described in volcanic soil deposits (Gomez-Alvarez et al., 2007; Weber & King, 2010) were more similar to the ice cave microbial communities, which suggests a potential for at least some background input of volcanic soils into the ice caves. Given the large number of sequences unique to the Hawaii ice caves, these caves may act as a reservoir of unique microorganisms.

Loose whitish powder was sampled from the upper tunnel in the back of MLIC (Fig. 1) and Actinobacteria were the most abundant phylum detected. Actinobacteria lineages are frequently reported as abundant, and sometimes dominant, members of cave environments (e.g. Cuezva et al., 2012; Hathaway et al., 2014; Riquelme et al., 2015; Içuş et al., 2016; Lavoie et al., 2017) and have previously been reported as a potential biocatalyst in the weathering and precipitation of minerals (e.g. Laiz et al., 2003; Miller et al., 2014). It has been proposed that Actinobacteria can use atmospheric CO₂ to dissolve the lava cave walls and subsequently precipitate CaCO₃ crystal during periods of low humidity and/or
CO₂ (Cuezva et al., 2012). A previously sequenced genome of an actinobacterium from the Frankineae suborder isolated from a calcareous stone surface gives insight into the potential metabolic features of the most abundant lineage in mineral sample M1b (Normand et al., 2012): it is possible that this ice cave lineage is radiation-resistant, capable of autotrophy, and may also be a facultative heterotroph. The potential to adjust their mode of carbon acquisition, radiation-resistance, and thick gram-positive cell wall that allows for increased resistance to desiccation makes it plausible to hypothesize that the lineage may successfully compete in an isolated ice cave environment with a potentially low level of organic matter input. It is also possible that the biogenic production of CaCO₃ is a process performed by the Actinobacteria during long bouts of little to no organic input into the lava caves.

Within the ice water sample, a larger diversity of microorganisms frequently found in isolated aquatic environments (i.e. lakes and ponds) and soils was detected. Although found in very low abundance, two different groups from the domain Archaea were found in the ice melt sample. The most similar sequence to both archael lineages found here, Marine Group II and Marine Benthic Group D (DHVEG-1), were to gene clones identified from serpentinized dunite in the Leka ophiolite complex (Daae et al., 2013); this result indicates potential common archael members in low temperature, terrestrial basaltic habitats. Further, this result suggests that these archael inhabitants may not be restricted to ice cave environments. Given the use of nested PCR and its impact on biasing microbial community diversity studies (e.g. Yu et al., 2015), the presence of these rare taxa should be validated by other techniques.

Potential for astrobiology
The study of lava cave microbial communities is particularly relevant to improving biomarker detection strategies that might be employed to search of extinct life on other planets (Northup and Lavoie, 2015). Large areas of Mars are covered by basaltic rocks with clear evidence of lava caves (Greeley, 1973; Cushing, 2012; Cushing & Okubo, 2015) that might act to retain water and provide protection from solar radiation (Williams et al., 2010; Northup and Lavoie, 2015). Putative autotrophic microbial lineages, such as the Actinobacteria found in the basaltic lava tubes studied here and in other cave environments (e.g. Lavoie et al. 2017), might serve as model organisms to explore chemolithoautotrophic processes, biomineralization, and radiation-resistance in an accessible environment analogous to Mars.

Conclusions
We have investigated the geochemistry and microbiology of two remote high-altitude lava tubes on Mauna Loa volcano that contain perennial ice.
The secondary minerals from the deep parts of the lava tube caves are multi-phase and predominantly amorphous silica, calcite, and gypsum. Based on stable isotope ratios, all of the analyzed calcite-rich samples are cryogenic in origin. An ice body that has melted away since 1978 has left behind a veneer of SiO$_2$ and cryptocrystalline calcite superposed on gypsum crystals. Gypsum is present at least ~1cm into the porous basalt surface, at least where the cave walls were in contact with an ice body. Al-rich silicate is found at one spot in Arsia Cave.

The isotopic composition of an ice pond sample from Arsia Cave suggests little evaporation has occurred, implying that the isotopic composition of the ice record still reflects that of the surface waters. Hence, the layered ice ponds may be useful as a proxy record of paleotemperature or moisture sources.

Analysis of microbial communities inhabiting the calcite-SiO$_2$ sample and melt ice water revealed unique microbial diversity in the Hawaii ice caves. Microorganisms from all three domains of life were detected, although Bacteria were the dominant community members in both samples. Putative heterotrophic and chemolithoautotrophic microbial groups are found in the ice caves, with likely allochthonous input of organic material. The ice caves serve as environments to understand microbial life inhabiting terrestrial low-temperature basaltic rocks and potential microbial biosignatures and life on other planets.

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Table 1. List of collected samples. Samples with ‘M’ are from Mauna Loa Icecave; samples with ‘A’ are from Arsia Cave.

<table>
<thead>
<tr>
<th>Sample Id</th>
<th>Sample Type</th>
<th>Analyses</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>mineral powder</td>
<td>Raman, EDS, C&amp;O isotopes, PCR, sequencing</td>
<td>2 × 1mL</td>
</tr>
<tr>
<td>M2-1</td>
<td>mineral veneer</td>
<td>Raman, SEM, EDS, C&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M2-3</td>
<td>mineral veneer</td>
<td>Raman, C&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M2-5</td>
<td>mineral veneer</td>
<td>Raman, C&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M3-1</td>
<td>mineral veneer</td>
<td>Raman</td>
<td></td>
</tr>
<tr>
<td>M4-1</td>
<td>icicle</td>
<td>ions, H&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M4-2</td>
<td>icicle</td>
<td>ions, H&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M4-3</td>
<td>icicle</td>
<td>ions, H&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M5-1</td>
<td>snow</td>
<td>ions, H&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>M5-2</td>
<td>snow</td>
<td>ions, H&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>icicle</td>
<td>ions, H&amp;O isotopes</td>
<td></td>
</tr>
<tr>
<td>(A5)</td>
<td>water blank</td>
<td>ions, PCR, sequencing</td>
<td>2 × 10mL</td>
</tr>
<tr>
<td>A6</td>
<td>mineral veneer</td>
<td>Raman, SEM, EDS</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td>melted ice (pond)</td>
<td>ions, H&amp;O isotopes, PCR, sequencing</td>
<td>2 × 10mL</td>
</tr>
<tr>
<td>A8</td>
<td>mineral powder</td>
<td>Raman, SEM, EDS, XRD, PCR</td>
<td>4 × 1mL</td>
</tr>
</tbody>
</table>
Table 2. Ions contained in the water samples. Molar concentrations are calculated from results given in mg/L (ppm). UHM tap water is added as illustrative comparison. n.a. = not found, 0 = <0.01mg/L, n.a. for nitrite and bromide.

<table>
<thead>
<tr>
<th>µmol/L (µM)</th>
<th>Cl⁻</th>
<th>F⁻</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>PO₄³⁻</th>
<th>K⁺</th>
<th>Na⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4-1 icicle</td>
<td>15</td>
<td>9</td>
<td>8</td>
<td>41</td>
<td>1</td>
<td>6</td>
<td>87</td>
<td>172</td>
<td>50</td>
</tr>
<tr>
<td>M4-2 icicle</td>
<td>11</td>
<td>4</td>
<td>3</td>
<td>21</td>
<td>1</td>
<td>4</td>
<td>43</td>
<td>255</td>
<td>26</td>
</tr>
<tr>
<td>M4-3 icicle</td>
<td>61</td>
<td>3</td>
<td>3</td>
<td>347</td>
<td>4</td>
<td>6</td>
<td>478</td>
<td>173</td>
<td>283</td>
</tr>
<tr>
<td>M5-1 snow</td>
<td>1</td>
<td>0.5</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.5</td>
<td>2</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>M5-2 snow</td>
<td>1</td>
<td>0.5</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.3</td>
<td>2</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>A1 icicle</td>
<td>15</td>
<td>4</td>
<td>24</td>
<td>11</td>
<td>n.a.</td>
<td>5</td>
<td>37</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>A5 blank</td>
<td>1</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0.8</td>
</tr>
<tr>
<td>A7 pond</td>
<td>18</td>
<td>27</td>
<td>9</td>
<td>160</td>
<td>3</td>
<td>5</td>
<td>338</td>
<td>544</td>
<td>90</td>
</tr>
<tr>
<td>UHM Water</td>
<td>2623</td>
<td>2</td>
<td>35</td>
<td>163</td>
<td>n.a.</td>
<td>68</td>
<td>2584</td>
<td>302</td>
<td>601</td>
</tr>
</tbody>
</table>
Table 3. List of mineral samples and their identities. Elements commonly detected on the EDS sample points are also listed.

<table>
<thead>
<tr>
<th>Sample Id &amp; Type</th>
<th>Appearance</th>
<th>Raman Identification</th>
<th>Common EDS Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1, deposit</td>
<td>fine white powder</td>
<td>Calcite</td>
<td>O, Si, C, Ca</td>
</tr>
<tr>
<td>M2-1, wall</td>
<td>white coating (veneer)</td>
<td>Calcite, Gypsum</td>
<td>Si, S, O, Ca, Mg</td>
</tr>
<tr>
<td>M2-3, wall</td>
<td>white coating (veneer)</td>
<td>Calcite, Gypsum</td>
<td></td>
</tr>
<tr>
<td>M2-5, floor</td>
<td>white coating (veneer)</td>
<td>Calcite, Gypsum</td>
<td></td>
</tr>
<tr>
<td>M3-1, wall</td>
<td>white coating (veneer)</td>
<td>Calcite, Gypsum</td>
<td></td>
</tr>
<tr>
<td>A6, wall</td>
<td>white coating (veneer)</td>
<td>Gypsum</td>
<td>Si, S, O, Ca, C</td>
</tr>
<tr>
<td>A8, deposit</td>
<td>white, clay-like, soft</td>
<td>Gypsum</td>
<td>Al, Ca, Si, O, Na</td>
</tr>
</tbody>
</table>
Figure 1. Plan view and extended profile of MLIC with approximate sampling locations (red points and red stars). The map is modified from Kempe (1979). True north is reconstructed from magnetic north, based on a difference of 11° in 1978.
Figure 2. A) The north side of Mauna Loa as seen from Mauna Kea, Hawaii. B) A person stands on the massive ice lake in branch A of Arsia Cave, looking back onto a stalagmite at the front end of the lake (Photo credit: Peter Bosted). C) The front end of branch B of Arsia Cave, where samples were collected. Photo taken April 20, 2014 in outward direction. D) Portion of the same ice lake; the entire pond is about 30m long. The white dusting on the ice has not been sampled, but is thought to be a cryogenic evaporite. Large air bubbles are discernible in the foreground. Photo taken April 22, 2012.
Figure 3. Photos of mineral samples and their contexts: M1 (macrophoto, left; context with 16cm photoscale, second from left), M2-1 (focus-stacked macrophoto of heterogeneous veneer on basalt rock, the smallest division on the ruler is 0.5mm; context, right), A6 (white veneer on basalt), and A8 (in-situ, middle; macrophoto, right). The small squares in the background are 1 mm². The mosaic in the middle shows the bright and sharply delineated veneer in MLIC’s Party Hall section that covers the floor and the lowest ~0.5m of the cave walls. Veneer samples M2-3, M2-5, and M3-1 (not shown) have similar appearance to M2-1.
Figure 4. Water isotopic compositions compared to global meteoric water line. External precision for H and O isotope measurements is ±0.73‰ and ±0.14‰ respectively.
Figure 5. Raman spectrum of the exterior of Sample M2-1 (black), which contains both calcite (red) and gypsum (blue).
Figure 6. Stable isotope concentrations $\delta^{18}$O and $\delta^{13}$C of calcite samples from MLIC relative to VBDP (Vienna Pee Dee Belemnite) standard. The sample data are compared to data from other caves as described in Zak et al. (2004, 2008). CCC = cryogenic cave calcite.
Figure 7. Left: Secondary electron (SE) image of surface of sample M1a with SiO$_2$ and calcite. Right: EDS spectrum for orange box.
Figure 8. (A) Secondary electron (SE) image of Sample M2-1. (B) Backscattered electron (BSE) image of blocky gypsum, cracking and parting along cleavage planes as a result of dehydration under high vacuum inside the EPMA instrument. The calcite crystals are on the scale of 1 μm, and hence cryptocrystalline. The ultrafine grained (featureless) cement between the (brighter) calcite blades is silica. Crystal identities are based on EDS spot analyses.
Figure 9. Principal coordinate analysis (PCoA) of binary Jaccard dissimilarity indices using 99% OTU clustered sequence reads. Percent variation explained by principal components one and two are indicated.
Panel (A) includes ice cave samples A7 and M1b, other samples from Hawaii volcanic soil deposits, Hawaii and Azores lava cave microbial mats, Icelandic volcanic glass, Antarctica ice caves and dry valleys, the cacti microbiome, Chinese wetland soil, Atacama desert soil, Canary Island volcanic rocks, and Alaskan volcanic soil. Panel (B) includes only ice cave samples A7 and M1b and other samples from Hawaii, including volcanic soil deposits and lava cave microbial mats. (C) Venn diagram of 97% and 99% OTU clustered Illumina sequence reads showing overlap in microbial communities from Hawaii ice caves (A7 and M1b) with previously described Hawaii samples and other non-Hawaii samples described above. The first number listed indicates results for OTUs clustered at 97% and the second number for 99%.
Figure 10. Microbial taxonomic fingerprint at phylum level from water sample A7 and mineral sample M1b. The fraction of Archaea and Bacteria in the sample is indicated by the colored heatmap and the absolute number of sequences is represented by three different shapes. Eukarya were present in sample M1b, but are not shown here. A maximum-likelihood phylogeny based on aligned sequence reads is shown in the inset. The scale bar corresponds to 0.1 substitutions per nucleotide position.
Figure 11. Phylogenetic relationships between the ten most abundant ice cave water (A7) and mineral (M1b) derived SSU rRNA gene sequences and their closest publicly available relatives. The relative
abundance of the amplicon sequence is indicated in parentheses. Black (100%), gray (>80%), and white (>50%) circles indicate nodes with bootstrap support, from 2000 replicates. The scale bar corresponds to 0.1 substitutions per nucleotide position.
Figure 12. Phylogenetic relationships within the phylum Actinobacteria showing relationships between the most abundant mineral-derived SSU rRNA gene sequences and cultivated lineages. The number of sequences in the collapsed groups is indicated. Other information is as in Figure 11.