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Office

6001 Pulaski Pike NW
Huntsville, AL 35810 USA
Tel: 256-852-1300
nss@caves.org

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National Center of Environmental
Assessment (8623P)
Office of Research and Development
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue NW
Washington, DC 20460-0001
703-347-8601 Voice 703-347-8692 Fax
field.malcolm@epa.gov

**Production Editor
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Knoxville, TN
225-281-3914
saecaver@gmail.com

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Front cover: Capshaw Cave main passage showing cave stream and location of flood-deposited sediments. See Hart in this issue.

CAVE RADON EXPOSURE, DOSE, DYNAMICS AND MITIGATION

Chris L. Waring^{1, c}, Stuart I. Hankin¹, Stephen B. Solomon², Stephen Long², Andrew Yule², Robert Blackley¹, Sylvester Werczynski¹, and Andrew C. Baker³

Abstract

Many caves around the world have very high concentrations of naturally occurring ^{222}Rn that may vary dramatically with seasonal and diurnal patterns. For most caves with a variable seasonal or diurnal pattern, ^{222}Rn concentration is driven by bi-directional convective ventilation, which responds to external temperature contrast with cave temperature. Cavers and cave workers exposed to high ^{222}Rn have an increased risk of contracting lung cancer. The International Commission on Radiological Protection (ICRP) has re-evaluated its estimates of lung cancer risk from inhalation of radon progeny (ICRP 115) and for cave workers the risk may now (ICRP 137) be 4–6 times higher than previously recognized. Cave Guides working underground in caves with annual average ^{222}Rn activity $> 1,000 \text{ Bq m}^{-3}$ and default ICRP assumptions (2,000 workplace hours per year, equilibrium factor $F = 0.4$, dose conversion factor $\text{DCF} = 14 \mu\text{Sv (kBq h m}^{-3})^{-1}$) could now receive a dose of $> 20 \text{ mSv y}^{-1}$. Using multiple gas tracers ($\delta^{13}\text{C-CO}_2$, Rn and N_2O), linked weather, source gas flux chambers, and convective air flow measurements a previous study unequivocally identified the external soil above Chifley Cave as the source of cave ^{222}Rn . If the source of ^{222}Rn is external to the cave, a strategy to lower cave ^{222}Rn by passively decreasing summer pattern convective ventilation, which draws ^{222}Rn into caves, is possible without harming the cave environment. A small net annual average temperature difference (warmer cave air) due to geothermal heat flux produces a large net annual volumetric air flow bias (2–5:1) favoring a winter ventilation pattern that flushes Rn from caves with ambient air. Rapid anthropogenic climate change over decades may heat the average annual external temperature relative to the cave temperature that is stabilized by the thermal inertia of the large rock mass. Relative external temperature increases due to climate change (Jenolan Caves, 2008–2018, 0.17°C) reduces the winter pattern air flow bias and increases Rn concentration in caves.

INTRODUCTION

^{222}Rn is an inert radioactive gas with a half-life of 3.8 days formed from ^{226}Ra as part of the ^{238}U decay series. ^{220}Rn formed from ^{232}Th decay may be measurable in trace concentrations in caves without adding significantly to total radon concentration. Further reference to radon in this paper refers to the more abundant isotope ^{222}Rn .

Many of the early cave Rn studies reported high, but variable, Rn concentration, with most expressing a strong seasonal and possible diurnal variation patterns (Gunn et al., 1991; Haki et al., 1997; Hyland and Gunn, 1994; Middleton et al., 1991; Solomon et al., 1996; Szerbin, 1996). In the absence of further environmental data, the seasonal pattern with high Rn in summer was often assumed to be due to greater winter air flow diluting underground Rn accumulation (Tanahara et al., 1997; Tremaine et al., 2011). Studies using continuous Rn monitoring in conjunction with environmental monitoring provided greater time resolution to the seasonal patterns, with the addition of caves with the reverse seasonal pattern (Lario et al., 2005) or no annual variation. The divergence in cave Rn patterns appeared to be associated with different cave configurations and consequent ventilation patterns leading to cave classification based on 3D configuration influencing ventilation. Addition of gas tracers such as CO_2 that may closely correlate with Rn activity (Gregorič et al., 2013; Kowalczyk and Froelich, 2010), and detailed cave temperature measurements also helped constrain the conceptual model of Rn in caves. However, for many studies, measurement of air flow through the cave is absent and the source of Rn in caves remains unresolved. To explain fully Rn dynamics in caves, a challenge remains to classify correctly the 3D void shape and dimensions, consequent ventilation regime and Rn source for the many complex cave variants. In this paper, we describe a typical chimney effect ventilated cave at Jenolan Caves, Australia and what are the causes that affect Rn activity in Chifley Cave (Waring et al., 2017). We also infer mechanisms to explain other cave Rn patterns from selected well described examples.

A cave worker may be exposed to elevated radon concentrations while working underground. The average of the variable radon concentration while working underground is expressed in units Bq m^{-3} (Becquerels per cubic metre) and is multiplied by the total time spent underground to estimate cumulative radon exposure, expressed as Bq h m^{-3} (Equation (1)). The International Commission on Radiological Protection (ICRP) advises (ICRP, 2019a) calculating the effective dose from inhaling radon involves multiplying the average radon level by the time exposed (Rn exposure) and by the dose coefficient (Equation (2)). Radon level may also be expressed as a radon activity or concentration.

¹Australian Nuclear Science and Technology Organisation, Environmental Research, Sydney NSW 2234, Australia.

²Australian Radiation Protection and Nuclear Safety Agency, Melbourne VIC 3095, Australia

³National Parks and Wildlife Service, Bathurst, NSW 2795, Australia

^cCorresponding author: clw@ansto.gov.au, chris.waring2@gmail.com

$$\text{Radon exposure} = \text{radon level} \times \text{time exposed} \quad (1)$$

$$\text{Effective dose} = \text{radon level} \times \text{time exposed} \times \text{dose coefficient} \quad (2)$$

The dose coefficient may be expressed as the dose conversion factor (DCF) in units μSv per kBq h m^{-3} for simple calculation of dose, incorporating assumptions of a default equilibrium factor $F = 0.4$. The recommended dose limit for occupationally exposed workers is 20 mSv y^{-1} , averaged over a defined period of 5 years, with no single year exceeding 50 mSv (ICRP, 2007). For cave visitors and the general public, the dose limit is 1 mSv y^{-1} (ICRP, 2007).

A comprehensive review of the health risk to cavers and cave workers (Field, 2007) is based on the known radiological health risk in 2007, ICRP 65 (ICRP, 1993; UNSCEAR, 2000) and dose estimates using the model software Lungdose 90 (Nikezic and Yu, 2001). The dose estimates for professional cavers (600 h y^{-1}) and full-time cave workers with an assumed time in caves of $2,000 \text{ h y}^{-1}$ is calculated based on a dose conversion factor (DCF) of $12.92 \mu\text{Sv (kBq h m}^{-3})^{-1}$ or $20.75 \text{ mSv per WLM}$ (Table 7, Field, 2007). This dose conversion factor is similar to the DCF ($15 \mu\text{Sv (kBq h m}^{-3})^{-1}$) tabulated in ICRP 137 for physically active cave workers, assumed to spend $\frac{2}{3}$ time in exercise and the recommended more general DCF of $14 \mu\text{Sv (kBq h m}^{-3})^{-1}$ for physically active indoor and cave workers in (ICRP, 2017).

Many cave managers will need to consider different strategies for mitigating cave worker exposure to high Rn concentrations in caves. We consider the merits and detriments for approaches based on limiting the time exposure of cave workers, passive modifications to cave ventilation and technologies available to reduce cave Rn concentration.

RADON EXPOSURE AND HEALTH EFFECTS

When radon gas undergoes radioactive decay, a series of radioactive elements, called radon decay products (RDP), are produced. Some of these RDPs have very short half-lives, and therefore, a significant probability of undergoing radioactive decay during their time in the lung. The energy deposited by the alpha-radiation emitted during these decays can damage cells, leading to an increased risk of lung cancer.

The ICRP has estimated that the cumulative risk of lung cancer up to 75 years of age for lifelong non-smokers is 0.4%, 0.5% and 0.7% radon activity of 0 (no radon exposure), 100 and 400 Bq m^{-3} , respectively (ICRP, 2010). It should be noted that the baseline risk of lung cancer for lifelong cigarette smokers is about 25 times that for non-smokers. Consequently, the corresponding cumulative risk for lifelong smokers is 10%, 12% and 15% radon activity.

The ICRP uses Effective Dose, usually measured in mSv, as a radiation protection quantity. The main uses of effective dose are the prospective dose assessment for planning and optimization in radiological protection, and demonstration of compliance with dose limits for regulatory purposes. The ICRP has evaluated the probability of the occurrence of a stochastic effect, such as cancer, after exposure to radiation at low dose rates of 4.2×10^{-5} per mSv for workers and 5.7×10^{-5} per mSv for the general public (ICRP, 1991). To calculate the effective dose due to exposure to radon, several key quantities must be known or estimated.

The airborne concentration of RDPs is usually quantified in terms of potential alpha energy concentration (PAEC), measured in units, J m^{-3} . The PAEC of RDPs in complete equilibrium with radon is $5.4 \times 10^{-9} \text{ J Bq}^{-1}$. However, radon and its progeny are rarely in equilibrium because the RDPs readily plate out onto the surrounding surfaces, removing them from the atmosphere. The ratio between the actual PAEC and the equilibrium equivalent value is known as the equilibrium factor. Equilibrium factors from 33 caves (Cigna, 2005) show a wide variation from 0.19 to 0.94. The measurement weighted average equilibrium factor is $F = 0.57$, $n = 880$ (Table 6, Cigna, 2005). The Jenolan Caves equilibrium factor $F = 0.55$ (Zahorowski et al., 1998) is very close to the global average (Cigna, 2005).

Exposure to RDPs is measured in terms of the product of PAEC, the breathing rate and the duration of the exposure. While the SI unit for RDP exposure is J h m^{-3} , an historical unit still used in some countries is the Working Level Month, where $1 \text{ WLM} = 3.54 \text{ mJ h m}^{-3}$. The unit of Working Level, popular in the U.S., is derived from mine literature for Rn exposure. $1 \text{ WL} = 101.3 \text{ pCi L}^{-1}$ or $3,746 \text{ Bq m}^{-3}$ of ^{222}Rn in equilibrium with its short-lived decay products.

Historic ICRP Guidelines and Reference Levels

In 1993, the ICRP recommended that dosimetric models should not be used for the assessment and control of radon exposure (ICRP, 1993). At that time, the ICRP concluded that the epidemiology of radon in mines was a more appropriate indicator of detriment than the more uncertain dosimetric models. The epidemiology led to the so-called conversion conventions wherein the recommended dose conversion factors (DCFs) were $1.43 \text{ mSv (mJ h m}^{-3})^{-1}$ for workers and $1.1 \text{ mSv (mJ h m}^{-3})^{-1}$ for members of the public. Using the standard value of 0.4 for the equilibrium factor yields a DCF in terms of radon concentration of $3.1 \mu\text{Sv (kBq h m}^{-3})^{-1}$ for workers (Table 1). If instead, the equilibrium factor for the average cave ($F = 0.57$) developed by Cigna (2005) or the average for Jenolan Caves ($F = 0.55$) Zahorowski et al. (1998) is used, the DCF is $4.4 \mu\text{Sv (kBq h m}^{-3})^{-1}$ or $4.2 \mu\text{Sv (kBq h m}^{-3})^{-1}$, respectively.

Based on these DCFs, the ICRP recommended that remedial measures should be instituted or its system of radiological protection adopted where radon concentration in workplaces exceeds an action level between 500 and $1,500 \text{ Bq m}^{-3}$.

Table 1. Summary of changes to International Commission on Radiological Protection; situation specific tabulation and recommendations for radon.

International Commission on Radiological Protection	Year	Location	Rn Exposure Action Level ICRP (Bq m ⁻³)	Assumed Equilibrium Factor <i>F</i>	Radon Dose Conversion Factors (μSv (kBq h m ⁻³) ⁻¹)	Radon Progeny Coefficients			Radon DCF Factor Increase from ICRP 65
						(mSv (mJ h m ⁻³) ⁻¹)	(mSv (WLM) ⁻¹)		
ICRP 65	1993	Home	200 – 600	0.4	...	1.1	4	...	...
		Workplace Cave	500 – 1,500	0.4	3.1	1.4	5	...	...
			...	0.4	...	...	...	...	...
ICRP 115 ^c	2010	Home	200 – 300	0.4	...	1.4	5	...	...
		Workplace Cave	1,000	0.4	...	...	...	...	...
			...	0.4	...	...	...	...	...
ICRP 126 ^d	2014	Home	200 – 300	0.4	...	3.0	10	...	...
		Indoor Workplace	1,000	0.4	...	3.0	10	...	...
		Physically Active Workplace Including Cave	1,000	0.4	...	...	...	...	...
ICRP 137 para (667) Recommendation	2017	Most Situations	...	0.4	6.9	3.0	10	2.2	2.2
ICRP 137 para (665) Specified Situation	...	Home ^a	200 – 300	0.4	8.5	3.7	13	2.7	2.7
ICRP 137 para (665) Specified Situation	...	Sedentary Indoor Workplace ^a	1,000	0.4	9.2	4.0	...	3.0	3.0
ICRP 137 para (665) Specified Situation	...	Mines ^a	1,000	0.2	7.6	3.3	12	2.4	2.4
ICRP 137 para (665) Specified Situation	...	Physically Active Indoor Workplace ^a	1,000	0.4	13.1	5.7	20	4.2	4.2
ICRP 137 para (665) Time in Exercise	...	Physically Active Cave Worker ^a	1,000	0.4	15.4	6.7	24	5.0	5.0
ICRP 137 para (668) Recommendation	...	Physically Active Indoor Workplace and Cave worker	1,000	0.4	14.0	6.0	20	4.5	4.5
ICRP 137 para (669) Annex A	...	Site Specific Cave known mJh m ⁻³ Jenolan Caves	1,000	0.55	19.3	...	...	6.2	6.2
Hypothetical Global Average Cave	...	Cigna 2005 ^b equilibrium factor <i>F</i> global average	...	0.57	20.0	...	...	6.4	6.4

^a Estimated dose conversion factors from ICRP specific situation Rn decay product dose coefficients.

^b Global average equilibrium factor 0.57, from 33 caves (Cigna, 2005).

^c ICRP 115 is based fundamentally on epidemiological evidence of risk factors.

^d ICRP 126 dose coefficients use ICRP reference biokinetic and dosimetric models with specified radiation and tissue weighting factors.

Other Dose Conversion Factors

Prior to adopting the conversion convention, the ICRP had recommended (ICRP, 1987) a DCF of $10 \mu\text{Sv} (\text{kBq h m}^{-3})^{-1}$ equilibrium equivalent radon concentration, based on a dosimetric model. Using the conversion factors given in ICRP Publication 50, this DCF is equal to $1.8 \text{ mSv} (\text{mJ h m}^{-3})^{-1}$.

The United Nations Committee on the Effects of Atomic Radiation (UNSCEAR) calculated a DCF of $1.6 \text{ mSv} (\text{mJ h m}^{-3})^{-1}$, also based on a dosimetric model (UNSCEAR, 1982). In its 2000 report, UNSCEAR recognized that more recent calculations with new dosimetric models resulted in higher values of dose conversion factor. However, UNSCEAR concluded that its calculated value was well within the range of possible dose conversion factors, and therefore, should continue to be used in dose evaluations (UNSCEAR, 2000, 2009). An equation in UNSCEAR (Annex B: p107, 2000) explicitly states the applied equilibrium factor separately from the dose conversion factor. For the equivalent equation, the ICRP incorporates the default equilibrium factor ($F = 0.4$) into published dose conversion factors, omitting explicit equilibrium factors to calculate dose from Rn exposure (ICRP, 2019a).

Field (2007) applied a dosimetric model from Nikezic and Yu (2001) to calculate doses to workers in caves. This model was based on the respiratory system model in ICRP Publication 66, "Human Respiratory Tract Model for Radiological Protection" (ICRP, 1994) and results in a dose conversion factor (DCF) of $9.5 \mu\text{Sv} (\text{kBq h m}^{-3})^{-1}$ (Field, 2007), significantly higher than the conversion convention in use by the ICRP in 2007 and different from $\text{DCF} = 12.92 \mu\text{Sv} (\text{kBq h m}^{-3})^{-1}$ applied in Field (2007, Table 7). An equilibrium factor of 0.366 is explicitly applied (Equation (9), Field, 2007) in addition to an assumed equilibrium factor of $F = 0.4$ incorporated in the DCF. Effectively equilibrium factor is applied twice for calculation of received dose (Field, 2007, Table 7, reproduced Table 2).

Current ICRP Guidelines and Reference Levels

In 2010, the ICRP published a review of more recent epidemiological studies (ICRP, 2010) and concluded that a lifetime excess absolute risk of 14×10^{-5} per (mJ h m^{-3}) should now be used as the nominal probability coefficient for radon and radon-progeny-induced lung cancer, replacing the previous value of 8×10^{-5} per (mJ h m^{-3}) (ICRP, 1993). Furthermore, the ICRP stated that radon and radon progeny should be treated in the same way as other radionuclides within the ICRP system of protection, that is doses from radon and its progeny should be calculated using ICRP biokinetic and dosimetric models.

RDPs are charged ions that rapidly combine with gasses and vapors in the atmosphere to form particles of a few nanometers in size. These particles may continue to combine with other sub-micron aerosol particles. When inhaled, the size of the particles to which the RDPs are attached determines the cells that are exposed to the alpha-radiation emitted by the RDPs. Therefore, the size distribution of the aerosols to which the RDPs are attached is a critical factor when calculating dose using the ICRP biokinetic and dosimetric models. Figure 1 indicates that the most important size range is those aerosols with diameters between 0.1 and 500 nm. Mines tend to have atmospheres with a greater proportion of larger particles compared with the much cleaner atmospheres of caves. A counter-intuitive consequence is that mines tend to have a lower equilibrium factor ($F = 0.2$, ICRP 137) and DCF than caves ($F = 0.57$, Cigna, 2005) resulting in a 3 times lower dose to mine workers than cave workers for the same Rn exposure.

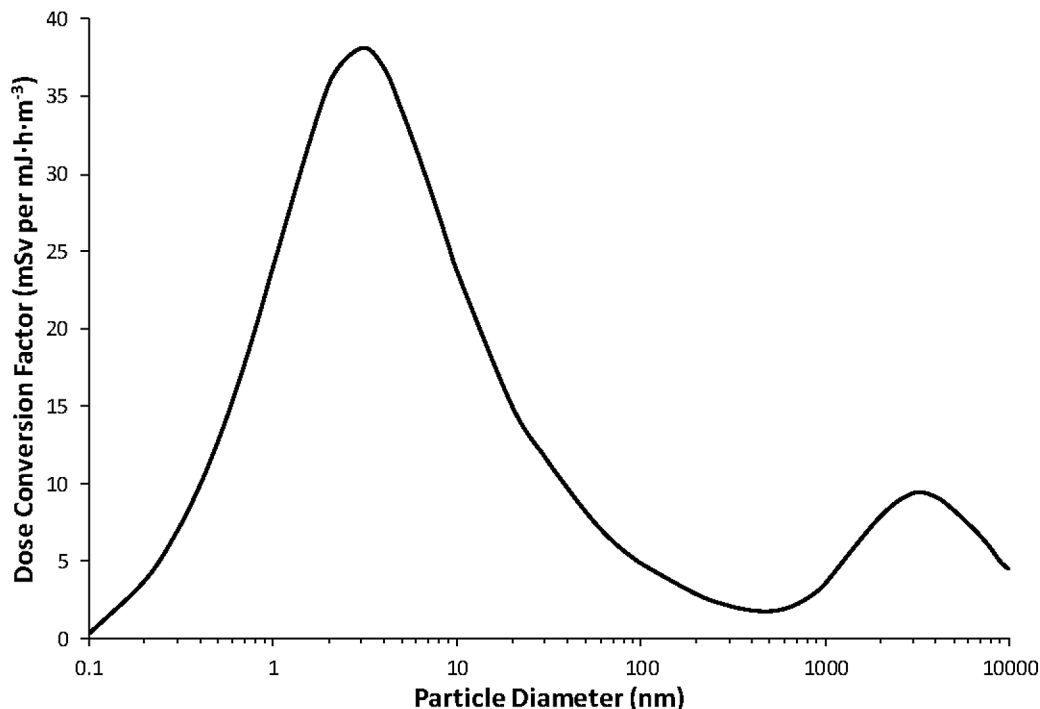


Figure 1. Effective dose per potential alpha energy exposure as a function of particle size of a monodispersed aerosol for a reference worker with an average breathing rate of $1.2 \text{ m}^3 \text{ h}^{-1}$ following exposure to radon (^{222}Rn) progeny. Unit density and a unit shape factor were assumed and hygroscopic growth was not taken into account (fitted values from Figure A5 in ICRP 137 (ICRP, 2017)).

In 2017, the ICRP published new DCFs for the inhalation of radon and its progeny (ICRP, 2017). A tabulation of situation specific DCFs in ICRP 137 (Table 12.7) lists indoor workplaces as $5.7 \text{ mSv (mJ h m}^{-3})^{-1}$ (20 mSv WLM^{-1}) and for the specific case of tourist caves $6.7 \text{ mSv (mJ h m}^{-3})^{-1}$ (24 mSv WLM^{-1} , $15.4 \text{ } \mu\text{Sv (kBq h m}^{-3})^{-1}$). In these calculations, the reference worker is assumed to spend two-thirds of the time in exercise. The ICRP now recommends a DCF of $3 \text{ mSv (mJ h m}^{-3})^{-1}$ for miners and sedentary workers and $6 \text{ mSv (mJ h m}^{-3})^{-1}$ ($14.0 \text{ } \mu\text{Sv (kBq h m}^{-3})^{-1}$) for active indoor workers and workers in tourist caves, over four times greater than that previously recommended. These recommendations assume standard particle distributions and an equilibrium factor of 0.4, which differs from the global average cave equilibrium factor of 0.57 (Cigna, 2005). The ICRP does note that in cases where aerosol characteristics are significantly different from typical conditions, sufficient, reliable aerosol data are available, and estimated doses warrant more detailed consideration, site-specific DCFs could be calculated.

The use of a site specific DCF in caves is challenging due to the high variability of parameters within a cave system. Jenolan Caves is one of a few cave systems where a site specific DCF may be calculated from known aerosol characteristics (Solomon, 2019) for a DCF of $13.27 \text{ mSv (mJ h m}^{-3})^{-1}$ for Temple of Baal cave or $5.67 \text{ mSv (mJ h m}^{-3})^{-1}$ for Katies Bower chamber in Chifley Cave. These same two caves, Chifley and Temple of Baal, were selected for their different ventilation patterns to provide site-specific continuous measurements of Rn, Rn progeny, condensation nuclei, and equilibrium factor (Zahorowski et al., 1998) to estimate an annual average equilibrium factor of $F = 0.55$, and therefore $\text{DCF} = 19.3 \text{ } \mu\text{Sv (kBq h m}^{-3})^{-1}$.

RADON MEASUREMENT METHODS

Active measurement of Rn implies an active flow of air past the Rn detector, which typically requires a solid-state electronic detector to achieve accurate equilibrated measurements in 5 minutes. Passive measurement of Rn relies on Rn diffusing through a filter or into a chamber to reduce sensor sampling anomalies. A common passive Rn sensor is polyallyl di-glycol carbonate (PADC) plaque, also known as CR-39 or a nuclear track etch detector, which is capable of recording cumulative α particle decay from Rn in the chamber leaving a microscopic track that can be made visible by etching. The number of α particle tracks in the exposed plaque is proportional to the average Rn concentration multiplied by the exposure time, reported as a cumulative exposure (Bq h m^{-3}) typically over some months. Each track etch detector has a characteristic diffusion time of up to 1–2 days (Tate and Long, 2016) to establish a Rn concentration diffusion gradient between external and chamber. The track etch detector is calibrated for a linear response to Rn, after establishing a diffusion gradient and above a threshold of counted tracks, the minimum reporting level (MRL). When track etch detectors are deployed as a static cave Rn monitor (Solomon et al., 1996), the time required to establish an equilibrium diffusion gradient is small (1–2 days) compared to the total exposure time (~ 90 days), resulting in a small error. Passive Rn detectors are not suitable for use as personal dosimeters for walk-through cave workers where the dosimeter diffusion time is large (up to 1–2 days) compared to the exposure time (1–2 hours) typical for a cave tour.

There are also many passive digital Rn monitors designed for static indoor household use with an unknown time lag to establish a Rn diffusion gradient for accurate measurement. These monitors may be deployed for static real-time Rn measurements within caves. There are a few active digital Rn monitors with a continuous air-flow rate (Rn sniffer) suitable for the mobile monitoring of walk-through cave workers in a highly dynamic cave environment ($10\times$ Rn concentration in 4 hours, Figs. 4 and 5). If air flow direction is changed by the usual ventilation change driven by the diurnal temperature cycle, the equilibrium factor changes independently of Rn concentration because of different aerosol and condensation nuclei characteristics for different air sources.

In this study, radon activity was measured by recording at 1-hour integrated intervals using a Saphymo Alphaguard PQ2000 Pro radon monitor. Environmental sensors were placed above the $2.0 \text{ m high} \times 0.5 \text{ m wide}$ passageway at Flitch of Bacon (FoB) drip water site near Chifley Cave exit to Grand Arch. Data was recorded at 15-minute intervals on a Datataker DT80 Series 2 data logger. Air flow direction and velocity data were obtained using a Gill Instruments Windsonic 2D sonic anemometer.

For Australian show caves including Jenolan Caves, Solomon et al. (1996) measured cave Rn exposure by placing passive track etch CR-39 detectors throughout the cave system, collected quarterly and calculated cave worker Rn exposure from time spent on particular cave tours to give seasonal, as well as cave specific, assessments. After 2001, subsequent Jenolan Caves Rn studies used active measurement techniques and targeted representative sites with differing ventilation characteristics in Chifley and Temple of Baal Caves (Barnes et al., 2001; Waring et al., 2017; Whittlestone et al., 2003; Zahorowski et al., 1998).

Average annual Rn concentration in Jenolan Caves, $1,021 \text{ Bq m}^{-3}$ (Solomon et al., 1996) is low to moderate in comparison with the Australian average 610 Bq m^{-3} and other caves worldwide, 11 Bq m^{-3} to $47,419 \text{ Bq m}^{-3}$ (Table 2). Average annual cave Rn concentration may mask the extreme variation from maximum to minimum of approximately two orders of magnitude (Field, 2007) and differ significantly from the actual average Rn exposure of cave workers.

Table 2. Summary of international ^{222}Rn literature (modified from Hyland and Gunn (1994) and Field (2007)) for average annual ^{222}Rn in caves and calculated dose to cave workers assuming hours worked underground (2,000 or 600 hours), equilibrium factor (0.4 or 0.57) and dose conversion factors. Figures for estimated dose reproduced from Field (2007) Table 7^a. Many of the national references include ^{222}Rn measurements from several sources^b.

International Summary Cave ²²² Rn Concentration and Received Dose		Table 7 ^a	DCF μSv (kBq h m ⁻³) ⁻¹ = 14	DCF μSv (kBq h m ⁻³) ⁻¹ = 20	
	Mean Annual ²²² Rn Conc. (Bq m ⁻³)	UG hours = 2,000 Field 2007 (mSv y ⁻¹)	UG hours = 2,000 ICRP 137 F = 0.4 (mSv y ⁻¹)	UG hours = 600 ICRP 137 F = 0.57c (mSv y ⁻¹)	References
Country Cave					
Australia	610	6 (16)	17	7	Solomon et al. (1996)
Jenolan Caves	1,021	...	29	12	Solomon et al. (1996)
Jenolan Caves	2,146	...	60	26	this study
Jenolan Caves, Chifley	838	...	23	10	Solomon et al. (1996)
Jenolan Caves, Chifley	4,578	...	128	55	this study
China	141	1 (4)	4	2	Wiegand et al. (2000)
Shawan Cave, China	47,419	...	1,328	569	Wang et al. (2019)
Czech Republic	1,235	12 (32)	35	15	Burian and Stelcl (1990)
Great Britain	2,907	27 (75)	81	35	Hyland and Gunn (1994)
Great Britain	35,890	339 (927)	1,005	431	Gunn et al. (1991)
Great Britain	9,306	88 (240)	261	112	Gillmore et al. (2000)
Great Britain	365	3 (9)	10	4	Gillmore et al. (2002)
Great Britain	315	3 (8)	9	4	Gillmore et al. (2002)
Greece	25,179	238 (650)	705	302	Papastefanou et al. (1986)
Hungary	3,300	31 (85)	92	40	Somogyi et al. (1989)
Hungary	2,468	23 (64)	69	30	Lenart et al. (1990)
Ireland	4,127	39 (107)	116	50	Duffy et al. (1996)
Japan	11	0 (0)	0	0	Miki and Iauthora (1980)
Malaysia	596	6 (15)	17	7	Gillmore et al. (2005)
Poland	1,166	11 (30)	33	14	Przylibski (1999)
Russia	2,390	23 (62)	67	29	Gunn (1991)
Slovenia	1,412	13 (36)	40	17	Kobal et al. (1988)
Slovenia	965	9 (25)	27	12	Kobal et al. (1987)
Postojna Cave LP, Slovenia	3,255	...	91	39	Gregorič et al. (2014)
Postojna Cave BC, Slovenia	2,315	...	65	28	Gregorič et al. (2014)
Postojna Cave GC, Slovenia	25,020	...	701	300	Gregorič et al. (2014)
Spain	108	1 (3)	3	1	Dueñas et al. (1998)
Spain	3,564	34 (92)	100	43	Lario et al. (2005)
Altamira Cave, Hall, Spain	3,041	...	85	36	Sainz et al. (2018)
Altamira Cave, PC room, Spain	3,286	...	92	39	Sainz et al. (2018)
Rull Cave, Spain	1,762	...	49	21	Pla et al. (2016)
South Africa	267	3 (7)	7	3	Gamble (1981)
Switzerland	25,000	236 (646)	700	300	Surbeck (1990)
United States	1,927	18 (50)	54	23	Yarborough (1976)
United States	2,589	24 (67)	72	31	Eheman et al. (1991)
United States	1,475	14 (38)	41	18	Ahlstrand (1980)
United States	11,678	110 (302)	327	140	Bashor (2004)
Carlsbad Cavern, USA	1,821	...	51	22	Cheng et al. (2007)
Hollow Ridge #2, Florida	4,733	...	133	57	Kowalczk and Froelich (2010)
Global average, Haki et al.	2,800	...	78	34	Haki et al. (1997)
Global average, this study	6,160	...	172	74	this study
total # of caves ^b in Table 2	39	...	...	...	...
# of caves > 1,000 Bq m ⁻³	29	...	...	...	...
# of caves > 20 mSv y ⁻¹	...	...	31	24	...

^a Doses of Table 7 in Field (2007) are derived by application of Eqn 9 (Field, 2007, $\text{DCF} = 12.92 \mu\text{Sv per kBq}\cdot\text{h}\cdot\text{m}^{-3}$, $F = 0.366$) citing Wiegand et al. (2000) where DCF was applied twice, contrary to (ICRP, 2019a). DCF assumes an equilibrium factor $F = 0.4$. Divide figures in Table 7, Field (2007) by 0.366 to correct values in parentheses and compare with adjacent dose estimates.

^b Data quality control likely varies for each study conducted for each country which should be regarded as problematic.

^c Global average equilibrium factor $F = 0.57$ (Cigna, 2005) is applied.

Annual average cave worker Rn exposure is a useful measure when converted to received dose for that individual to comply with workplace health standards. However, individual cave worker Rn cumulative exposure does not measure Rn concentration variations in different parts of the cave system, during different seasons or time of day to inform cave monitoring and mitigation measures.

Average annual Rn activity in a significant majority of caves from all countries, 28 from a total of 38 exceed the ICRP recommended action level of $1,000 \text{ Bq m}^{-3}$ to reduce Rn exposure in the workplace (Table 2). Cave Rn activity and the ICRP recommended action level of $1,000 \text{ Bq m}^{-3}$ have remained the same since 1994. However, the calculated received dose to cave workers has changed markedly from 1994 with a significant majority of full-time cave workers now expected to receive a radiation dose from Rn in excess of 20 mSv y^{-1} , whether calculated with an ICRP assumed 2,000 workplace hours and equilibrium factor of 0.4 or a more likely 600 hours underground and equilibrium factor of 0.57. Table 2 is a guide to Rn exposure and consequent dose using available annual averages. Actual Rn exposure and dose to cave workers need to be assessed for each cave and cave worker.

SOURCE OF RADON IN CAVES

Radon is known to accumulate in some homes or poorly ventilated basements where the underlying rock has an elevated concentration of U necessary to maintain a high Rn flux to sustain elevated Rn concentrations in enclosed spaces. The short half-life of Rn (3.8 days) and short diffusion length through solid rock (Cigna, 2005) dictate that high Rn in an enclosed space must be replenished by direct diffusion from a high U host rock with permeability (micro-cracking) or air flow from a relatively high U or Ra source. Some granites and sandstones may have relatively high U concentrations due to magmatic fractionation or hydrothermal concentration effects. In contrast, unaltered marine limestone typically has low U concentration, yet may host cave chambers with very high Rn concentrations. The simple mechanism of direct diffusion or seepage from a high U host rock applicable to enclosed poorly ventilated spaces in homes is unlikely to apply to the common high Rn found in limestone caves. The implicit direct Rn accumulation from cave host-rocks (Rn source) assumption in many studies (Fairchild and Baker, 2012; Fernandez-Cortes et al., 2015; Sainz et al., 2018; Wang et al., 2019) is not supported.

Soil Source of Rn

Cigna (2005) points to three fundamental mechanisms that favor a soil or cave sediment source of Rn compared with emanation from limestone hosting the cave void:

1. Low concentration of ^{238}U and ^{226}Ra in limestone host rock and relatively high concentration of ^{238}U and ^{226}Ra in clays,
2. Inability of Rn to escape the CaCO_3 mineral lattice, unless very close to the surface ($0.02\text{--}0.07 \mu\text{m}$), and
3. The effective diffusion length of Rn in soil via inter-granular pore space to open cracks and fissures is large enough to effectively transport Rn to the cave void with convective air transport.

A further uranium concentration mechanism favoring residual soils above karst is the concentration effect by dissolution of CaCO_3 leaving the less soluble oxides and silicates including clays, effectively concentrating U. Cave sediments may also form in-situ from limestone dissolution, however, an unknown proportion of the less soluble clays, oxides, and silicates may be transported to the cave and deposited by streamflow. Uranium concentration in seven cave sediment profiles from the UK (Bottrell, 1991) were found to be generally low ($2\text{--}4 \text{ mg kg}^{-1}$) with one exception at $15\text{--}16 \text{ mg kg}^{-1}$ compared to external detrital sediments ($16\text{--}24 \text{ mg kg}^{-1}$).

Discriminating Between Cave Accumulation and Soil Source of Rn

The most likely sources of Rn in caves are (1) an external soil source requiring transport of Rn to the cave or (2) the *in-situ* accumulation of Rn from internal cave sediments, limestone, drip-waters, or (3) from connected cracks, faults or open fissures to a high U source rock. All sources of cave Rn are influenced by cave-air convective ventilation that may produce very similar seasonal cave Rn activity patterns. Cave Rn alone is not a good source discriminant. Time series correlation of Rn and CO_2 showing a strong seasonal pattern (summer high) is used (Pla et al., 2016) to infer a common accumulation of Rn ($513\text{--}3,500 \text{ Bq m}^{-3}$) and CO_2 ($565\text{--}4,065 \text{ ppm}$) from a cave source, assuming CO_2 outgassing from speleothem growth air-flow Rn emanation from cave walls or sediments. An alternate soil source for CO_2 and Rn transported to the cave by top-down dominant cave ventilation in summer is also possible. Isotopic measurement of $\delta^{13}\text{C}\text{--CO}_2$ is used (Waring et al., 2017) to discriminate between CO_2 derived from speleothem outgassing in a cave ($\delta^{13}\text{C} = -19 \text{ ‰ VPDB}$) and CO_2 transported from soil ($\delta^{13}\text{C} = -25 \text{ ‰ VPDB}$) by summer chimney effect convective ventilation. In addition to $\delta^{13}\text{C}\text{--CO}_2$, another conservative gas tracer N_2O is relatively abundant in soil gases and not formed or destroyed in caves. Together these gas tracers unequivocally attributes the source of Rn in Chifley Cave to the soil above (Waring et al., 2017). Synchronous measurement of Rn source flux, external and cave temperature, cave air flows, and multiple gas tracers can discriminate between Rn sources and Rn air-flow transport or dilution function.

Drip-Water Source of Rn

Radon may also be transported from surface soils to the cave void by dissolved groundwater flow through joints, fissures and faults (Surbeck, 2005). For this mechanism to be effective groundwater flow to the cave must be rapid compared to the Rn half-life of 3.8 days. A further constraint on groundwater transport is the requirement for Rn solubility to change causing the outgassing of Rn, potentially by a temperature increase of groundwater along the flow path.

CAVE VENTILATION

Models of Cave Ventilation Patterns

Cave physiography and temperature contrasts between cave air and external air temperature have a major influence over cave ventilation, particularly for caves with an upper and lower entrance separated by an elevation difference (Fig. 2). This configuration may be described as a chimney effect cave with the physics driving cave ventilation described in detail in the literature (Atkinson et al., 1983; Badino, 2010; Covington and Perne, 2016; Wigley and Brown, 1971; Wigley and Brown, 1976). In winter, cave air temperature is typically warmer than external air causing the less dense and relatively warm cave air to rise and expel to the external atmosphere. The converse summer air flow is in the opposite direction due to relatively cool cave air sinking to expel through the lower cave opening. Bi-directional convective air flow through the cave is proportional to the elevation difference between upper and lower openings, as well as the magnitude of the temperature contrast. On any single day air flow through the cave may be in both directions due to relative temperature being both higher and lower than cave temperature. Diurnal bi-directional cave air flow is most likely in spring and autumn where external temperature contrast is both positive and negative with respect to cave temperature. A chimney effect summer ventilation pattern from top to bottom along the major cave void path also induces a slight suction in the cave to draw air slowly into the cave through minor openings, cracks, and fissures.

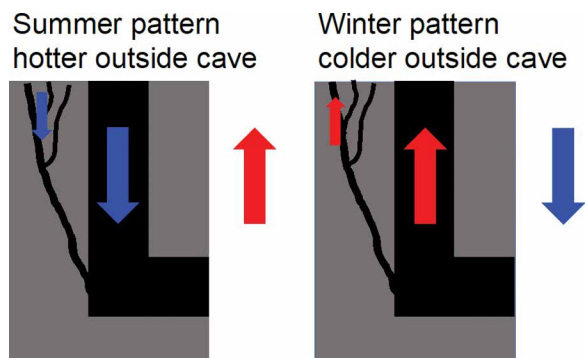


Figure 2. Illustration of chimney effect ventilation pattern where ventilation through a cave with 2 unequal elevation openings to the external atmosphere. A relatively constant cool cave temperature implies a dominant downward air flow in summer, when the external temperature is normally hotter than the cave temperature, driving convective ventilation through the cave. The converse pattern holds in winter. On any day, the temperature difference between cave and the external atmosphere may drive convective cave ventilation in either or both directions.

be described as either a summer cold air trap (SCAT), if the entrance is higher, or a winter warm air trap (WWAT), if the entrance is lower. Another mechanism to induce cave airflow is barometric pumping (Barometric), where a large cave chamber or network is connected to the surface via a single opening.

For single opening caves where the air mass is warmer than the external temperature, typically in winter, warm air may exit the cave through a major upper opening with replacement dense cool air seepage into the cave through soil, restricted openings, or fissures. The term seepage, or air seep, is used to describe slow air flow caused by air density contrast along a temperature gradient between different temperature air masses, not as a substitute term for diffusion. In winter, relatively dense cold air on the ground surface and in soil with high Rn may seep into near surface caves along small cracks and fissures if cave temperature is greater than external temperature, typical for a SCAT configuration.

A summer cold air trap type cave may be close to the surface with a single entrance or exit above the main chamber, as shown in Figure 3. Having less air exchange in summer for a SCAT configuration is likely because of a stable temperature profile. The stable temperature profile through a SCAT cave in summer is from relatively cold air at the base of the isolated SCAT chamber to relatively warm on the surface above. This stable temperature profile does not induce Rn transport from the soil above a SCAT cave in summer. In winter, the surface temperature is often colder than the relatively warm cave below. This winter temperature profile is unstable causing cold, dense (soil) surface air to seep into the cave below through minor cracks and fissures, transporting Rn from soil to SCAT type cave in winter, resulting in a summer low, winter high seasonal Rn pattern for SCAT type cave morphologies.

Temperature contrast between cave and external air drives convective cave ventilation on different physical and temporal scales. The magnitude of air mass flow under chimney effect ventilation is much greater and ubiquitous than

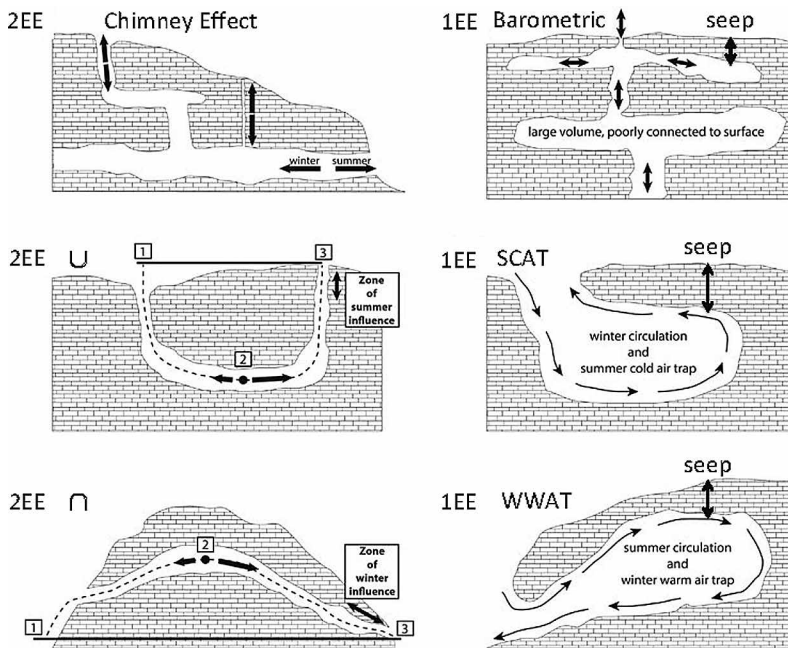


Figure 3. A modification of cave classification from Covington and Perne (2016) with morphology affecting ventilation pattern.

coming off the Grand Arch and an upper opening on the ridge in a surface doline (Elder Cave) 62 m above the lower opening. The minimum path length between openings is 536 m and cave volume 9,587 m³ along this path (Zlot and Bosse, 2015). Radon and CO₂ measurements shown in Figures 4 and 5 are from a cave chamber, Katies Bower, 120 m from the lower cave entrance, in the Grand Arch and one quarter of the total path length (536 m). Measurements of air flow, cave temperature, and pressure are from an instrument site in a 2 m high × 0.5 m wide passage called the Flitch of Bacon, 75 m from the Grand Arch (Figs. 4, 5, and 6). There are many extended side passages including Jubilee Cave without a direct opening to the surface. An alternate lower passage connection to the Grand Arch through Imperial cave has a sealed door except for tour groups to pass, approximately three visits per day.

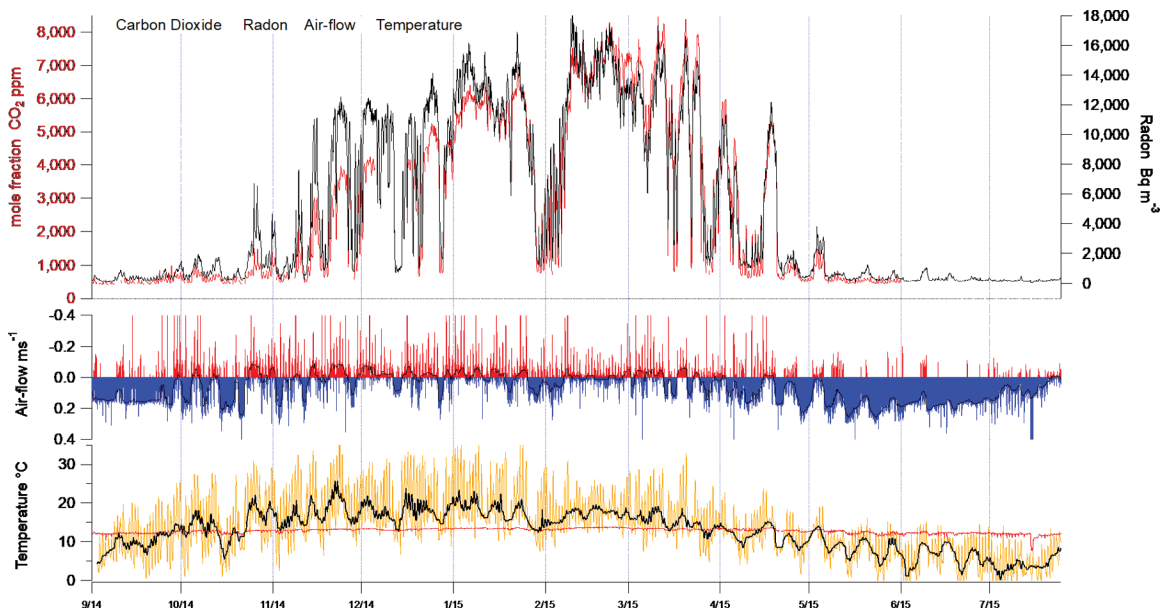


Figure 4. Radon and CO₂ annual cycle. Synchronous seasonal variation at Lower Katies Bower, in Chifley Cave from September 2014 to September 2015. Rn continuous active integrated air sample recorded at 1-hour intervals, CO₂, and temperature at 15-minute intervals. Winter air-flow pattern (blue) reduces Rn concentration and summer pattern (red) increases Rn. The air-flow magnitude and direction is determined by the temperature contrast between cave temperature (red line) and external temperature (yellow line). The diurnal temperature range is often greater and less than cave temperature in a single day, causing a diurnal reversal of air flow. A 24-hour moving average of external temperature (black) removes the diurnal temperature variation to reveal a synoptic temperature pattern.

net air exchange via other ventilation patterns due to through flow (Badino, 2010; Luetscher and Jeannin, 2004; Wigley and Brown, 1976). For caves with large entrances or on exposed hillsides, prevailing wind effects may also contribute to cave ventilation.

The complexity of natural cave systems suggests that in different parts of an extended cave network air flow may be behaving in hybrid or complex patterns that only direct measurement can reveal. Combining knowledge of likely cave air flow patterns and the probable source of cave Rn permits first pass estimation of cave radon patterns.

Chifley Cave Example of Chimney Effect Ventilation Pattern

Chifley Cave is part of an interconnected group of show caves including Imperial, River, Jubilee and Elder Cave on the northern side of the large Grand Arch at Jenolan Caves, Australia. Another group of interconnected caves on the southern side of the Grand Arch includes Temple of Baal Cave. Chifley Cave has a lower opening in a cleft

the surface. An alternate lower passage connection to the Grand Arch through Imperial cave has a sealed door except for tour groups to pass, approximately three visits per day.

The Jenolan Caves complex has been mapped at high resolution (3 cm) using mobile LIDAR (Zlot and Bosse, 2014) with the full 3D dataset available (Zlot and Bosse, 2015). An excerpt from the 3D data (Zlot and Bosse, 2015) shows the north side cave

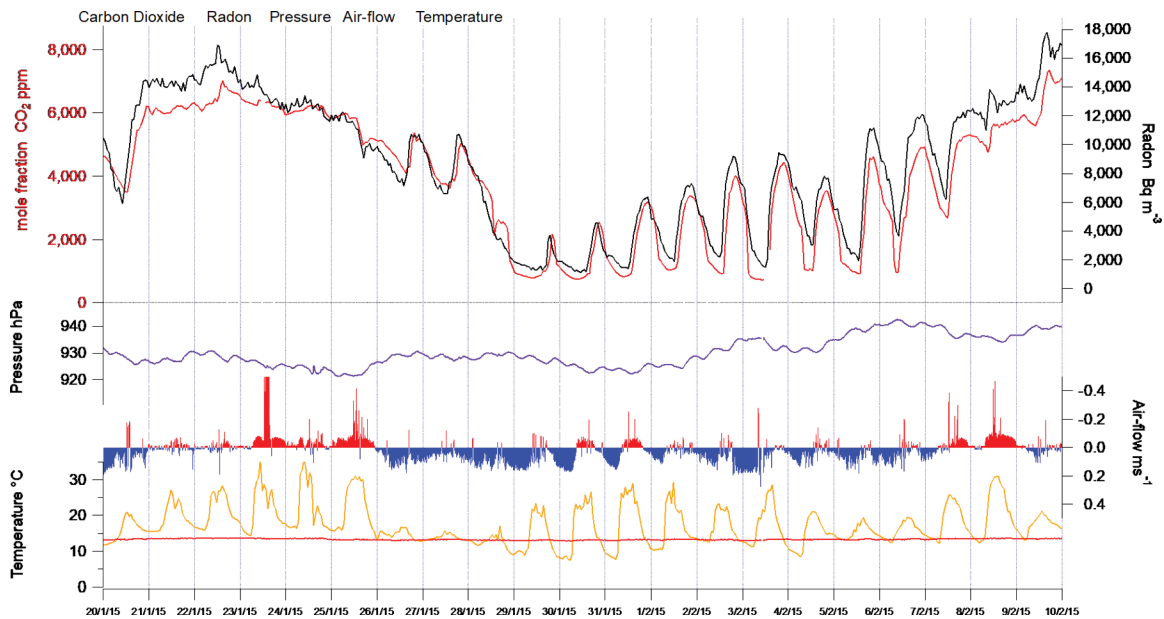


Figure 5. Radon and CO₂ diurnal cycle. Synchronous variation over 20 days for Rn and CO₂ at Lower Katies Bower, in Chifley Cave from January 20, 2015 to February 10, 2015. Rn continuous integrated air sample recorded at 1-hour intervals, CO₂, air flow, temperature, pressure at 15-minute intervals. An unusual 3-day period of cold external temperatures in summer causes air flow to reverse direction (blue) reducing Rn concentration then returning to a diurnal pattern of bi-directional air flow. The air-flow magnitude and direction is determined by the temperature contrast between cave temperature (red line) and external temperature (yellow line). The diurnal temperature range is often greater and less than cave temperature in a single day, causing a diurnal reversal of air flow. A 24-hour moving average of external temperature (black) removes the diurnal temperature variation to reveal a synoptic temperature pattern.

summer and low Rn in winter as a result of chimney effect ventilation (Table 3). The common summer-high pattern of cave Rn activity is also represented in common summer-high estimated doses to Guides (Solomon et al., 1996). The alternate winter-high pattern of cave Rn is observed in Shawan (Wang et al., 2019) and Altimira Caves (Sainz et al., 2018). Both caves are very close to the surface with a single entrance-exit. Seasonal preferential seepage of cold air in soil through the epikarst above the

cave due to temperature contrast in winter may explain the winter high Rn pattern for these shallow caves (Table 3). Both seasonal patterns show a strong correlation between cave Rn and CO₂, suggesting a common external soil source of both gases.

Links between cave Rn activity and temperature contrast (external T–cave T) are well known and widely attribut-

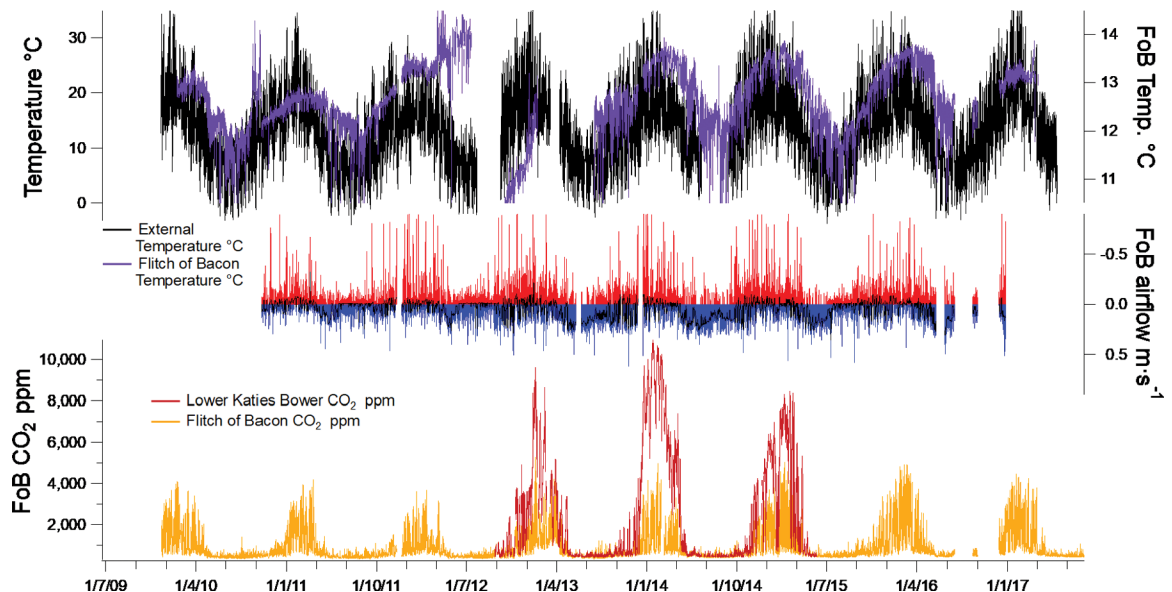


Figure 6. Temperature difference, cave airflow, and CO₂ multi-year comparison. Synchronous variation (15 min) over 8 years for CO₂ at Fitch of Bacon (FoB) and Lower Katies Bower (LKB) sites with 45-meter separation in Chifley Cave from 1 July 2009 to 20 August 2017. Cave temperature measured at FoB (purple) has a seasonal variation of < 3 °C compared to the external seasonal variation of around 30 °C. Cave temperature recorded between February 2012 and July 2013 may be unreliable due to water saturation of co-hosted humidity sensor. Short data gaps < 2 months due to sensor failure are interpolated by modeling with adjacent years for construction of Table 4.

complex and the L shape geometry between Elder and Chifley Cave (Fig. 7) with an upper and lower entrance or exit (2EE) necessary to achieve strong chimney effect cave ventilation.

RADON SEASONAL PATTERN IN CAVES

A summary of key findings from international literature shows radon in caves varies widely (Table 2) between years and also seasonally within a cave system, favoring high Rn in sum-

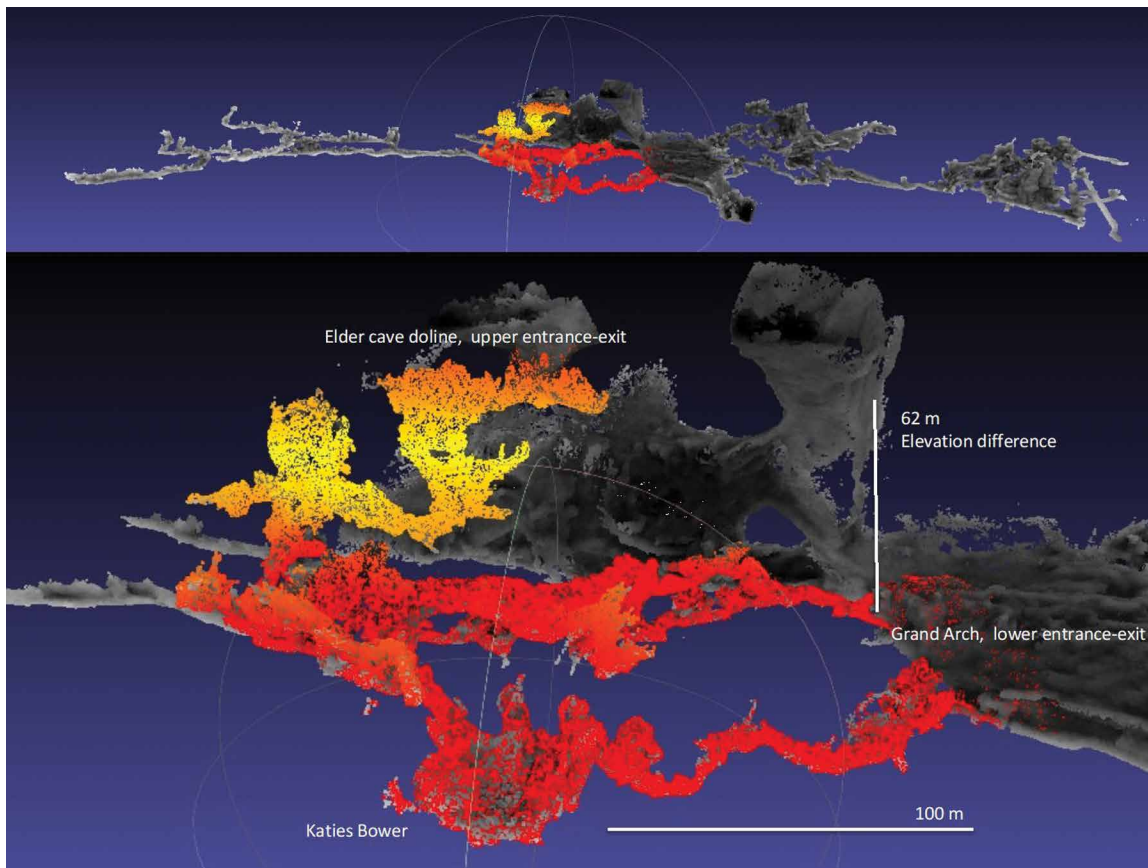


Figure 7. Oblique view of air-flow path between Elder cave doline upper entrance-exit and the lower Grand Arch entrance-exit.

closely correlates with CO_2 concentration on a time scale of < 1 hour (Fig. 5) for a 20-day period in summer 2015 suggesting a common source (Waring et al., 2017). Both Rn and CO_2 cave concentrations are a function of soil source concentration and chimney effect ventilation. The 1 month delay in CO_2 rise in the cave is likely caused by a delayed soil CO_2 respiration response to an increase in soil biological primary productivity from spring soil warming compared to inorganic constant rate production of Rn in the soil.

Temperature Difference and Air Flow Influence on Cave CO_2 and Rn

Rn activity in Chifley Cave is dependent upon the temperature difference between cave and external air to drive cave ventilation measured as bi-directional air mass transfer, shown as weekly integration increments in Figure 8. When measured at the 'Flitch of Bacon' (FoB) passage, the weekly integrated seasonal temperature pattern is symmetrically distributed with external temperature in excess of FoB cave temperature in summer and similarly in deficit in winter (Fig. 8). If integrated over a year from July 1st to June 30th inter-annual comparisons of temperature difference are very close to evenly balanced with a very small bias to warmer cave temperatures (Table 4).

Air velocity into or out of Chifley Cave is also measured at 'Flitch of Bacon' passage. The orthogonal hewn passage at FoB is 2.0 m high by 0.5 m wide simplifying calculation of air mass transfer from the Grand Arch entrance and exit. In stark contrast to the even temperature distribution, air flow driven by temperature difference is highly biased favoring winter air flow (Fig. 8). The ratio of volume of air moving into Chifley Cave from the Grand Arch (winter pattern) ranges from 2–5 times greater (Table 4) than the volume of air flowing out of Chifley Cave (summer pattern). The seasonal asymmetrical airflow bias implies cave temperature is approximately 5°C warmer than the average external temperature (Fig. 8).

Karst rock mass temperature is balanced by a geothermal heat flux from below and heat loss (winter) from above that approximates surface temperature. Caves provide air flow, water, and heat transfer pathways for temperature equilibration to extend the depth of average surface temperature influence to the depth of the cave system (Luetscher and Jeannin, 2004). In karst, a negative geothermal gradient close to the surface where temperature decreases with depth, has a deeper inflection point before resumption of a positive geothermal gradient to the Earth's interior. Air flow through caves and karst aquifers provide heat transfer mechanisms to establish a long-term stable near surface geothermal gradient as a bal-

able to cave ventilation (Gregorič et al., 2014; Kowalczyk and Froelich, 2010; Wigley and Brown, 1976). However, the source of Rn is often assumed to be by accumulation inside the cave with seasonal cave Rn concentration patterns caused by dilution with winter dominant cave ventilation of external low Rn air.

Lower Katie's Bower chamber (Chifley Cave) Rn activity has a very strong seasonal bias with high Rn in summer and low Rn in winter (Fig. 4). Rn concentration

Table 3. Summary of selected cave literature for average annual Rn activity association with assumed Rn source, known cave geometry, diurnal and seasonal patterns, correlation with CO₂ and winter biased airflow.

Cave, Country	Mean Annual ²²² Rn Activity (Bq m ⁻³)	Measurement Method	Interpreted or Assumed ^a Rn Source	Cave Geometry				Cave Ventilation Diurnal Pattern	Cave Rn Seasonal Pattern	Correlation with CO ₂	Winter Air-Flow Bias	Reference
				# Entrance – Exit Shape 2EE L U n	Shape 1EE Baro SCT	WWT						
Jenolan Caves all	1,021	Passive Etch Track	...	...			...	...	...	...	...	Solomon et al. (1996)
Jenolan Caves all	2,146	Passive Etch Track	...	...			...	...	...	...	...	this study
Jenolan Caves, Temple of Baal	2,850	Active Si SSD	...		1EE WWAT			none	Summer low Autumn high	no	...	Whittlestone et al. (2003)
Jenolan Caves, Chifley Cave	838	Passive Etch Track	...	...	2EE L			...	Summer high Winter low	...	...	Solomon et al. (1996)
Jenolan Caves, Chifley Cave	4,578	Active Si AlphaGuard	Soil		2EE L			strong	Summer high Winter low	yes	yes	this study
Postojna Cave LP, Slovenia	3,255	Passive Si	Cave		2EE L			strong	Summer high Winter low		yes	Gregorič et al. (2014)
Postojna Cave BC, Slovenia	2,315	Passive Si	...		2EE L			...	Summer high Winter low	...	...	Gregorič et al. (2014)
Postojna Cave GC, Slovenia	25,020	Passive Si	Cave		2EE L			strong	Summer high Winter low	yes	yes	Gregorič et al. (2014)
Hollow Ridge #2, Florida ^b	4,733	Active Si RAD 7	Cave		4EE L			yes	Summer high Winter low	yes	yes	Kowalczk and Froelich (2010)
Shawan Cave, China	47,419	Active Si RAD 7	Cave		1EE SCAT			weak	Summer low Autumn high	unknown	unknown	Wang et al. (2019)
Altamira Cave, Hall, Spainc	3,041	Passive Si Scout	Cave		1EE			yes	Summer low Winter high	yes	no	Sainz et al. (2018)
Altamira Cave, PC room, Spain	3,286	Passive Si Scout	Cave		1EE WWAT			no	Summer low Winter high	yes	no	Sainz et al. (2018)
Rull Cave, Spain	1,762	Active Si Radim 5WP	Cave		1EE			no	Summer high Winter low	yes	yes	Pla et al. (2016)

^a Cave Rn source is assumed to be by cave accumulation with winter air-flow dilution.

^b Hollow Ridge #2 annual average calculated from seasonal averages.

^c Altamira annual average calculated from monthly averages Dec 13–16.

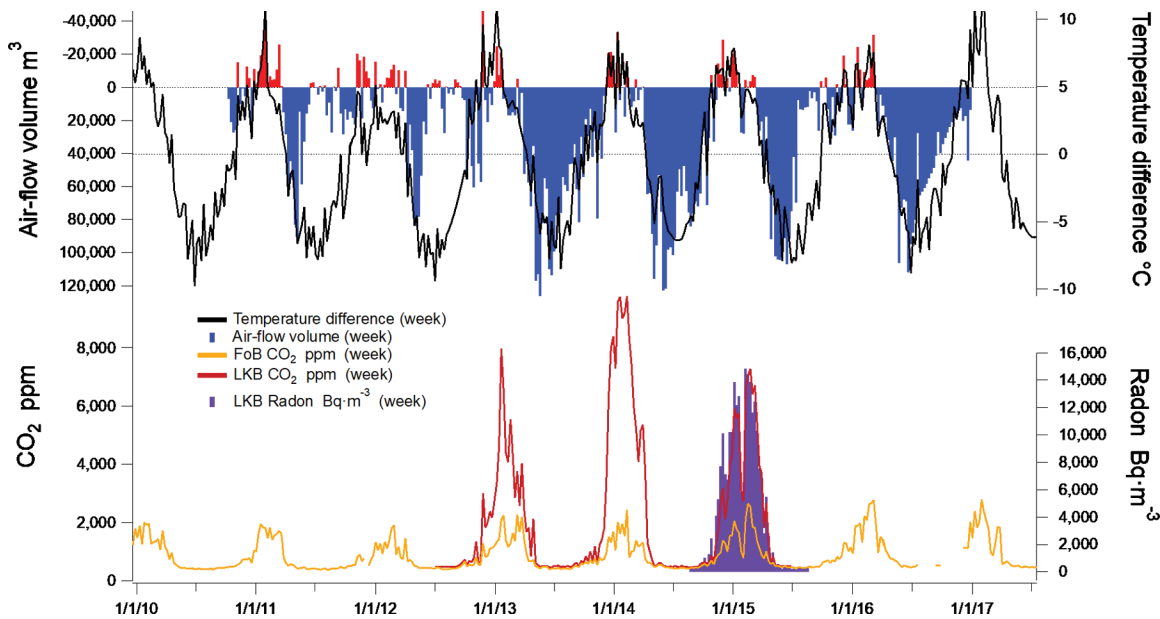


Figure 8. Weekly aggregates of 15 minute and hourly sensor data illustrates a balanced seasonal temperature difference (external temperature—cave temperature) compared to a biased seasonal airflow through Chifley Cave. The strong winter pattern airflow bias from the bottom up flushes Rn and CO₂ from Chifley Cave. A warmer cave temperature of approximately 5 °C is implied by the biased seasonal airflow. Rn and CO₂ are drawn into Chifley Cave from the soil above during top down summer pattern airflow. Average weekly CO₂ measured at Flitch of Bacon passage during summer has a maximum close to 2,000 ppm whereas CO₂ measurements at Lower Katies Bower, 45m further into Chifley Cave has a maximum that ranges between 7,000–10,000 ppm.

vertical profile varies from 12°C at the lowest elevation in Chifley Cave (FoB) to a 16°C soil temperature above Chifley Cave (Table 4). The average temperature for a column of air through Chifley Cave is 2–5 °C warmer than temperature at FoB due to geothermal heating. Similarly, external temperature varies depending on position of the weather station with respect to local topography, potentially creating a temperature offset from the true average temperature of the column of air in a cave or externally due to thermometer location.

The small net annual average temperature difference between a column of air inside the cave and an external column of air produces a large net annual volumetric air flow bias (2–5:1) favoring the winter ventilation pattern that flushes Rn from caves with ambient air (Fig. 8). A model (Greene et al., 2019) of external temperature variation at Jenolan Caves shows a small temperature increase (0.17°C) from 2008 to 2018 consistent with an increase in temperature from global warming. Cave temperature from Flitch of Bacon increases by a smaller amount (0.1°C) over the same period, though with less certainty due to cave temperature data record gaps (Fig. 6).

Comparison of average annual CO₂ concentration measured at Lower Katies Bower (Table 4) from 2013–2014 (1,812 ppm) to 2014–2015 (2,909 ppm) shows an inter annual increase of 60% before reducing by 30% in 2015–2016 (2,195 ppm). A small variation in annual average external temperature, amplifies net air flow causing a large impact on cave CO₂ concentration (Table 4). Inter-annual variations in Rn activity of approximately 60% may also be expected from a small ~0.5 °C change in average external temperature. Warming external temperature due to climate change over decades will decrease the cave minus external temperature difference causing a decrease in winter pattern airflow and an increase in cave Rn activity.

Cave CO₂ as a Proxy for Rn

Chifley Cave Rn and CO₂ concentration closely correlates (Fig. 9, $R^2 = 0.94$) within a measurement timescale of < 1 hour, over 20 days because of a mutual soil source. Over a longer seasonal time frame, the soil source CO₂/Rn ratio changes slightly due to primary biological productivity only affecting CO₂ from bacterial and root respiration and not Rn. Individual rain events and longer low rainfall periods may also affect soil moisture, and consequently, soil CO₂ concentration and the CO₂/Rn source ratio (Waring et al., 2017). In caves where there may not be a mutual source of Rn and CO₂ or where chimney effect ventilation is not evident, a Rn–CO₂ correlation is unlikely to apply (e.g., Temple of Baal, Jenolan Caves). CO₂ in caves may also have an inorganic source from limestone dissolution in the epikarst, speleothem growth, or from the breath from high numbers of cave visitors to change the cave CO₂/Rn ratio.

With the exception of the Temple of Baal, increases in CO₂ associated with visitation at Jenolan are typically relatively short-lived and quickly return to the pre-tour level (Baker, 2014). Consequently, the CO₂ from cave visitors would not

ance between average surface temperature and the geothermal heat flux from below.

The annual average surface temperature is therefore expected to be the same as the annual average cave air temperature, plus a component due to heat flux from the Earth's interior. Cave air temperature varies depending on position within the cave and the geothermal gradient. For Jenolan Caves, cave air temperature

Table 4. Annual (July 1 – June 30) external and cave temperature, temperature difference (external – cave), airflow volume (m³) into Chifley Cave from the Grand Arch (up) and in the opposite direction (down) from the surface and soil above Chifley Cave, the ratio of air volume (up / down), CO₂ ppm concentration measured in the passage at Fitch of Bacon, CO₂ concentration measured at Lower Kates Bower chamber 45 m further into Chifley Cave, and average annual Rn activity (Bq m⁻³) measured at Lower Kates Bower. Cave temperature measured at Fitch of Bacon is near the lowest point in Chifley Cave which also has the lowest temperature.

Year Starting July 1	External Temperature (°C)	Soil Temperature (°C)	Cave FoB Temperature (°C)	Temperature Difference (°C) xt. – Cave	Ratio				Average Annual CO ₂ ppm FoB	Average Annual CO ₂ ppm LKB	Average Annual Rn Bq m ⁻³ LKB
					Annual Airflow Volume m ³ Up	Annual Airflow Volume m ³ Down	Direction	Volume Up / Down			
2010–2011	12.11	...	12.34	-0.23	...	...	...	...	...	...	...
2011–2012	11.63	...	12.37	-0.74	857,534	-434,969	...	1.97	771	...	...
2012–2013	11.22	...	12.82 ^a	-1.60 ^a	1,088,932	-504,167	...	2.16	722	...	...
2013–2014	12.23	...	12.14 ^a	0.08 ^a	2,026,444	-600,469	...	3.37	861	1,812	...
2014–2015	12.76	15.60	12.76	-0.01	2,679,207	-511,373	...	5.24	835	2,909	...
2015–2016	13.02	15.97	12.80	0.22	2,634,826	-549,638	...	4.79	913	2,195	4,861
2016–2017	13.03	16.06	12.74	0.30	...	...	...	...	...	...	...
2017–2018	12.90	15.80	12.82	0.08	...	...	...	...	...	...	...

^a Cave temperature sensor pattern for 2012 and 2013 less reliable (see Figure 8).

be expected to significantly skew the CO₂/Rn ratio if averaged more broadly across a 24 hour or weekly timeframe. Ten years of monitoring exhibition caves at Jenolan shows that 8 caves exhibit strong seasonal variations in CO₂ with a summer maximum and a winter minimum, with the exception the Temple of Baal (Baker, 2014).

For most exhibition caves at Jenolan, including Chifley Cave, the breath from cave visitors and soil source CO₂/Rn variation is likely to be small, permitting CO₂ to be used as a proxy measurement for Rn.

POTENTIAL MITIGATION STRATEGIES TO REDUCE RADON EXPOSURE

The ICRP (ICRP, 2019b) provides summary recommendations for workplaces:

If cave Rn activity levels are anticipated to be elevated, cave managers are expected to monitor cave Rn activity, if possible reduce workplace Rn activity and take action to reduce worker exposure to Rn in the workplace (cave).

ICRP 126 (ICRP, 2014) Executive summary:

“(m) Characterisation of the exposure situation is also a prerequisite for application of the optimisation principle. This principle is the driver for controlling radon exposure in order to maintain or reduce exposure to a level that is as low as reasonably achievable [ALARA], taking the prevailing economic and societal circumstances into account. As with the control of other sources of radiation, the Commission recommends the use of a source-related individual dose restriction in conjunction with the optimization of protection.”

Prerequisite characterization of the exposure situation implies that cave Rn monitoring is sufficient for applying optimization principles (mitigation measures). Continuous real-time Rn monitoring to track diurnal, seasonal, and cave location specific variable Rn activity is absent from most tourist caves. Reduction of Rn in the workplace (optimization) may be possible, applying ALARA principles, before restriction of cave worker hours exposed to elevated Rn activity.

For workplaces where radon levels exceed 1,000 Bq m⁻³, ICRP Publication 126 (ICRP, 2014) states:

“In workplaces where, despite all reasonable efforts to reduce radon exposure, individual doses persist above 10 mSv y⁻¹, the workers should be considered as occupationally exposed and their exposure should be managed using the relevant radiological protection requirements established for occupational exposure: identification of the exposed workers, information, training, dose monitoring (in doses or potential alpha energy concentration) and recording, and health surveillance. In any case, the individual doses should not exceed the upper value of the 1–20 mSv band.”

Limiting Time Exposed to Rn

Different approaches to mitigating worker exposure to high Rn in caves may be achieved by limiting the underground hours of Rn exposure for cave workers. Some cave systems may be able to adjust cave worker hours / schedule to lower cave worker dose within an acceptable range, for others this may be very difficult. Some workers may not wish to work greater or fewer underground hours or assume a greater perceived health risk.

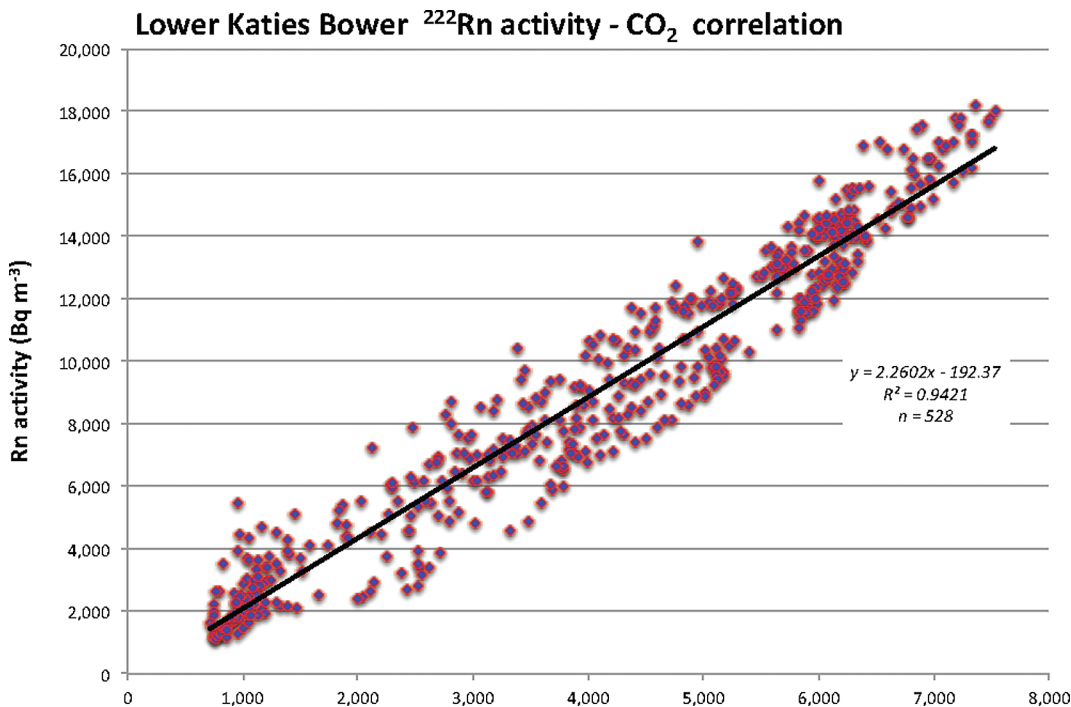


Figure 9. Radon activity vs. CO₂ concentration correlation during the time period January 20, 2015 to February 10, 2015, shown in Figure 4.

Passive Modifications to Cave Ventilation

If cave managers wish to reduce the worker exposure to high Rn concentration by controlling a cave's natural ventilation, the source(s) of Rn makes a material difference, whether Rn is excluded from the cave and reduced, or trapped in the cave and increased. The natural ventilation regime in many show caves may have been significantly altered by existing facilities, air flow enhanced by passage widening or restricted by construction of sealed doors. Intervention in the ventilation regime for the purpose of reducing Rn exposure needs to consider the

possible consequences on cave environments, speleothems, and biota to prevent deleterious effects.

Based on a detailed knowledge and understanding of Rn dynamics in a typical chimney effect cave (Chifley Cave), it is possible to devise a strategy to exclude Rn from caves originating from the soil above and enhance the natural flushing of air by opening and closing sealed doors in synchrony with external temperature changes. For example, when the external temperature is greater than the average cave temperature an air-tight door remains closed, and when the external temperature is lower than the average cave temperature the door is open. An automated door opening and closing regime may be sufficient to passively reduce Rn in the cave to acceptable levels ($\ll 1,000 \text{ Bq m}^{-3}$).

While many caves have some proportion of bi-directional convective ventilation there are other cave configurations, often with only one significant entrance or exit where Rn ingress is not driven by convective air flow as a result of warmer external temperatures. A passive Rn exclusion strategy is less likely to be successful in reducing Rn for these cave configurations with one entrance and no convective air flow. Increased convective air flow from opening a door may provide a second entrance or exit and have a complex material impact on Rn, Rn progeny, equilibrium factor (Zahorowski et al., 1998). An example from Jenolan Caves system is the Temple of Baal, which has a winter high, summer low Rn pattern (Barnes et al., 2001; Whittlestone et al., 2003) and no correlation with CO₂ concentration (Baker, 2014).

Technologies Available to Reduce Cave Rn Concentration

Simple room air-conditioning demonstrates effective lowering of indoor Rn (Yu et al., 1995), however air-conditioning is not feasible to implement for most caves. Air conditioning and central heating coupled with door opening and closing reduces indoor Rn (Marley and Phillips, 2001) by stimulating indoor air-exchange similar to cave chimney effect ventilation. Both indoor and cave systems show an inverse correlation between indoor-cave Rn and indoor-cave temperature, a corollary to winter pattern air flushing. Mitigation measures that clean cave air with an electrostatic air cleaner (EAC), an ion generator/fan system (IG/F), or a filtration unit (Hopke et al., 1993) may significantly reduce cave Rn progeny by 63%, 34%, and 66%, respectively. However, air conditioners and air cleaners may also change cave humidity and significantly detract from the cave visitor experience by addition of background noise.

Cave Rn and Rn Progeny, Relative Humidity

Cave Rn activity monitoring is essential to implement management controls for the reduction of Rn exposure to cave workers. Modifying passive cave ventilation to reduce Rn may also affect the size distribution of aerosol particulate (Fig. 1) and equilibrium factor with Rn progeny (Zahorowski et al., 1998), which materially affects received dose of Rn. If the estimated received dose from Rn in a cave warrants further consideration (may approach dose limits) monitoring cave Rn progeny will provide a more accurate assessment of cave worker received dose, rather than rely on an assumption of a default Rn-Rn progeny equilibrium factor. This is an important subtle distinction that may change the cave worker

received dose estimate by 50%. Direct measurement of Rn progeny is more accurate than estimation of dose from Rn alone because approximately 90% of the received dose is from Rn progeny retention in the lungs.

Modifying cave ventilation patterns to reduce Rn may also impact cave humidity producing either condensation or drying effects. Monitoring relative humidity in caves is necessary to manage and to avoid potential deleterious effects on speleothems.

SUMMARY AND CONCLUSION

At Jenolan Caves, detailed cave monitoring has measured many parameters including continuous Rn concentration patterns in Chifley Cave to identify the source of the Rn as the soil above the cave. Bi-directional convective ventilation draws Rn and CO₂ into Chifley Cave when external temperature is greater than cave temperature, typically during summer. Air flow in the opposite direction typically during winter dilutes cave Rn and CO₂ with ambient air. This common seasonal convective ventilation pattern explains the major variation of Rn in caves with further cave Rn variation due to a cascade of dependencies from external weather (temperature) driving diurnal variation, site specific soil cover above the cave (Rn source concentration), and climatic zone driving convective air flow through major passages of complex 3D voids with multiple possible connections to isolated passages. Within a cave, Rn concentration may vary over a few tens of meters due to exposure to different internal micro-ventilation patterns that also extends to time of day and season.

Small inter-annual average surface temperature variability (~ 0.5 °C) amplifies the net convective air-mass exchange to transport $\sim 60\%$ more CO₂ and implicitly Rn into Chifley Cave. Decadal average surface temperature increase due to climate change may also significantly increase CO₂ and Rn in caves by diminishing the annual average difference between relatively warm cave air.

The received radiation dose to cave workers exposed to high Rn in caves has changed significantly since initial surveys of cave Rn in the 1990's, principally because of changes to appreciated health risk by the ICRP and recommended dose conversion factors. As a result, received dose has increased by a factor of 4–6 for a similar Rn concentration. Most caves have an annual average Rn activity above the ICRP recommended 1,000 Bq m⁻³ reference or action level.

Cave managers face difficult decisions on what actions may mitigate Rn exposure without detriment to workers or cave formations with incomplete monitoring or dosimetry data. Rescheduling of worker hours underground and limiting tours or time in caves with high Rn concentration may achieve regulatory compliance but may not achieve significant reduction of total dose received collectively by all cave workers. Changing natural air flow patterns by closing air-tight doors when external temperature is greater than cave temperature is likely to reduce Rn being drawn into caves. The converse action of opening air-tight doors when external temperature is cooler than cave temperature is likely to increase the natural convective flushing of Rn from caves. Changing air flow in caves also carries a perceived risk of potential impact on speleothems through drying or condensation corrosion. Lowering cave Rn while maintaining cave humidity through passive intervention of natural ventilation patterns, such as opening airtight doors when colder outside the cave and closing when hotter, is a promising mitigation measure that may lower Rn for caves with chimney effect ventilation.

Most show caves will require monitoring to accurately measure the highly dynamic Rn and Rn decay product variability in the workplace and demonstrate minimal health risk to cave workers. If cave Rn monitoring shows an estimated potential dose to cave workers in excess of 10 mSv y⁻¹ for radiation exposed workers, mitigation of Rn in show caves needs to be instigated.

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DATING AND INTERPRETATION OF RECENT CLASTIC SEDIMENTS IN AN URBAN CAVE

Evan A. Hart¹

Abstract

Capshaw Cave functions as a major stormwater runoff channel for the city of Cookeville, Tennessee, receiving inputs from several large sinkholes. Sediments deposited in the cave reflect the history of erosion and runoff from the city as it grew over the last century. At various locations in the cave, ~1 m thick sequences of flood-deposited, laminated fine sediments were observed along the modern stream. Alternating laminations observed in the upper ~40 cm of the sediment profile varied between 0.5 cm thick (10Y 7/6 yellow, fine sand) and 2.0 cm thick (10Y 3/2 very dark grayish brown silty sand) layers. Based on measurements of ¹³⁷Cs activity, the upper 35 cm of sediment was deposited between 1963 (the peak year of ¹³⁷Cs fallout from nuclear testing) and 2013 (the year samples were collected), at an average rate of 0.7 cm y⁻¹. A total of 23 alternating pairs of layers indicate an average flood recurrence interval of ~2.2 years between 1963 and 2013. Total Pb concentrations measured in cave sediments showed a peak at the 45 cm depth, suggesting that sediments above this level were deposited after the decline in Pb emissions in the 1970s, and showing general agreement with the timing of deposition suggested by ¹³⁷Cs. Below 40 cm, the dark silty sand layers were fewer in number and increased in thickness (up to 10 cm), possibly due to changes in cave hydrology or sediment erosion from the surrounding watershed. These findings suggest that, before the 1960s, sedimentation rates were higher and floods were less frequent. After the 1960s, sedimentation rates decreased and floods became more common, probably as a result of urbanization in the watershed.

INTRODUCTION

Cave deposits are important paleo-environmental records and include 1) chemical deposits (speleothems); 2) clastic sediments (fine-grained infill); 3) breakdown (Bosch and White, 2004); and 4) biologic accumulations (e.g., bat guano) (Onac et al., 2015). The potential of speleothems as paleo-environmental records is well-established (Spötl and Mangini 2002; Drysdale et al., 2006; Meyer et al., 2008; Asmerom et al., 2010; Boch et al., 2011; Badertscher et al., 2014), also see excellent reviews in McDermott (2004), Sasowsky and Mylroie (2007), and White (2007). However, clastic cave sediments have received less attention in the scientific literature and their potential as paleo-environmental proxies remains under-utilized. Clastic cave sediments may be derived from external (allogenic) sources or may originate from within the cave itself (autogenic). The protected environment inside caves may preserve intact sequences of clastic sediment, which are not typically well-preserved outside caves, for example on floodplains. Some caves contain clastic sediment sequences deposited by main valley rivers that overflow into them or simply by cave streams that bring sediment through the karst system. Clastic cave sediments have been dated using cosmogenic isotopes and paleomagnetism to estimate long-term river incision rates (Sasowsky et al., 1995, Granger et al., 2001, Stock et al., 2004). Estimating river incision rates is usually done by dating sediments found in caves along valley walls some height above the modern stream level, and then comparing the age of these sediments to younger sediments preserved in caves at lower elevations.

Studies of more recent clastic sediments have sought to better understand sediment transport in caves and to identify allogenic sediment pollution sources (Mahler et. al 1998; Mahler and Lynch, 1999; Modrá et al., 2017). Dogwiler and Wicks (2004) estimated that up to 85 % of stream substrates in some Missouri and Kentucky caves can be transported during flows having relatively short return periods (<1 year). Other work has focused on the lithology, grain size, and roundness of clastic sediments in caves and related these to sediment provenance (Bull, 1978, 1981; Brinkman and Reeder, 1995). Gillieson (1986) showed that clastic sediments formed diamictos in some New Guinea caves, and that these sediments originated as debris flows from allogenic source areas.

Paleoclimate and paleoenvironmental studies have successfully applied several dating methods to cave sediments including U-Th dating of speleothems (Thompson et al., 1976), paleomagnetism of clastic sediments (Sasowsky et al., 1995), magnetic susceptibility (Šroubek et al., 2001; Šroubek et al., 2007), cosmogenic isotope analysis of cave gravels (Stock, et al., 2004), OSL (optically stimulated luminescence) (Clark-Balzan, et al., 2012), ¹⁴C (Surić, et al., 2016), as well as pollen and ¹³C analysis of organic material extracted from bat guano (Onac et al., 2015). For younger cave sediments deposited over the last few centuries, a suite of other dating methods were tested. Feist (2017) used the isotope ²¹⁰Pb to date recent (~100 years) sediments contaminated with heavy metals in Hidden River Cave, Kentucky. Very recent sedimentation (two to three months) in caves has been traced using ⁷Be (Broderick et al., 2017).

¹ Department of Earth Sciences, Tennessee Tech University, Cookeville, TN 38505; ehart@tntech.edu

The fallout nuclide ^{137}Cs , generated by atomic testing in the mid-1900s, has been used to date cave sediments deposited since that period (Stanton et al., 1992; Klimchouk and Gudzenko, 1996; Curry, 2003). Cesium-137 is a radioactive isotope and fission byproduct, having a half-life of 30.17 years (Poreba, 2006). Atomic testing beginning in the 1950s released radioactive ^{137}Cs into the atmosphere around the globe (Walling and He, 1999). The resulting fallout of ^{137}Cs was incorporated into surface soils and water bodies throughout most of the world, reaching a peak in 1963 (Zhang, et al., 2016). Cesium-137 has been used widely to date lake sediments by assuming that the peak ^{137}Cs activity in a lake sediment core is equivalent to the year 1963, the peak year of atomic testing.

In the United States, Pb emissions, mostly from leaded gasoline vehicles, reached a peak in the 1970s and declined after being phased out of use (USEPA, 2000). The peak and decline of Pb emissions can be seen in Pb concentrations measured in lake sediments (Callender and Van Metre, 1997; Juracek and Ziegler, 2006). In this study, ^{137}Cs and total Pb are used to investigate the timing of flood-deposited sediments in an urban cave system. Whatever method is used to date cave sediments, care should be taken to assure that site-specific conditions are consistent with the assumptions of the technique. For example, Schiegl et al. (1996) found that waterlogging may lead to significant chemical alteration of cave sediments, making dating and interpretation problematic.

STUDY AREA

The study area is located on the East Highland Rim, a low plateau (280–340 m.a.s.l.) located west of the Cumberland Plateau escarpment in central Tennessee (Fig. 1, inset), and underlain by carbonate and siliclastic rocks of Mississippian age. That portion of the Highland Rim underlain by the St. Louis limestone typically forms a well-developed

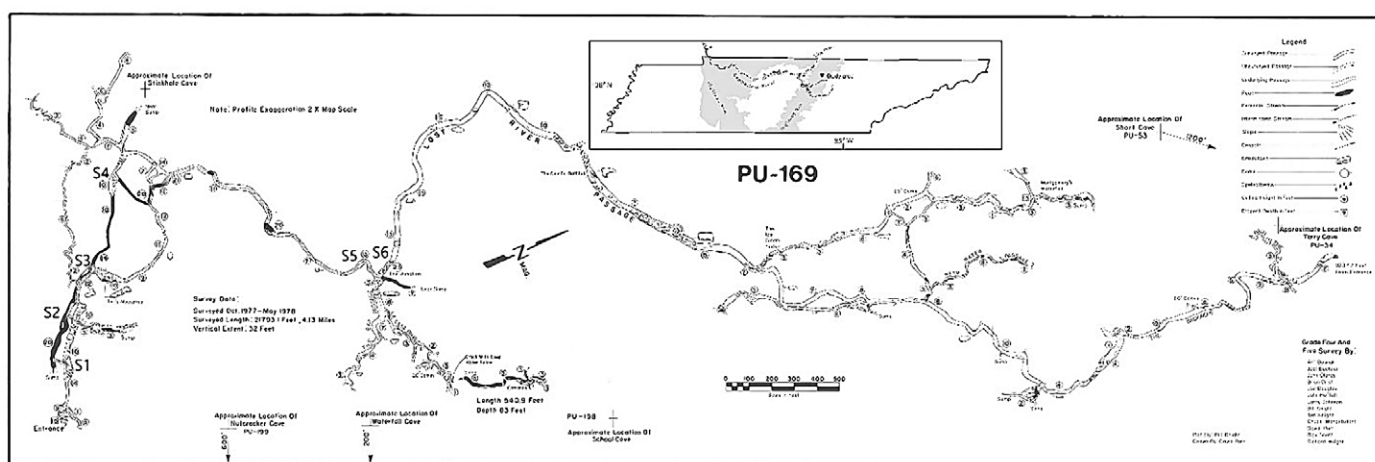


Figure 1. Map of Capshaw Cave showing sampling location S-4. Other sites with similar deposits are shown but were not sampled. The East Highland Rim, Tennessee (inset).

sinkhole plain, mantled with residual, clay-rich soils, and crossed by only a few surface streams. However, areas underlain by the siliclastic-carbonate Warsaw unit support a fluviokarst drainage network, with numerous sinking streams and resurgences. This study focuses on the stream in Capshaw Cave within the city limits of Cookeville, Tennessee (Fig. 1). The cave is formed in the middle portion of the Warsaw unit, has a 6.6 km total surveyed length, and consists of a borehole-type passage with diameters ranging from 4 m to 7 m. Capshaw Cave ends in the downstream direction at a sump and apparent cave constriction. Dye tracing at the sump in Capshaw Cave showed a direct connection to a resurgent spring about 0.5 km downstream. The sump and the resurgence were also recently connected by a successful cave dive, which revealed that the cave constricts to a height of approximately 1.5 m at a point just downstream from the sump.

The watershed contributing to Capshaw Cave covers an area of approximately 8 km² and was originally covered with hardwood forest before European settlement. After 1800, cropland and pasture began to replace forest cover. The population of Cookeville grew from 1848 in 1910 to 23,923 by 2000, which growth changed the watershed by increasing peak runoff from roads and parking lots, as well as introducing contaminants from roads and industry. A coal-burning powerplant was built in the watershed in 1929 and remained in operation until 2017. The Capshaw Cave watershed consists of approximately 39 percent impervious surfaces and thus has very flashy lag times, generally less than one hour, resulting in cave-filling floods (Hart, 2006). The cave is directly connected to the overlying watershed by a series of large swallets, some with conduit openings through which large amounts of sediment move into the cave during floods (Fig. 2) (Hart and Schurger, 2005). Capshaw Cave serves as the de-facto stormwater drain for the city of Cookeville, thus trash and debris from the city is commonly found in the cave.

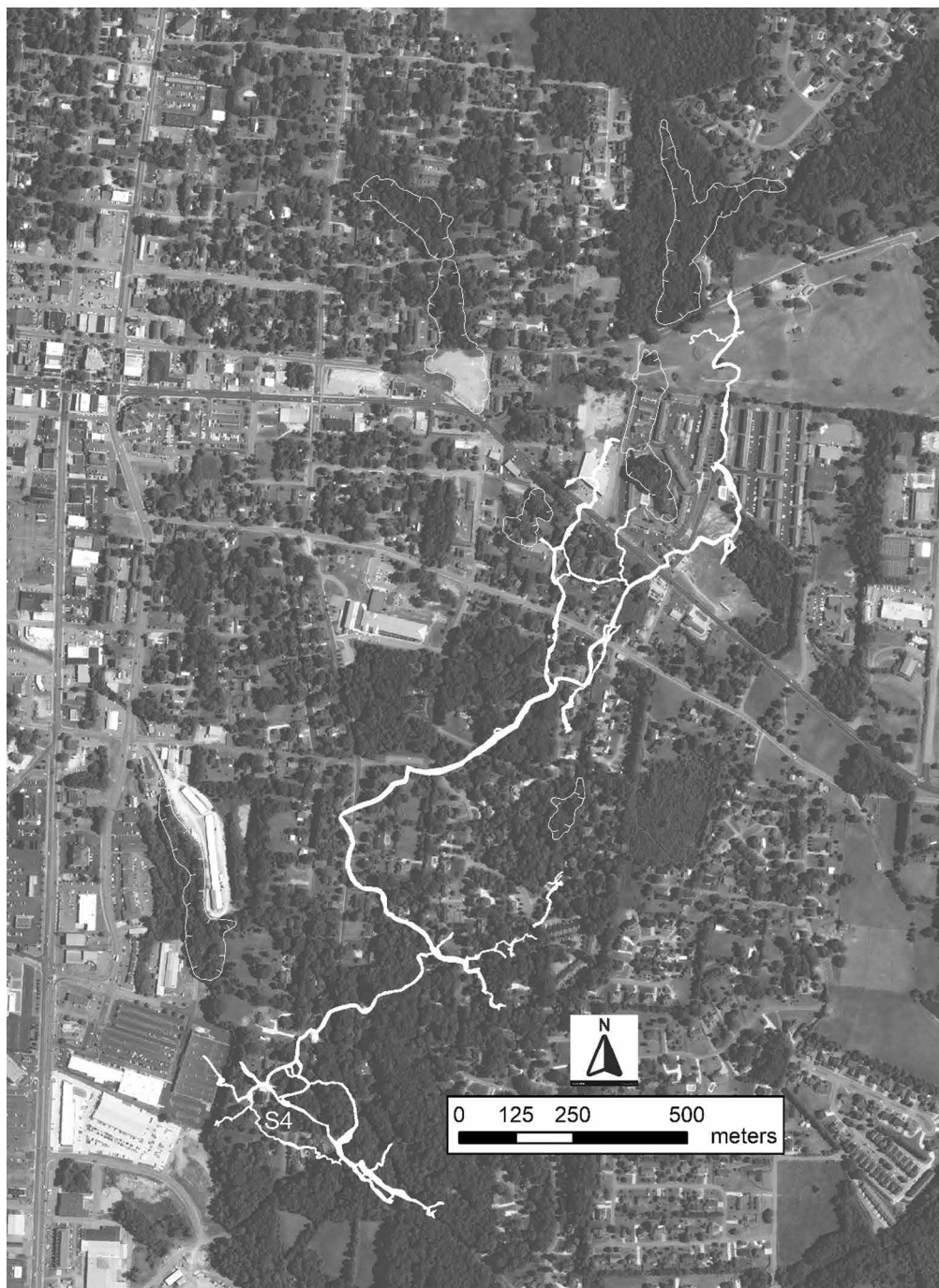


Figure 2. Aerial photograph of Cookeville, TN in 2017 showing location of Capshaw Cave and major swallets that drain into the cave. Cave stream runs from north to south, with sump at south end. Sampling location S-4 shown.

laminations were too thin to be collected separately without mixing. It was possible to sample individually three of the thicker layers making a total of six (three light layers and three dark layers) at depths of 15 cm, 38 cm, and 86 cm. Mixed samples of both light and dark layers were also collected. For all samples, particle size distribution was determined by hydrometer analysis (Ashworth et al., 2001).

To establish the timing of the cave sediment deposition, additional box samples were collected directly from the sediment profile in 10 cm sections for chemical analysis. These samples were shipped to Flett Research Ltd, Laboratory, Winnipeg, Canada where ^{137}Cs activity was measured at each section mid-point (5 cm, 15 cm, etc.) by gamma spectrometry using high purity germanium detectors. Additional samples from site S-4 were also collected at 10 cm intervals (midpoints at 5 cm, 15 cm, etc.) and tested for total elemental Pb concentration at the Tennessee Tech Univer-

sities. Piles of fine sediment approximately 1 m above the modern stream are found in at least six locations in Capshaw Cave, mostly in protected bedrock alcoves (Fig. 3), the locations of which are indicated in Figure 1. Each time after visiting Capshaw Cave between flooding events, it was noticed that a layer of light-colored sand was deposited on top of a darker, more fine-grained layer at all sites shown on Figure 1. This alternation between dark- and light-colored layers appeared to continue down into the profile. Our aim was to sample sediments at one of these sites to better understand the timing of deposition as it relates to flooding in the cave. One of these six locations (site S-4, shown in Fig. 1 and Fig. 2), with especially good preservation of laminations, and a deep profile (140 cm), was chosen for detailed study.

METHODS

Samples were collected from site S-4 by excavating a fresh surface vertically through the deposit to a depth of approximately 1.4 m, at which point channel gravel was encountered. Plastic sample boxes were inserted horizontally into the sediment profile and removed carefully to preserve the laminations. In the lab, we attempted to separate the light and dark laminations to test these independently for particle size distribution, however, most of the

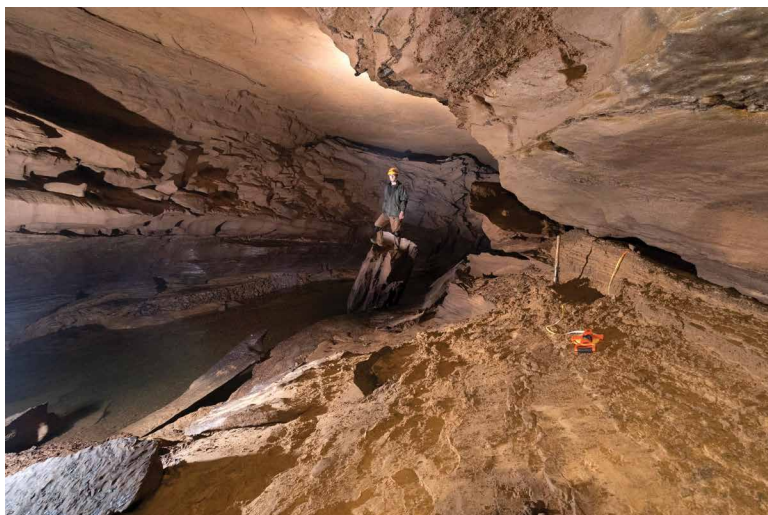


Figure 3. View of Capshaw Cave main passage showing cave stream and location of flood-deposited sediments to the right of stream channel. Photo taken near site S-6 in Figure 1.

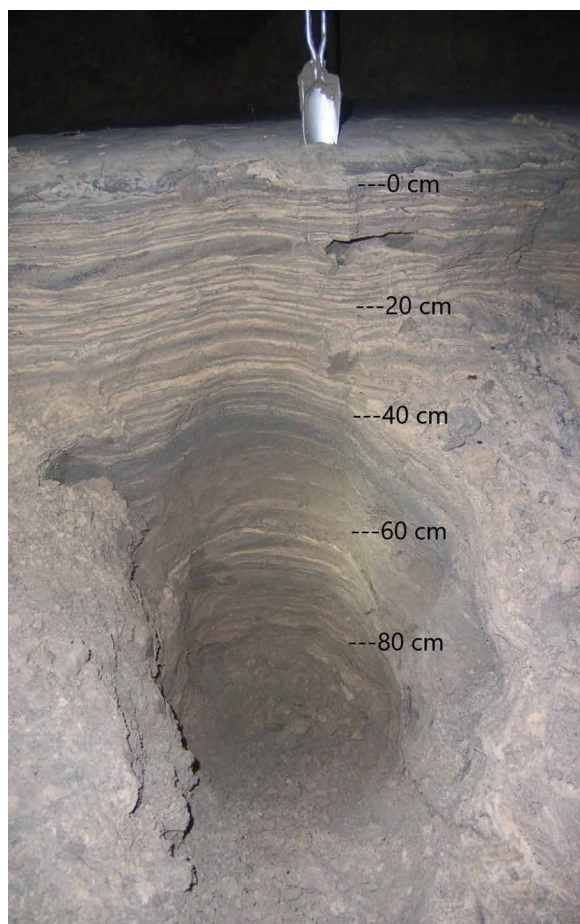


Figure 4. Sediment exposure at site S-4 in Capshaw Cave, showing alternating layers of fine sand (yellow) and silty sand (very dark grayish brown). Fine sand layers have average thickness of 0.5 cm in the upper 40 cm of the profile. Below 40 cm, silty sand layers increase to 5 to 10 cm in thickness and are separated by only a few fine sand stringers. Only ~80 cm of the entire 140 cm of sediment at this site is shown here. See Figure 5 for entire stratigraphic column of site S-4.

sity Water Center using inductively coupled plasma atomic emission spectroscopy (USEPA, 1994). Total Pb concentration in lake sediments has been shown to track closely with the rise and fall of leaded gasoline use in the United States (Juracek and Ziegler, 2006). To shed light on the timing of cave sediment deposition, Pb concentrations measured in cave sediments in this study were compared to Pb emissions data from the 1900s, for which the timing is well-established. Recent sedimentation in the cave was measured by driving rebar pins vertically downward into the sediment and left for a period of two years. After flood events, the rebar stakes were checked to estimate the amount of deposition or re-working.

RESULTS AND DISCUSSION

Character of the Sediment

The alternating light and dark colored laminations at site S-4 have Munsell colors of 10Y 7/6 (yellow) and 10 Y 3/3 (very dark grayish-brown), respectively, and these colors are consistent throughout the deposit (Fig. 4). There is evidence of cross bedding and termination of layers, suggesting a complex depositional history and at least some degree of re-working of sediments. The rebar stake measurement at site S-4 indicated that approximately 0.6 cm of vertical accretion occurred between 2013 and 2015 (0.3 cm y^{-1}), but this was likely too short of a period to capture long-term trends.

A total of 23 alternating laminations in the upper 40 cm of the profile vary in thickness from 0.5 cm to 2.0 cm and alternate in dominant particle size between fine sand (yellow) and silty sand (very dark grayish-brown). The median particle size diameter for the fine sand samples is 0.2 mm and 0.08 mm for the silty sand (Fig. 5). Between 40 cm and 80 cm, silty sand layers increase in thick-

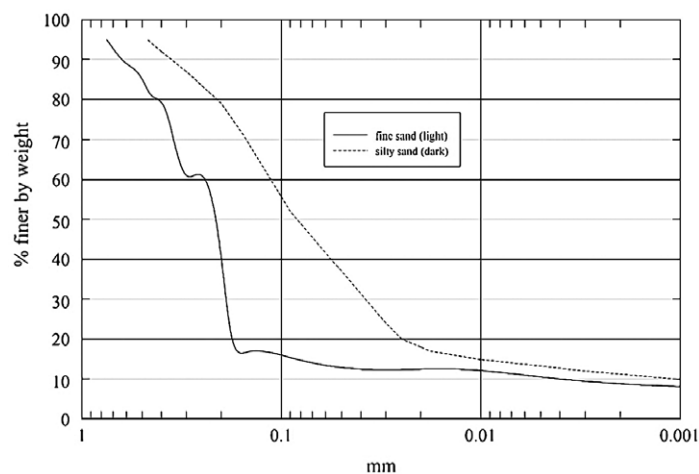


Figure 5. Plot of grain size frequency distribution for fine sand (yellow) and silty sand (very dark grayish brown) for cave sediment samples at site S-4. Three separate samples were collected from fine sand and silty sand layers at depths of 15 cm, 38 cm, and 86 cm. Data plotted are averages of these three samples taken from each dark and light layer that was sampled. The median particle size diameter varied from 0.2 mm for fine sand to 0.08 mm for silty sand. The theoretical settling velocities for fine sand (2.0 cm s^{-1}) are 40 times greater than for silty sand (0.05 cm s^{-1}).

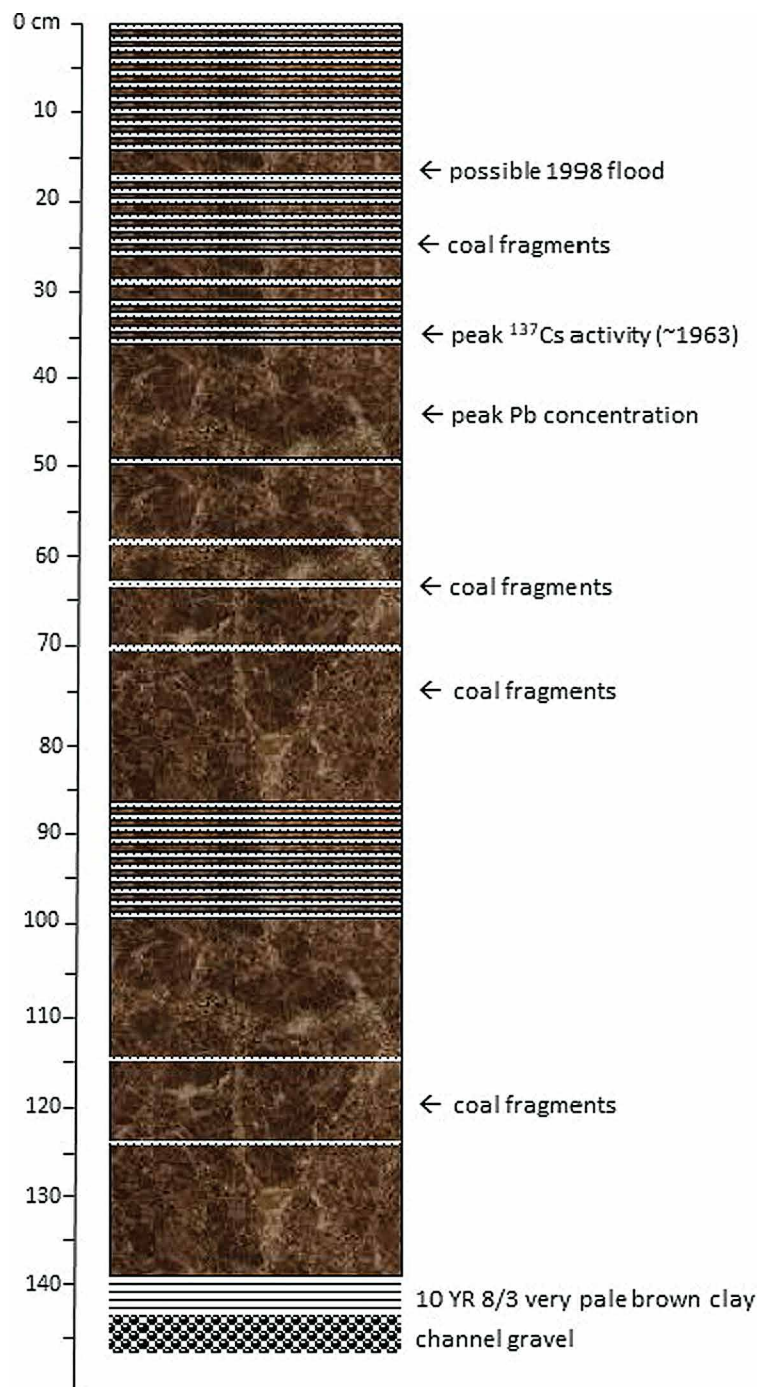


Figure 6. Generalized stratigraphic column for site S-4. Dark pattern shows 10Y 3/3 (very dark grayish brown) silty sand and light stippled pattern shows layers of 10Y 7/6 (yellow) fine sand. Cross bedding and truncation of some layers does occur. Sediments above 35 cm were deposited after the 1963 ^{137}Cs peak fallout. Coal fragments found throughout the deposit come from anthropogenic sources that became abundant after 1900. We interpret the 0 cm to 40 cm section to have more frequent flood events (note the many fine sand layers) and a lower sedimentation rate, both of which may result from urbanization. A 2 cm thick silty sand layer at 15 cm depth may represent an extreme flood event in 1998 that is known from the local area. From 40 cm to 140 cm, floods appear less common and fine sand layers are missing. This pre-urban period likely had fewer cave-filling floods, but a greater rate of sedimentation, perhaps due to erosion from gullied farmland. Photo in Figure 4 shows the same site, but only down to ~80 cm.

ness (5 cm to 10 cm) and are separated by only four (<0.5 cm) fine sand stringers (Fig. 6). This change in layer thickness may reflect changes in cave hydrology or changes in sediment availability within the cave, possibly associated with land use changes in the watershed. Between depths of 85 cm and 100 cm, another set of approximately 10 fine sand stringers occur in close succession. From 100 cm to 140 cm, the pattern again changes and the dark silty-sand layers increase in thickness and are separated by only four thin, fine sand layers. Finally, at a depth of 140 cm a firm 2.5 Y 8/3 (pale yellow) clay overlies channel gravels.

SEDIMENTATION RATES AND FLOOD FREQUENCY

Cesium-137 activity in the Capshaw Cave sediments shows a defined spike at approximately 35 cm depth and a steep decline below that point (Fig. 7A and Table 1). This pattern is consistent with ^{137}Cs activity measured in lake sediments across North America (Callender and Van Metre, 1997), and in other areas, and matches the peak atomic testing year of 1963. If this is the case, then the upper 35 cm of sediments were deposited after 1963, at a minimum rate of 0.7 cm y^{-1} . Background ^{137}Cs activity was reached at the 45 cm measurement, and so no further ^{137}Cs measurements were made on sediments below that level.

Total Pb concentrations from the Capshaw Cave sediments show an obvious spike at 0.15 mg kg^{-1} between 40 cm and 50 cm (Fig. 7B). All values for Pb concentrations reported here are below USEPA (1997) threshold level for possible adverse biological effects (30.2 mg kg^{-1}). The entire total Pb curve matches well with the Pb emissions curve, which has a peak at around 1970 (USEPA, 2000) (Fig. 7C). This match between Pb concentration in the cave sediments and Pb emissions suggests that sediments can be dated based on the Pb emission curve. The timing of deposition suggested by Pb concentration tracks closely with the timing indicated by ^{137}Cs activity in the same sediments (Fig. 7A and 7B).

If each silty sand-fine sand sequence represents one flood event, then approximately 23 flood events are recorded in the upper 35 cm to 40 cm of sediments in Capshaw Cave. If the upper 40 cm was deposited between 1963 (the peak ^{137}Cs fallout) and 2013 (the sampling year), this would compute to a flood return interval of 2.2 years for cave-filling flood events. Between 40 cm and 85 cm, only 4 fine sand stringers are found, suggesting that cave floods may have been less common before the 1960s;

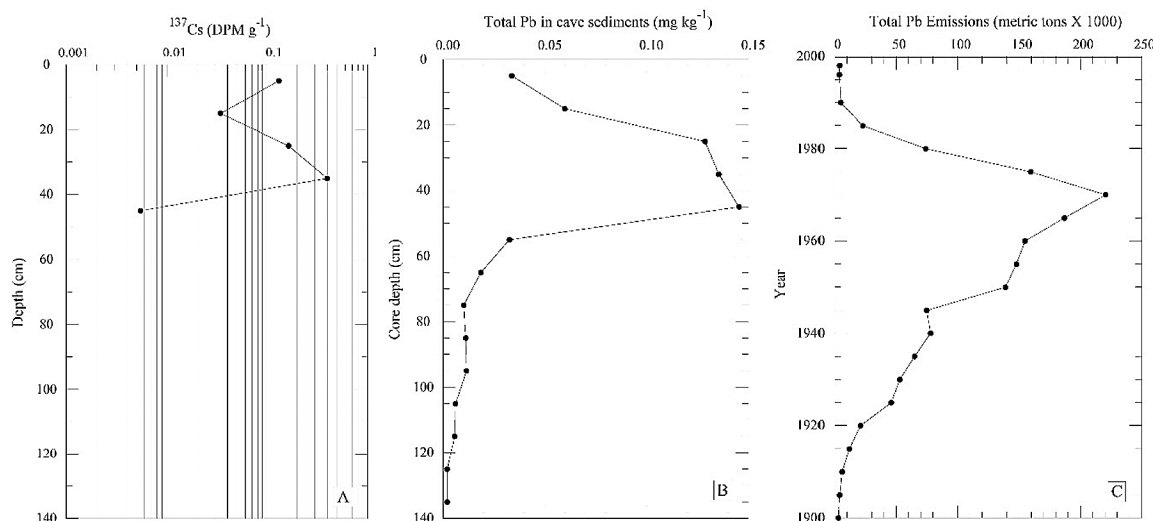


Figure 7. (A) Activity level of the fallout radionuclide ^{137}Cs in the Capshaw Cave sediment profile S-4. Measurements were made every 10 cm beginning at 5 cm. Cesium-137 units are disintegrations per minute per gram (DPM g^{-1}). The peak of atomic testing that released ^{137}Cs into the atmosphere occurred in 1963 and is shown to occur at approximately 35 cm in this profile. Background ^{137}Cs activity was reached at a depth of 45 cm, and thus no further samples were tested below that depth. (B) Total Pb concentration measured in sediments at site S-4, at 10 cm intervals beginning with 5 cm depth. (C) Total Pb emissions from all sources 1900 to 2000. Data after 1970 are from USEPA (2000) and data before 1970 are from Callender and Van Metre (1997).

however, between 86 cm and 100 cm depth 10 fine sand layers are observed, indicating a short interval of more frequent floods (Fig. 6). There is no control on dating of sediments at this depth, so an approximate flood return interval cannot be calculated for these layers.

Sand-sized coal fragments are found throughout the Capshaw sediment profile S-4. Under mag-

Table 1. Data from ^{137}Cs measurements, Flett Research Ltd, Laboratory, Winnipeg, Canada where ^{137}Cs activity was measured at each section mid-point (5 cm, 15 cm, etc.) by gamma spectrometry using high purity germanium detectors. DPM is disintegrations per minute. ^{137}Cs activity is plotted on Figure 7A.

Sample depth (cm)	Dry sample weight (g)	Sample thickness (mm)	Count time (sec)	Gammas ($\text{min}^{-1} \text{g}^{-1}$)	^{137}Cs Activity (DPM g^{-1} dry weight)	Error DPM (g^{-1})
5	7.837	2.475	240000	0.112571	0.132436	0.043704
15	12.154	2.8	240000	0.028879	0.033975	0.064636
25	5.996	1.575	240000	0.139525	0.164146	0.062376
35	6.949	1.9	240000	0.338068	0.397726	0.091082
45	9.065	2.425	240000	0.004679	0.005505	0.085876

inification, coal fragments can be easily distinguished from quartz grains that make up most of the cave sediments. Coal does not occur naturally in this watershed and its likely source is a coal-burning power plant that was in operation between 1929 and 2016. When in operation, a large pile of coal sat in the open on the plant parking lot, located 1.5 km upstream from a swallet that feeds the cave. A small channelized stream runs behind the plant and leads directly to the swallet. Another source for coal could have heating of private homes, however, this source may not have produced a large enough volume of coal that would later be preserved in cave sediments. In either case, the presence of coal in the watershed does not extend back any earlier than approximately 1900, and so the presence of coal in cave sediments shows that deposition occurred sometime after 1900.

To summarize, ^{137}Cs activity indicates that the upper 35 cm of sediment was deposited after the decade of the 1960s, at an approximate rate of 0.7 cm y^{-1} . These sediments were likely deposited during floods with recurrence interval of approximately two years, assuming each flood layer represents one flood event. It is possible that sediments receiving ^{137}Cs fallout outside the cave, were then temporarily stored (in sinkholes, for example) before arriving in the cave at their present locations. However, the year 1963 marks the earliest possible period of deposition for sediments at the 35 cm depth. The total Pb concentration measured in cave sediments at site S-4 provides good agreement that the upper 35 cm to 40 cm was deposited since the 1960s or 1970s. Finally, based on the presence of coal fragments, the entire sequence of sediments at site S-4 was likely deposited after 1900. If this timing is true, then deposition of sediment between 140 cm and 40 cm occurred at a rate no slower than 1.6 cm y^{-1} , which is more than twice the rate from 1963 to 2013 according to ^{137}Cs activity (0.7 cm y^{-1}).

INTERPRETATION OF FLOOD EVENTS FROM SEDIMENT LAYERS

The median particle diameters for the fine sand (0.2 mm) and silty sand (0.08 mm) layers (Fig. 5) correspond to an approximate settling velocity of 2.0 cm s^{-1} for the fine sand and 0.05 cm s^{-1} for the silty sand, a 40-fold difference. These velocities indicate that fine sand and silty sand layers are deposited at different flood stages. The exact sequence of deposition is difficult to interpret without acquiring additional data, for example, direct measurement of water velocity during floods and cave passage geometry would be helpful. While a fining-upward sequence is thought to be typical in many flood deposits, observations in Capshaw Cave suggest another scenario. Evidence comes from the simple observation that fine sand layers are always found on top of the sediment banks in Capshaw Cave after a flood, indicating that it must be the last material deposited in a flood (for example, see the fine sand on the surface of Site-4 in Fig. 4). Based on these results, there is possibly more than one explanation for the nature of laminated sediments in Capshaw Cave.

As water rises in the cave from runoff, velocities increase quickly, and some re-working of sediment may occur. This re-working may be the cause of the cross bedding seen in some layers in Fig. 4. As the flood progresses, the rate of flow into the cave exceeds the discharge leaving the cave, due to the narrow downstream constriction below the sump. The constriction creates a damming effect and water is temporarily ponded in the cave, causing it to leave behind flotsam plastered on the cave ceiling that is regularly observed after floods. During these times of ponding and lower velocity, the finer silty sand is likely deposited. As the cave drains, velocities increase again and the fine sand is left behind, often as very thin stringers that may only partially cover the dark silty sand.

The above interpretation is speculative, but perhaps represents the best understanding of these particular cave sediments, and only applies to floods that fill the entire cave. Smaller floods that never reach pipe-full conditions would not necessarily have the same sequence of deposition. These smaller floods may also re-work some of the lower sediments, distorting the overall interpretation. Thus, due to the fact that the cave fills to different heights depending on flood volume, the assumption that each sequence of fine sand/silty sand layers represents one flood event may not be valid. It is possible that some layers are the result of multiple events. The fact that the dark, silty sand units are thicker below 40 cm may be the result of different cave hydraulics that existed previously, for example, a tighter constriction below the sump could cause ponding in the cave to last longer. However, it seems unlikely that the 5 cm to 10 cm thick silty sand layers like those seen below 40 cm in Figure 6 each represent one flood event. It may be that several flood events are represented in one of these thick silty sand layers, and the fine sand is missing either because it was reworked or was not available to be deposited at that time.

CONCLUSIONS

Laminated sediments found in Capshaw Cave have two distinct mean particle size populations that indicate deposition at different velocities. Truncated beds and cross bedding indicate that re-working of sediments does occur in the cave, however, the amount of re-working must be minor since the regular pattern of laminations is preserved (Fig. 4). Cesium-137 activity measured in the sediments shows a general trend of gradual vertical accretion at a rate of approximately 0.7 cm y^{-1} from 1963 to 2013. The concentration of total Pb in the cave sediments tracks with the rise and fall of Pb emissions in the United States and corresponds generally to the timing of deposition indicated by ^{137}Cs activity. If each of the 23 fine sand/silty sand sequences between 0 cm and 40 cm represents one flood event, this equates to an average flood return interval of ~ 2.2 years. In the section from 40 cm to 80 cm, only 4 fine sand layers are found between 5 cm to 10 cm beds of silty sand. This finding suggests that flooding was less frequent before the 1960s and has increased with the growth of urbanization. Changes in cave hydrology that affect the drainage rate of the cave could also have changed over this period. In addition, re-working of sediment and sediment availability may challenge the assumption that each layer was deposited by a separate flood event. Anthropogenic coal fragments found throughout the entire depth of the deposit at site S-4 show that these sediments were deposited after coal burning became common in the watershed, about 1900 based on historical records. The rate of sedimentation in Capshaw Cave appears to have declined after the 1960s, at the same time that cave floods became more common (Fig. 6). This could be the result of urban growth after the 1960s, which led to more runoff and flooding and less sediment erosion, as agricultural fields were replaced by pavement. Pre-1960s land use was primarily agriculture and early air photos (1939) show a landscape scarred by gullies, capable of producing large volumes of sediment. More detailed sedimentological analysis of sediments and more precise dating methods (e.g., ^7Be) could aid in better understanding the nature of recent cave sediment deposition.

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DIVERSITY OF SIDEROPHORE-PRODUCING BACTERIAL CULTURES FROM CARLSBAD CAVERNS NATIONAL PARK CAVES, CARLSBAD, NEW MEXICO

Tammi R. Duncan^{1,C}, Margaret Werner-Washburne², and Diana E. Northup²

Abstract

Siderophores are microbially-produced ferric iron chelators. They are essential for microbial survival, but their presence and function for cave microorganisms have not been extensively studied. Siderophores are classified based on the common functional groups (catechols, hydroxamates, carboxylates, and mixed) that coordinate to ferric (Fe^{3+}) iron. Cave environments are nutrient-limited and previous evidence suggests siderophore usage in carbonate caves. We hypothesize that siderophores are likely used as a mechanism in caves to obtain critical ferric iron. Cave bacteria were collected from long-term parent cultures (LT PC) or short-term parent cultures (ST PC) inoculated with ferromanganese deposits (FMD) and carbonate secondary minerals from Lechuguilla and Spider caves in Carlsbad Caverns National Park, NM. LT PC were incubated for 10–11 years to identify potential chemolithoheterotrophic cultures able to survive in nutrient-limited conditions. ST PC were incubated for 1–3 days to identify a broader diversity of cave isolates. A total of 170 LT and ST cultures, 18 pure and 152 mixed, were collected and used to classify siderophore production and type and to identify siderophore producers. Siderophore production was slow to develop (>10 days) in LT cultures with a greater number of weak siderophore producers in comparison to the ST cultures that produced siderophores in <10 days, with a majority of strong siderophore producers. Overall, 64% of the total cultures were siderophore producers, with the majority producing hydroxamate siderophores. Siderophore producers were classified into *Proteobacteria* (*Alpha*-, *Beta*-, or *Gamma*-), *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* phyla using 16S rRNA gene sequencing. Our study supports our hypothesis that cave bacteria have the capability to produce siderophores in the subsurface to obtain critical ferric iron.

INTRODUCTION

The semi-arid caves in the Guadalupe Mountains, considered to be extreme environments for life, are excellent examples of aphotic and oligotrophic (low nutrients) carbonate cave systems (Northup and Lavoie, 2001). Despite resource limitations due to oligotrophic conditions, biodiversity is rich in the bacterial and archaeal domains and the fungal kingdom in cave environments (Tomczyk-Żak and Zielenkiewicz, 2016). Along with adapting to oligotrophic conditions, microorganisms in carbonate cave systems need mechanisms to acquire essential soluble ferrous iron. Ferric (Fe^{3+}) iron is more available than ferrous (Fe^{2+}) iron in Carlsbad Caverns National Park (CCNP) caves and can be hundreds to thousands of times more available in enriched FMDs in comparison to underlying bedrock (Northup et al., 2003; Spilde et al., 2005). Microorganisms deal with the limited availability of soluble ferrous (Fe^{2+}) iron by producing siderophores that break down insoluble ferric oxide-containing compounds to acquire essential soluble ferrous iron.

Siderophores are a unique class of small molecules that chelate and provide access to insoluble ferric (Fe^{3+}) iron with high binding affinity from their environments (Dave et al., 2006; Chu et al., 2010; Hider and Kong, 2010; Ahmed and Holmström, 2014). Siderophores are classified based on the common functional groups (catechols, hydroxamates, carboxylates, and mixed) that coordinate to ferric (Fe^{3+}) iron (Sandy and Butler, 2009; Khan et al., 2018). Bacteria, archaea, and fungi produce and excrete siderophores in response to low intracellular iron levels (Dave et al., 2006; Chu et al., 2010; Ahmed and Holmström, 2014). Siderophores secreted into the environment can be used either by individual bacteria and fungi, or shared and competed for by multiple organisms in a community (i.e., microbial consortia) (Glick, 2003; Chu et al., 2010; Hider and Kong, 2010; Ahmed and Holmström, 2014). Because siderophores are valuable in acquiring and processing insoluble ferric (Fe^{3+}) iron, individual or mixed culture microorganisms are predicted to use siderophores in an oligotrophic environment.

Siderophore production by isolated bacteria has been shown in several low nutrient oligotrophic (<2 mg/L total organic carbon (Barton and Jurado, 2007)) environments, such as the open ocean (Mawji et al., 2008; Boiteau et al., 2016; Velasquez et al., 2016; Bundy et al., 2018), cloud water (Vinatier et al., 2016), oligotrophic lakes (Sorichetti et al., 2014), cold deserts (Yadav et al., 2015), and recently, in a few carbonate caves (Hershey et al., 2014; Venkadesaperumal et al., 2014; Qin et al., 2017). Siderophores were shown to be produced by *Pseudochrobactrum kiredjianiae* strain A4 that was isolated from Karaulnaya-2 Cave, Russia (Qin et al., 2017) and by seven isolates collected from Mud Volcano

¹Science & Wellness Division, University of New Mexico-Valencia, 280 La Entrada Road, Los Lunas, New Mexico, 87031

²Department of Biology, MSC03-2020, 1 University of New Mexico, Albuquerque, NM, 87131

^CCorresponding author: tammid31@unm.edu

and Lime Cave, Baratang Island, India (Venkadesaperumal et al., 2014). Siderophore production was also detected from Lechuguilla Cave bacterial isolates in New Mexico (Hershey et al., 2014). The relative lack of information about isolates or mixed culture microorganisms that produce and use siderophores in oligotrophic caves, therefore, provides a rich area for discovery.

Caves in semi-arid areas have no phototrophy and any products of phototrophy are limited to what can filter down from surface soils. For example, drip water (seepage water) from within Lechuguilla Cave has shown that a limited amount of organic carbon is entering through this infiltration (Levy, 2007). In addition, Lechuguilla and Spider Caves, two semi-arid oligotrophic caves in CCNP, NM, also provide a variety of excellent microbial habitats, including wet carbonate secondary mineral deposit surfaces (Hill and Forti, 1997) and ferromanganese deposits (FMDs) (Northup et al., 2000; Spilde et al., 2005). FMDs, classified by color, were documented to have high iron oxide levels in red orange/red brown (8.18–30.30 wt %), chocolate brown (17.07–23.79 wt %), and black (10.66–17.07 wt %) FMDs relative to bedrock (0.01–0.03 wt %) (Northup et al., 2003; Spilde et al., 2005). FMDs with documented high ferric iron levels relevant to bedrock could be good sources of ferric iron that would require the use of siderophores by microorganisms in these microenvironments.

We hypothesized that cave bacteria would possess the ability to produce siderophores to acquire ferric iron to process for critical ferrous iron needed for cellular processes. Supporting our hypothesis, we found that siderophores were produced by cultured bacteria from cave deposits in Lechuguilla and Spider Caves. To our knowledge, this siderophore study is also the first to identify cave mixed bacterial cultures that have the ability to produce siderophores.

MATERIALS AND METHODS

Cave Site Description and Sampling

We sampled from two caves, Spider Cave and Lechuguilla Cave, CCNP (Fig. 1A). Spider and Lechuguilla Caves have relatively stable air temperatures of 17.0–18.4 °C (Spider) and ~19–20 °C (Lechuguilla), and a relative humidity of 99–100 % once you reach the deep cave zone (Northup et al., 2003). Both caves are oligotrophic. Brusseau et al. (2019) characterizes the unsaturated subsurface vadose zone as having a very low organic carbon content, usually less than 0.1 %, or <2 mg/L total organic carbon (Barton and Jurado, 2007). Spider Cave has <0.104 % organic carbon in the deposits that have the most biodiversity (i.e., Ferromanganese deposits) and punk rock (Northup et al. 2003). Several pool water samples from Lechuguilla had dissolved organic carbon measurements of less than 1 mg/L, except for one that was 1.7 mg/L (Levy 2007).

Both caves are formed in the Permian Reef Complex, a moderately dissected, gently tilted plateau of reef to back-reef strata above the Guadalupe Escarpment (Hayes, 1964; Palmer and Palmer, 2009). Spider Cave is located within dolomite bedded back-reef strata, has 9.8 km of mapped passage, and a maximum depth below the surface of 42.4 m (Gulden, 2019). Lechuguilla Cave, in contrast, is located in both the calcite to dolomite massive reef and bedded back-reef strata, has 222.6 km of mapped passage, and a maximum depth below the surface of 489 m (Gulden, 2019).

Isolation of LT Parent Cultures

LT cultures were utilized as a major part of our siderophore research because long-term survival of microorganisms on low nutrients is an adaptation by microorganisms to oligotrophic environments, such as found in our caves. We hypothesized that the LT cultures would have selected for actively-growing cave bacteria capable of showing long-term

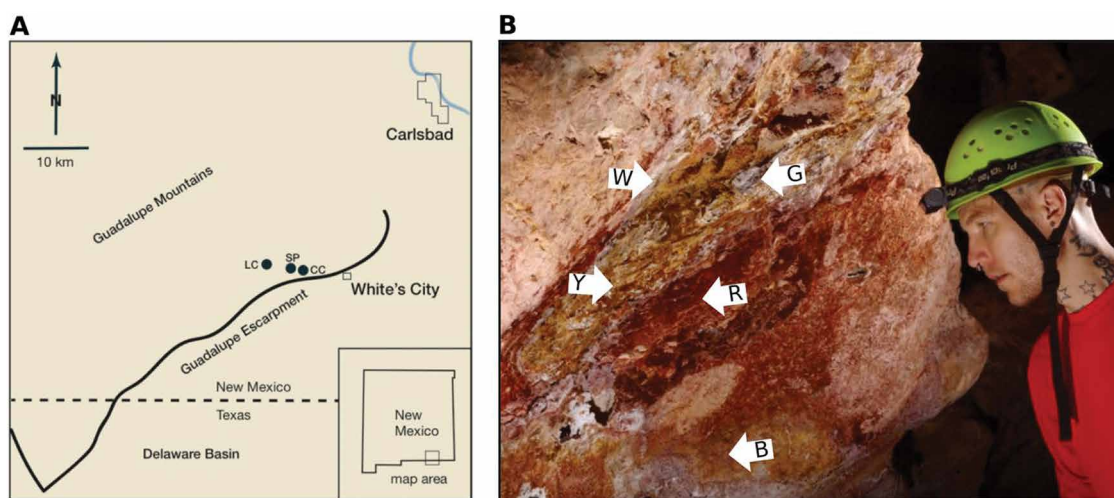


Figure 1. (A) General map area of the Carlsbad Caverns National Park (CCNP) near the New Mexico and Texas border (dashed lines). Approximate locations of Lechuguilla Cave (LC), Spider Cave (SC), and nearby Carlsbad Cavern (CC) along the Guadalupe Escarpment (solid line) south east of Carlsbad, NM. Map was released under the Attribution 4.0 International (CC-BY 4.0) license. Redrafted by A. Winter from the original in (Hayes, 1964). (B) Representative in-cave sample site of varying colors of ferromanganese deposits (FMD) and punk rock: W-white punk rock, brown FMD-B, grey FMD-G, red FMD-R, and yellow FMD-Y. Photo by Kenneth Ingham.

survival in media with inorganic and minimal organic energy sources. Therefore, LT cultures would likely be cultures of chemolithoheterotrophic bacteria able to utilize inorganic chemicals for their energy and rely on organic chemicals for their carbon sources and possibly energy from the cave walls and secondary deposits. LT parent cultures, LT PC-1 to LT PC-4, were inoculated with yellow FMD (Spider Cave), brown and orange FMD (Lechuguilla Cave), and punk rock (Lechuguilla Cave) (Table 1). Punk rock is a zone of soft altered bedrock (Hill, 1987). The representative colors of ferromanganese deposits are shown in Figure 1B.

Table 1. Long-term (LT) parent cultures, sample location and type, and total subcultured bacterial cultures (n=80).

Parent Culture No.	Parent Cultures	Parent Media	Cave	Parent Sample type	No. of cultures collected from subculture			
					R2A ^a	R2A ^b	AIA ^{a,b}	Total
LT PC-1	011505-14	Reduced Mn-supplement	Spider	Yellow FMD	21	14	5	40
LT PC-2	Fe+C'011101-72	Reduced Fe-supplement	Lechuguilla ^c	Brown and Orange FMD	5	8	0	13
LT PC-3	Mn+P ₀ 011101-63	Reduced Mn-supplement	Lechuguilla ^c	Punk Rock	6	6	2	14
LT PC-4	Mn-C ₀ 011101-48	Reduced Mn-supplement	Lechuguilla ^c	Brown and Orange FMD	4	6	3	13

Subculture media: ^a FeCl₃ added

^b MnCO₃ added

^c EA sample site in Lechuguilla Cave.

Parent sample types: Ferromanganese deposit (FMD).

Parent cultures descriptions: Fe+ has iron tack and cofactors, Mn+ has cofactors, Mn- contains no cofactors, P₀ is a 2×10^{-1} dilution of white punk rock with brown flecks sample, C' is a 2×10^{-2} dilution of FMD sample, and C₀ is 2×10^{-1} dilution of FMD.

To collect cave deposits for inoculation of potential chemolithoheterotrophic LT parent cultures, sterile loops were used to collect cave samples that were stabbed into parent screw-cap test tubes (16mm diameter, 100 mm length) filled with sterile basal medium and taken to the lab in the dark. Test tube agars all consisted of the following basal medium (per liter): 0.5 g NaCl, 0.5g CaCO₃, 0.5g MgSO₄·7H₂O, 0.75g K₂HPO₄, 0.25g NaH₂PO₄, 0.1g KNO₃, 4g Bacto agar (contains linear polysaccharide agarose and heterogenous agaropeptin), and sterile Milli-Q H₂O. This basal medium was enriched with reduced manganese (Mn) for LT PC-1, LT PC-3, and LT PC-4, and enriched with reduced iron (Fe) for LT PC-2 (Table 1) to represent cave conditions and as a possible energy source. For Mn supplements, 0.02 g/L of MnCl₂ was added and for iron supplements, one sterile reduced iron carpet tack was added to the bottom of the test tube.

To promote the selection of potential chemolithoheterotrophic cave bacterial cultures capable of LT survival in media with inorganic energy sources, LT parent cultures were incubated for years in the cave, lab, or both. Additional in-cave incubation parameters were tested using were lightly sealed test tubes placed in a container on the ground in an area remote from the travel trails in the dark zone. The Spider Cave LT PC-1 was incubated in the cave for six years and subsequently transferred to a lab incubator and incubated for five additional years (20 °C, dark, and no added humidity) (Table S1). Lechuguilla Cave LT PC-2 to LT PC-4 were incubated in the cave for one to three days, transferred to lab incubator (20 °C, dark, and no added humidity), and incubated for 10 years (Table S1).

Subculture of LT Bacterial Cultures

Eighty cultures collected from LT parent cultures are shown in Table 1. LT bacterial cultures were subcultured from LT PC-1 to LT PC-4 by collection at three depths from each LT parent culture using a sterile loop and aseptically streaked onto three isolation media that contained carbon: R2A+FeCl₃, R2A+MnCO₃, and AIA+FeCl₃+MnCO₃ agar plates targeting potential chemolithoheterotrophs and heterotrophs. The Reasoner's 2A agar medium (R2A, Difco, Sparks, Md.) and Actinomycetes Isolation Agar (AIA, Difco, Sparks, Md.) were made according to manufacturer's instructions. For added supplements, 0.01 FeCl₃ g/L and 0.1 MnCO₃ g/L were added to the media and autoclaved. The inoculated agar plates were incubated at 20 °C (no added humidity and in the dark) and individual bacterial colonies were collected upon first appearance four to 25 days (Table S1). The bacterial colonies were streaked once again for individual colonies and 20 % glycerol stocks were prepared with R2B Medium (R2B, Millipore Sigma, Burlington, Mass., same as R2A with no agar) for long-term preservation at -80 °C.

Isolation of ST Parent Cultures

Five different media types were utilized with ST cultures to cultivate a broader diversity of cave bacterial cultures with chemoheterotrophic metabolic capabilities to complement LT cultures. ST PC-5 to ST PC-44 were inoculated with FMD (gray, brown, red, yellow), carbonate speleothem, and wet carbonate cave deposits collected from Lechuguilla Cave (Table 2).

To collect cave deposits for inoculation of ST parent cultures, a sterile BD polyester fiber tipped application swab (Falcon) moistened with sterile Ringer's solution pH 7.3–7.4 (Inglis, 2008) was used to collect cave deposits followed by

Table 2. Short-term (ST) parent cultures from Lechuguilla Cave, sample location and type, and total subcultured bacterial cultures ($n = 90$).

Location	Parent Culture No.	Parent Cultures	Parent Sample Type	Parent Media	Total No. Cultures
EA Junction	ST PC-5	L120303-2 ^a	Grey FMD	½ R2A + rock flour	3
	ST PC-6	L120303-3 ^a	Grey FMD	BG11	3
	ST PC-7	L120303-5 ^a	Grey FMD	AIA	3
	ST PC-8	L120303-6 ^a	Brown FMD	½ R2A	3
	ST PC-9	L120303-10 ^a	Brown FMD	AIA	3
	ST PC-10	L120303-12 ^a	Carbonate speleothem	½ R2A + rock flour	3
	ST PC-11	L120303-14 ^a	Carbonate speleothem	AIA + nystatin	3
	ST PC-12	L120303-17 ^a	Yellow FMD	½ R2A + rock flour	3
	ST PC-13	L120303-18 ^a	Yellow FMD	BG11	3
	ST PC-14	L120303-20 ^a	Yellow FMD	AIA	3
Lake Chandelar	ST PC-15	L120303-21	Wet Carbonate	½ R2A	2
	ST PC-16	L120303-22	Wet Carbonate	½ R2A + rock flour	2
	ST PC-17	L120303-28	Wet Carbonate	BG11	2
	ST PC-18	L120303-30	Wet Carbonate	AIA	2
	ST PC-19	L120303-32	Wet Carbonate	½ R2A + rock flour	2
	ST PC-20	L120303-35	Wet Carbonate	AIA	2
	ST PC-21	L120303-36	Carbonate speleothem ^a	½ R2A	2
	ST PC-22	L120303-37	Carbonate speleothem ^a	½ R2A + rock flour	2
	ST PC-23	L120303-38	Carbonate speleothem ^a	BG11	2
	ST PC-24	L120303-40	Carbonate speleothem ^a	AIA	2
Tower Place	ST PC-25	L120304-41	Wet Carbonate	½ R2A	2
	ST PC-26	L120304-43	Wet Carbonate	BG11	2
	ST PC-27	L120304-47	Wet Carbonate	½ R2A + rock flour	2
	ST PC-28	L120304-48	Wet Carbonate	BG11	2
	ST PC-29	L120304-49	Wet Carbonate	AIA + nystatin	2
	ST PC-30	L120304-52	Red FMD	½ R2A + rock flour	2
	ST PC-31	L120304-53	Red FMD	BG11	2
	ST PC-32	L120304-55	Red FMD	AIA	2
	ST PC-33	L120304-57	Wet Carbonate	½ R2A + rock flour	2
	ST PC-34	L120304-58	Wet Carbonate	BG11	2
Briny Pool	ST PC-35	L120305-81	Carbonate speleothem	½ R2A + rock flour	2
	ST PC-36	L120305-82	Carbonate speleothem	BG11	2
	ST PC-37	L120305-84	Carbonate speleothem	AIA	2
	ST PC-38	L120305-85	Carbonate speleothem	½ R2A	2
	ST PC-39	L120305-87	Carbonate speleothem	BG11	2
	ST PC-40	L120305-88	Carbonate speleothem	AIA + nystatin	2
	ST PC-41	L120305-89	Carbonate speleothem	AIA	2
	ST PC-42	L120305-91	Carbonate speleothem	½ R2A + rock flour	2
	ST PC-43	L120305-94	Carbonate speleothem	AIA	2
	ST PC-44	L120305-95	Carbonate speleothem	½ R2A	2

^aDusting of soil.

Media-Reasoner's 2A medium (R2A), BG11 (Atlas, 2004), and Actinomycetes Isolation Agar (AIA).

streaking onto five types of sterile parent agar media (Table 2). Parent media types, all containing organic carbon, were made as follows (per liter): $\frac{1}{2}$ Reasoner's 2A medium (R2A), $\frac{1}{2}$ R2A with 5 g rock flour (Lower Guadalupe Mountains surface limestone that was pulverized in a rock crusher and autoclaved), BG11 (Atlas, 2004), Actinomyces Isolation Agar (AIA), and AIA with nystatin 0.072 mg/mL (Sigma-Aldrich, St. Louis, Mo.). These media target oligotrophic ($\frac{1}{2}$ R2A), bacteria adapted to Guadalupe Mountains caves (rock flour), and *Actinobacteria* (AIA).

To enhance the growth of a broad diversity of cave bacterial cultures, ST PC-5 to ST PC-44 were incubated in the cave for 1–3 days initially to give cave-adapted bacteria a head start under conditions to which they are adapted, followed by further growth time in the lab. Plates were incubated in the cave 1 to 3 days and transported in a cooler to a lab incubator (20 °C, dark, and no added humidity) for 23 days before subculturing (Table S1). Both pure (one organism) and mixed (2 or more organisms) cultures of siderophore producers were confirmed with sequencing.

Subculture of ST Bacterial Cultures

Ninety cultures were collected from ST parent cultures (Table 2). ST bacterial cultures were subcultured by collecting cells from ST parent cultures, ST PC-5 to ST PC-44, using a sterile loop and aseptically streaking onto $\frac{1}{2}$ R2A agar plates, targeting oligotrophs. The inoculated $\frac{1}{2}$ R2A agar plates were incubated in the lab at 20 °C (no added humidity and in the dark) and subsequent individual bacterial colonies were collected upon first appearance within a period of two to seven days (Table S1). The individual bacterial colonies were streaked once again for isolated colonies and 20 % glycerol stock were prepared with $\frac{1}{2}$ R2B Medium for long-term preservation at –80 °C. Both pure (one organism) and mixed (two or more organisms) cultures of siderophore producers were confirmed with sequencing.

Iron-limiting Growth of LT and ST Bacterial Cultures

For iron-limiting growth conditions, a three-step process was completed. First, bacterial cultures were streaked from 20 % glycerol freezer stocks onto sterile isolation subculture media and incubated at 20 °C (no added humidity and in the dark) for four days (Table S1). Second, a heavy streak of the bacterial culture was transferred aseptically to modified ISP4 agar with Fe (Lee et al., 2012) and grown at 20 °C (no added humidity and in the dark) for seven days. ISP4 medium was developed for detection of siderophores produced by soil *Actinobacteria*. Modified ISP4 agar plates with Fe contained (per liter): 4 g/L soluble starch, 0.3 g/L casein, 2 g/L KNO₃, 0.5 g/L MgSO₄·7H₂O, 0.02 g/L CaCO₃, 0.01 g/L FeSO₄·7H₂O and 25 g/L agar. Lastly, for subsequent Fe-depletion, the bacterial cultures were then transferred to iron-limiting ISP4 media that was modified by removal of FeSO₄·7H₂O and casein and addition of 1g/L of yeast extract and incubated at 20 °C (no added humidity and in the dark) for seven days (Table S1).

Siderophore-induction of LT and ST Bacterial Cultures

All cultures were screened with the Chrome Azurol S (CAS) assay then further tested for specific siderophores. Iron-limiting growth conditions were used to induce siderophore production and iron-starved bacterial cultures were plated on sterile CAS agar plates. CAS siderophore indicator plates were prepared as previously described (Schwyn and Neilands, 1987). Bacterial cultures were examined over time from the day of plating to the first day of halo. A CAS-color (blue) change to purple, described in the traditional CAS assay, indicated production of catechol-type siderophores. A CAS-color change to orange indicated hydroxamate siderophore production, while CAS-color change to completely clear probably indicated carboxylate siderophores (Pérez-Miranda et al., 2007; Sullivan et al., 2012). Negative siderophore production was monitored for approximately an additional month to verify no halo CAS-color change appearance surrounding the bacterial colonies. Representative phenotypes of CAS positive detection were categorized as siderophore-strong (>2mm zone of CAS-color change), -weak (<2mm zone of CAS-color change), -none (no CAS-color change), or no growth of isolate (Fig. 2).

Arnow's and FeCl₃ assays (Arnow, 1937; Neilands, 1981) were used to test for production of catecholate- and hydroxamate-type siderophores, respectively, according to protocols detailed in Lee et al., (2012). The bacterial cultures were exposed to iron-limiting conditions listed above and subsequently transferred to 2 mL of liquid ISP4 with no added iron for 53 days.

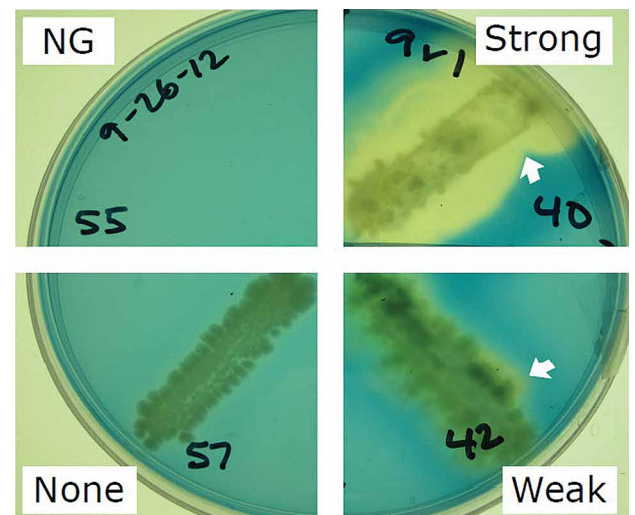


Figure 2. Chrome Azurol S (CAS) phenotypes showing varying production of siderophores by Fe-depleted cave cultures. Siderophore production is shown by the presence of color change around growth on CAS agar (arrows). Moving clockwise from top left: No growth (NG), Strong production (>2mm), weak production (<2mm), and no production (None). Numbers indicate cave culture identification number.

Two mL of inoculated medium were centrifuged for 20 min at 13K rpm and the supernatant collected for Arnow's and FeCl_3 assays. Catecholate (6 $\mu\text{g/mL}$) and hydroxamate, Desferrioxamine B (1mM), were used as positive controls in Arnow's and FeCl_3 assays, respectively. Milli-Q water was used as a negative control for both assays. A positive result for Arnow's assay was a color change to pink and a negative result remained colorless. Positive results for FeCl_3 assays were color changes to dark red (1mM Desferrioxamine B), orange (trihydroxamate), or pink (dihydroxamate) (Lee et al., 2012). Broad spectra (250–700 nm in 50 nm increments) were measured for positive and weak positive results of FeCl_3 assay using a Biomate 3 Spectrometer (Thermo Spectronic, Houston, TX, USA). 1mM Desferrioxamine B had a λ_{max} at 500 nm. Hydroxamate positives had a $\lambda_{500\text{nm}}$ greater than 0.260; weak hydroxamate positives were between 0.1 and 0.260; and hydroxamate negatives were less than 0.1.

16S rRNA Gene Sequencing

DNA was extracted from siderophore-positive pure and mixed cultures and purified using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). The pure or mixed pool 16S rRNA gene was amplified with universal primers 46F (5'- GCYTAAYACATGCAAGTCG-3') and 1409R (5'-GTGACGGGCRGTGTGTRCAA-3') bacterial primers (1362 BP) (Northup et al., 2010). Reactions were carried out in a 25 μL volume with $10\times$ PCR buffer with 15 mM Mg^{2+} , 0.3 μM of each primer, 0.25 mM of each dNTPs, 5 μg of 50 mg/mL BSA (Ambion Austin, TX, USA) and 1U Ampli-Taq LD (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed with an MJ thermocycler with the program, 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55.5 °C for 30 s, 72 °C for 1.5 min and final extension at 72 °C for 7 min. Amplicons were purified using MoBio PCR Clean Up Kit (MoBio, Carlsbad, Calif.) and cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.).

The 16S rRNA genes of all pure cultures and twelve clones from each mixed culture, randomly selected by coin flip, were sequenced with BigDye 1.1 using the T3 or T7 for the sense direction of the 16S rRNA gene. Sequencing was performed with the ABI Prism 3130 Automated DNA Sequencer at UNM-Molecular Biology Facility, Albuquerque, NM and an ABI Prism 3730xl Automated DNA Sequencer when sequenced at GENEWIZ Genomics, Danvers, Mass.

Phylogenetic Analysis

A total of 1102 16S rRNA gene sequences were analyzed for siderophore-strong and siderophore-weak, respectively (Table S2). Using Sequencer 5.1 (Gene Codes Corp., Ann Arbor, Mich.), ambiguous portions were trimmed to a minimum of 500 BP and edited. Chimeras were identified with QIIME v.1.9.1 (Caporaso et al., 2010) and removed. Sequences from each bacterial culture were assembled with 97 % sequence similarity to identify unique clone types. Within the siderophore-strong and siderophore-weak groupings the sequences were assembled with 98 % similarity to identify Operational Taxonomic Units (OTU). Using the longest representative sequence for each OTU, taxonomic assignments were identified using BLAST (Altschul et al., 1990). Representative sequences were assigned accession numbers in GenBank (Tables S3 and S4). A sequence alignment was performed using default parameters of EMBL-EBI webPRANK (<http://www.ebi.ac.uk/goldman-srv/webprank/>) and subsequently a similarity matrix was determined using a custom MATLAB script (Table S5). A maximum likelihood tree was constructed by using IQ-TREE web server: fast and accurate phylogenetic trees under maximum likelihood (<http://iqtree.cibiv.univie.ac.at/>) with 1000 replicates (Minh et al., 2013). The tree files were visualized and annotated with the Interactive Tree of Life (iTOL) v.3.5.3 program (<http://itol.embl.de/>) and vector-drawing program Inkscape v.0.92.1 (<https://inkscape.org>).

RESULTS

The bacterial cultures from cave deposits collected from both the LT and ST parent cultures represented a potentially rich diversity of chemoorganoheterotrophic microorganisms exposed to multiple days or years of incubation for testing for the capability to produce siderophores. One hundred and seventy bacterial cultures, pure and mixed, were gathered from subcultures of LT and ST parent cultures (Tables 1 and 2). Eighty LT bacterial cultures were collected from top, middle, and bottom sample depths from four LT parent cultures. The majority of bacterial cultures collected from the same depth grouped together to form a representative sequence (data not shown). Forty-five percent ($n = 36$) of LT bacterial cultures were subcultured on $\text{R2A} + \text{FeCl}_3$, 42.5 % ($n = 34$) were subcultured on $\text{R2A} + \text{MnCO}_3$, and 12.5 % ($n = 10$) of were subcultured on $\text{AIA} + \text{FeCl}_3 + \text{MnCO}_3$ (Table 1). Ninety ST bacterial cultures were collected from 40 ST parent cultures. All ST bacterial cultures were subcultured on $\frac{1}{2}$ R2A (Table 2).

Siderophores are Produced by LT and ST Bacterial Cultures

The CAS assay was used to classify bacterial cultures according to detection zones of siderophore production that was measured from plating of a culture to the first day of CAS color change (Table 3). There was a range of color changes, including a dominant change to orange (hydroxamate) or clear. A CAS-color change to completely clear probably indicated carboxylate siderophores (Pérez-Miranda et al., 2007; Sullivan et al., 2012), but was detected using an agar overlay with a color change to white. We believe the addition of the indicator to the medium resulted in a white color in our study. We did not observe complete clearing. There were no color changes to purple (catechol). Thirty-six percent

Table 3. Siderophore detection in LT (*n* = 80) and ST (*n* = 90) bacterial cultures.

Group	Siderophore Detection Level ^a	Days					Total Cultures (%)
		<10	11–15	16–20	21–25	>26	
LT	Strong (>2mm)	...	7 (9)	1 (1)	2 (3)	18 (23)	28 (36)
	Weak (<2mm)	...	2 (3)	...	...	10 (13)	12 (16)
	None	...	...	...	...	...	13 (16)
	NG ^b	...	...	...	...	...	27 (32)
	Total (%)	0 (0)	9 (12)	1 (1)	2 (3)	28 (36)	80 (100)
ST	Strong (>2mm)	11 (12)	21 (23)	10 (11)	6 (7)	20 (22)	68 (75)
	Weak (<2mm)	...	1 (1)	...	...	...	1 (1)
	None	...	...	...	...	...	15 (17)
	NG ^b	...	...	...	...	...	6 (7)
	Total (%)	11 (12)	22 (24)	10 (11)	6 (7)	20 (22)	90 (100)

^a Zone of color change.^b No growth (NG)- live bacteria cultures that did not grow on CAS media when transferred from Fe-limiting ISP4 media. Numbers within parentheses are percentages (%).

of the total LT bacterial cultures were strong siderophore producers, 16 % were weak, and 16 % had no detectable siderophore production, while 32 % did not grow on the CAS assay (Table 3). In contrast to the LT cultures, the majority of ST bacterial cultures were strong siderophore producers (Table 3) with 75 % percent of the total ST bacterial cultures strong siderophore producers, 1 % were weak, and 17 % had no detectable siderophore production. Seven percent of the ST bacterial cultures did not grow on the CAS assay (Table 3).

The period of time it took for the microbial siderophore production to manifest itself fell into five categories: less than 10 days, 11–15 days, 16–20 days, 21–25 days and greater than 26 days. The majority, 36 % of LT siderophores-producers, took greater than 26 days to manifest in contrast with no siderophores produced in less than 10 days. For ST siderophore-producers 24 % took 11–15 days to manifest with the lowest production of siderophores, 7 %, by cultures in 21–25 days (Table 3). Overall, the ST siderophore production occurred more quickly than LT and may be related to their overall faster growth.

Hydroxamate Rather than Catecholate Siderophores are Produced by LT and ST Bacterial Cultures

To determine the preference of siderophore use by cave bacteria, the bacterial cultures were exposed to iron-limiting growth conditions and siderophore type was assessed from the supernatant after 53 days. Hydroxamate siderophores, determined by the FeCl₃ assay color change of the supernatant, (color phenotypes shown in Fig. 3C) were the dominant type of siderophores detected in our study. Hydroxamate siderophore detection was fairly even between ST and LT cultures (Fig. 3B), while catecholate siderophores, which were determined by the Arnow's assay color change from clear to pink of the supernatant, were rarely detected in our study (Fig. 3A).

Siderophore-producing Bacterial Cultures are Closely Related to Members of *Proteobacteria* (Alpha-, Beta-, and Gamma-), *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*

A total of 982 16S rRNA genes were sequenced for strong siderophore producers and 120 16S rRNA genes for weak siderophore producers (Table S2). The total (982 sequences) siderophore-strong 16S rRNA gene sequences within the siderophore-strong class clustered into 85 representative bacterial operational taxonomic units (OTUs) (Fig. 4, Table S3). In contrast, the total (120 sequences) siderophore-weak class clustered into 14 representative bacterial OTUs (Fig. 5, Table S4). Together, the siderophore producing representative OTUs classified into *Proteobacteria* (Alpha-, Beta-, and Gamma-), *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Figs. 4 and 5).

Proteobacteria was the most abundant phylum for siderophore producers. *Gammaproteobacteria*, found only in the siderophore strong producers, had the most OTUs overall. Within the strong *Proteobacteria* siderophore producers there were 44 OTUs, classified as 12 Alpha-, 14 Beta-, and 18 Gamma- (Fig. 4, Table S3). Siderophore weak producers only had six OTUs, which were classified as four Alpha- and two Beta- (Fig. 5, Table S4). No Delta-, Zeta-, or Epsilon-proteobacteria were classified in either group.

Alphaproteobacteria had the most genera represented, with eight strong siderophore producers and three weak siderophore producers, even though *Gammaproteobacteria* overall had the most OTUs (Figs. 4 and 5). *Sphingopyxis* was the only genus identified in both siderophore producing groups (strong and weak), as opposed to the following genera, which were only identified in the siderophore strong group: *Mycoplana*, *Sinorhizobium*, *Phenylobacterium*, *Pheny-*

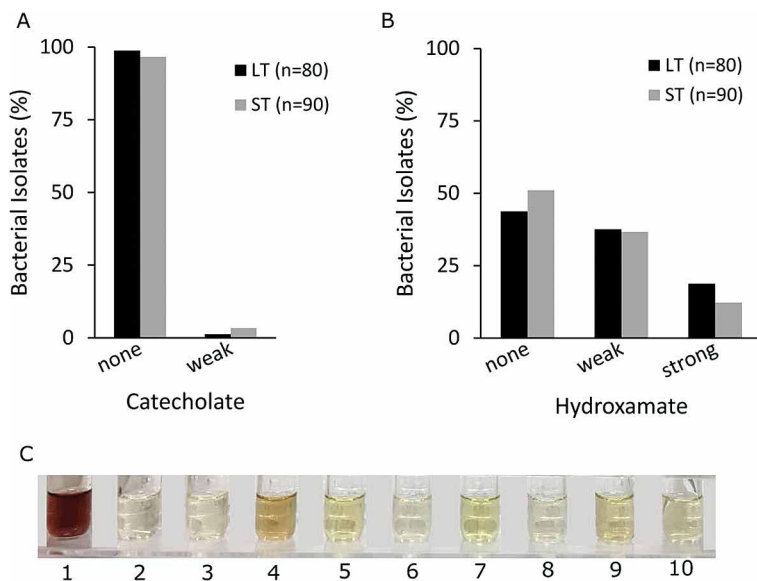


Figure 3. The percentage of Long-term (LT) and Short-term (ST) bacteria cultures producing Catecholate (A) and Hydroxamate (B) siderophores. C) Varying color phenotypes of Hydroxamate-type FeCl₃ Assay. Lane 1-positive control, Desferrioxamine B (1mM), 2-negative control, Milli-Q H₂O, 3-negative control, uninoculated media, 4-strong-positive, 5-weak-positive, 6-no hydroxamate, 7-weak positive, 8-no hydroxamate, 9-weak positive, and 10-weak positive.

Gammaproteobacteria in the siderophore strong group (Fig. 4, Table S3). The other three most abundant *Proteobacteria* were two *Betaproteobacteria* and one *Alphaproteobacteria* (Table S3). None of the siderophore weak producers had a representative OTU with greater than 20 sequences (Table S4).

Overall, *Proteobacterial* classes *Beta*- and *Gamma*-, in the strong siderophore producers group, include genera that are known as siderophore producers. In contrast, *Alphaproteobacteria* only had two genera, *Sinorhizobium* and *Sphingomonas*, related to known siderophore producers in the siderophore-strong and -weak, respectively (Figs. S1 and S2). The majority of the seven most abundant sequences that had greater than 20 sequences each are known siderophore producers, except for *Sphingopyxis*, which was not previously known to produce siderophores. Most of the close relatives of *Proteobacteria* siderophore producers can inhabit low nutrient environments, including other carbonate caves, can break down and recycle aromatic-containing structures, are possibly endosymbionts, and can thrive in heavy metal environments (Figs. S1 and S2).

Actinobacteria was the second most abundant phylum of siderophore producers. Within the strong and weak siderophore producers there were 16 and three representative OTUs, respectively, with the majority identified as closely related to known siderophore producers (Figs. 4 and 5). In the siderophore strong group, *Rhodococcus*, *Norcardia*, *Microbacterium*, *Arthrobacter*, and two uncultured bacteria: an uncultured Yanshan Mountain clone and a crude oil-degrading strain TPKD2 were among the genera identified (Fig. S1, Table S3). In the siderophore weak group, *Lentzea* and *Knoellia* were among the genera identified (Fig. S2, Table S4). *Streptomyces* was identified in each group (Tables S3 and S4), however it was not the most abundant genus. Instead, *Norcardia coeliaca*, *Curtobacterium luteum*, *Microbacterium* sp., *Arthrobacter methylotrophus*, *Arthrobacter siccitolerans*, and *Lentzea violacea* all had greater than 20 sequences each (Tables S3 and S4). Surprisingly, not all *Actinobacteria* genera identified are known siderophore producers. *Lentzea* (EU593719) and *Knoellia* (LN774289), not previously described as siderophore producers, were identified in karst limestone and cave air, respectively, and are possibly common to aphotic subsurface environments (Fig. S2, Table S4).

Bacteroidetes was the third most abundant phylum for siderophore producers. Within the strong and weak siderophore producers there were 14 and 2 total representative OTUs, respectively, with the majority identified as unknown siderophore producers (Figs. 4 and 5). There were no similar genera identified in each group, however *Chitinophaga*, was identified within the weak siderophore producing group (Table S4). In contrast, for the siderophore strong group, *Hymenobacter*, *Olivibacter*, *Flaviumibacter*, and four uncultured clones: an uncultured mineral photocatalysis clone, uncultured clone NDB4, uncultured soil diffusion chamber system CCRA clone, and uncultured *Sphingobacteriales* bacterial clone were all identified (Fig. S2, Table S3). The most abundant *Bacteroidetes* OTUs that had greater than 20 sequences in

lobacter, *Aminobacter*, *Devosia*, *Afipia*, two uncultured clones, an uncultured *Brevundimonas*, and an uncultured arsenic-contaminated soil clone in the siderophore strong group (Fig. 4, Table S3). Genera identified only in the siderophore weak group were *Erythrobacter*, *Sphingomonas* and one uncultured nonylphenol-degrading clone (Fig. 5, Table S4).

Betaproteobacteria were represented by six genera in the siderophore strong producers and a single genus, *Achromobacter*, in the siderophore weak group. *Achromobacter* was identified in both the strong- and weak-siderophore producing groups, along with *Cupriavidus*, *Ralstonia*, *Telluria*, *Duganella*, *Acidovorax*, and one uncultured *Betaproteobacteria* clone in the strong siderophore producers (Figs. 4 and 5, Tables S3 and S4).

Although *Gammaproteobacteria* were represented by the least number of genera, which included *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, and *Pseudoxanthomonas*, and one uncultured bacterial clone B18 (Fig. 4, Table S3), *Gammaproteobacteria* had the most overall representative OTUs (18). The seven most abundant *Proteobacteria* OTUs were represented by more than 20 sequences in each sequence cluster of which four were

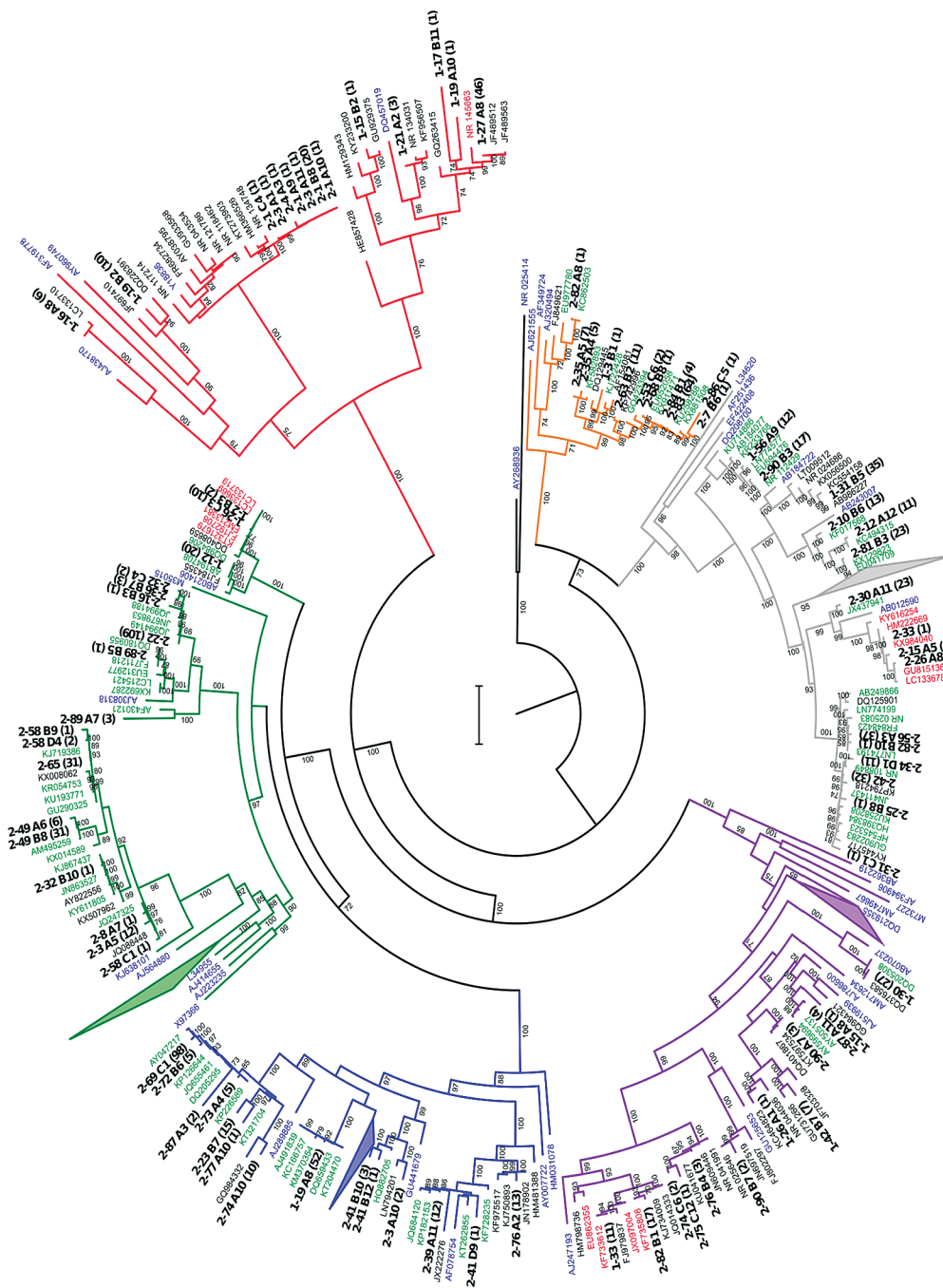


Figure 4. Phylogenetic tree of the closest bacterial relatives to siderophore-strong representative OTUs. Ultrabootstrap values are >70% or otherwise stated on 1000 replicates. The scale bar corresponds to 0.1 nucleotide substitutions per site. Representative OTU sequences code: 1-Long-term and 2-Short-term, culture number, letter-clone number, and numbers of clones per representative sequence are in parenthesis (Supplemental Table S3). The numbers of clones per representative sequence are in parenthesis. Color code of branches: Grey-Actinobacteria, Orange-Firmicutes, Purple-Alphaproteobacteria, Blue-Betaproteobacteria, Green-Gammaproteobacteria, and Red-Bacteroidetes. Name color code: Blue-reference strain, Green-a member of the same genus is known as a siderophore producer, Red-a member of the same genus is known as a non-siderophore producer, and Black-strain is uncultured (UC) or has not been reported as a siderophore producer. Triangles are collapsed reference strain clades.

bacteria), and the weak siderophore producer was *Chitinophaga* sp. (*Bacteroidetes*) (red text, Figs. 4 and 5). These bacteria may possibly produce novel siderophores or have additional microbial recycling roles, such as metabolism of aromatic rings (Figs. S1 and S2, Tables S3 and S4).

each representative sequence were uncultured bacterial clones (Table S3). Many of the closest relatives are related to bacteria that live in water, are inhabitants of low-nutrient environments, and can live in low radioactive and chemical waste sites (Tables S3 and S4).

Firmicutes was the least abundant phylum for siderophore producers. Within the strong and weak siderophore producers there were 11 and 3 total representative OTUs, respectively, with the majority identified to be related to known siderophore producers (Figs. 4 and 5, Tables S3 and S4). *Bacillus* was identified in both siderophore producing groups, along with *Paenibacillus*, *Geobacillus*, and one uncultured clone, an uncultured urban aerosol clone in the siderophore strong producer group (Fig. 4, Table S3). Additionally, *Bacillus* was the most abundant *Firmicutes* OTU with greater than 20 sequences (Table S3). Many of the closest relatives are related to bacteria that live in the ocean near geothermal mineral deposits and hydrocarbon seeps, are inhabitants of low nutrient clean rooms, are endophytic, or live in the soil (Figs. S1 and S2, Tables S3 and S4).

Interestingly, there were genera identified that were not previously known as siderophore producers in both the siderophore-strong and -weak groups. Genera of the siderophore strong producers were identified as *Brevundimonas* sp. (*Alphaproteobacteria*), *Pseudoxanthomonas* spp. (*Gammaproteobacteria*), and *Microbacterium* spp. (*Actino-*

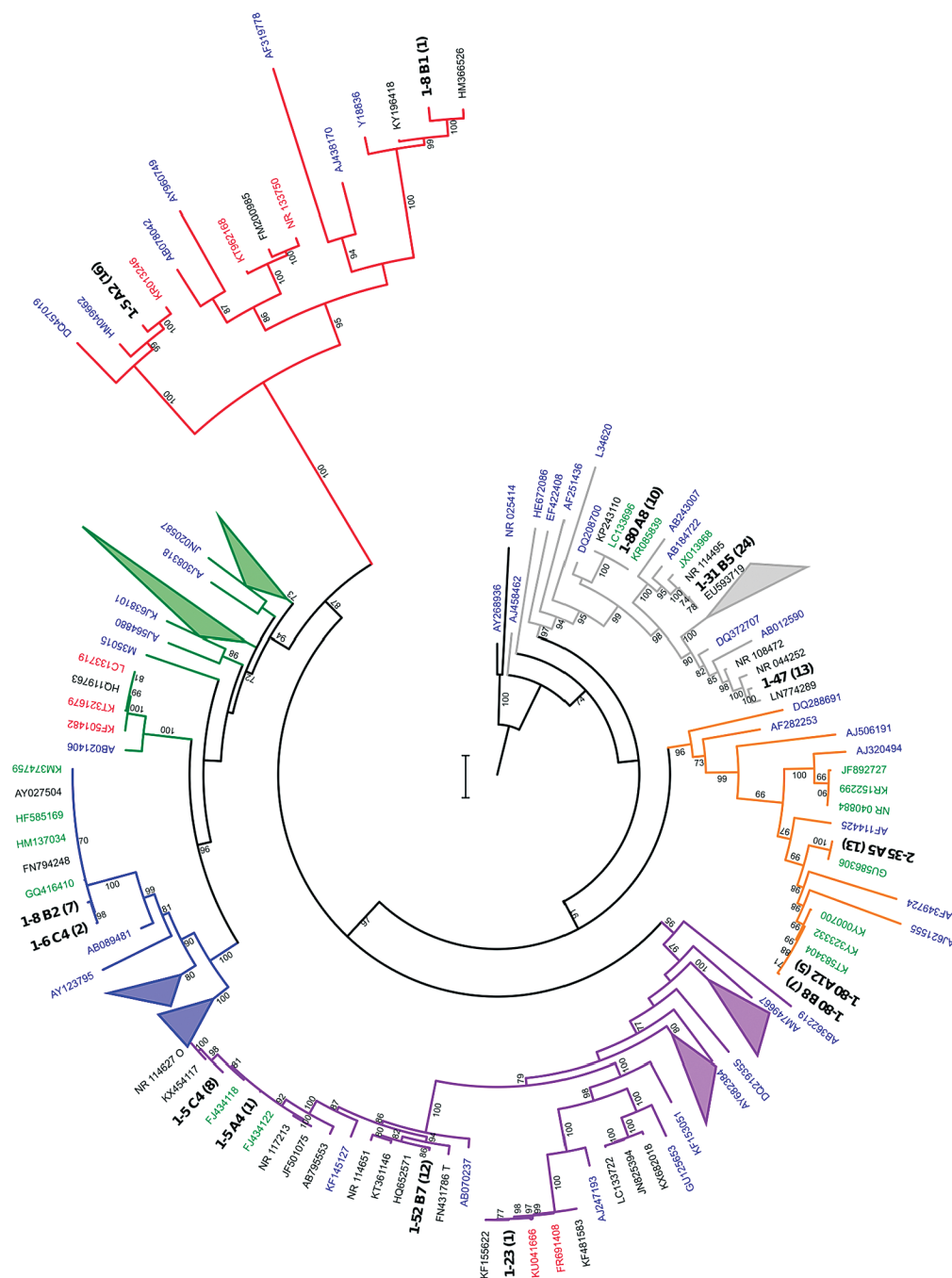


Figure 5. Phylogenetic tree of the closest bacterial relatives to siderophore-weak representative OTUs. Ultrabootstrap values are >70% or otherwise stated as 1000 replicates. The scale bar corresponds to 0.1 nucleotide substitutions per site. Representative OTU sequences code: 1-Long-term and 2-Short-term, culture number, letter-clone number, and numbers of clones per representative sequence are in parenthesis (Supplemental Table S4). The numbers of clones per representative sequence are in parenthesis. Color code of branches: Grey-Actinobacteria, Orange-Firmicutes, Purple-Alphaproteobacteria, Blue-Betaproteobacteria, Green-Gammaproteobacteria, and Red-Bacteroidetes. Name color code: See Figure 4 legend.

bacteria), and *Chitinophaga* sp. (*Bacteroidetes*). These bacterial genera have all been identified in oligotrophic caves or other cave studies (Barton et al., 2007; Chen et al., 2009; Ghosh et al., 2017) and along with potential siderophore production could contribute to the survival of an oligotrophic-dwelling community by offering non-siderophore related characteristics. The non-siderophore characteristics are metabolism of aromatic ring-containing compounds by *Microbacterium* sp. (de los Cobos-Vasconcelos et al., 2012; Jin et al., 2017), the production of proteases that are able to function in highly alkaline conditions by *Pseudoxanthomonas* sp. (Salwan et al., 2010), the ability to mobilize Fe, Al, Si,

DISCUSSION

In this study we found that 64% of the cave cultures obtained from Spider and Lechuquilla Caves were capable of siderophore production. Within this niche of CCNP cave deposits, the siderophore-positive bacterial cultures spanned four phyla, including *Proteobacteria* (*Alpha*-, *Beta*-, and *Gamma*-), *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, which are similar to the groups of bacteria frequently present in caves (Tomczyk-Zak and Zielenkiewicz, 2016). The siderophore-producing bacterial diversity that was identified in our study is generally similar to soil microbial siderophore-producing diversity (Purushotham et al., 2018; Schmidt et al., 2018; Hanaka et al., 2019; Wagner et al., 2019). The soil phyla are often categorized as plant growth-promoting bacteria in the rhizosphere of roots or as plant endophytes (Biessy et al., 2018; Gong et al., 2018; Purushotham et al., 2018; Hanaka et al., 2019; Wagner et al., 2019). What the roles of these phyla are in caves, which lack plants, remains to be discovered, but we have made progress in understanding the nature of siderophores in caves and expanding our knowledge of bacteria capable of producing siderophores.

Potential novel siderophore producers from pure or mixed cultures were identified in our study, including *Brevundimonas* sp. (*Alphaproteobacteria*), *Pseudoxanthomonas* spp. (*Gammaproteobacteria*), *Microbacterium* spp. (*Actino-*

an K from rock by *Chitinophaga* sp. (Wang et al., 2014), and the ability to adapt to oligotrophic environmental conditions of *Brevundimonas* sp. (Dworkin, 2002).

Survival in an oligotrophic deep cave environment appears to require several non-phototrophic microbial metabolic strategies (Tomczyk-Zak and Zielenkiewicz, 2016). We selected for chemolithoheterotrophic and heterotrophic bacteria from Lechuguilla and Spider Caves, CCNP. Chemolithoheterotrophic bacteria, also known as mixotrophs, derive their energy from inorganic chemicals, but rely on organic chemicals in the environment for carbon needs. No obligate chemolithotrophs were identified in this study, but four genera, *Ralstonia* sp. (Libert et al., 2004), *Burkholderia* sp. (Libert et al., 2004), *Cupriavidus* sp. (van Houdt et al., 2009) and *Achromobacter* sp. (Ehsani et al., 2019), were identified and are known to oxidize hydrogen as an energy source (chemolithotrophic). These bacteria were isolated from metal-rich deep oligotrophic basin water (*Ralstonia* sp. and *Burkholderia* sp.) and sediments (*Cupriavidus* sp.) and soil (*Achromobacter* sp.) environments, but what their specific metabolic roles are in the cave is an aim for future research.

We observed that cave bacteria prefer hydroxamate siderophores, possibly due to the overall oligotrophic nature of CCNP caves. Catecholate siderophore production was rarely preferred by cave microorganisms in our study, which most likely is due to the higher energy cost of production. The preference for the hydroxamate siderophores is possibly due to the less complex biosynthesis of hydroxamate siderophores. Hydroxamate siderophores are generally made from available amino alcohol building blocks; derivatives from amino acids (Challis, 2005), which are often present in the environment (Moura et al., 2013). In addition, previous work showed directly that hydroxamate siderophores can be recycled (Emery, 1971; Hartmann and Braun, 1980; Braun et al., 1984; Hannauer et al., 2010). By utilizing readily-available compounds in their local environments that have been shown to be recycled, bacteria are potentially able to limit the energetic cost to produce and recycle hydroxamate siderophores. Whether siderophore production by cave microorganisms is used primarily for ferric iron acquisition, or for nutrient exchange within a mixed culture, or both, remains to be determined.

Hydroxamate siderophores have a binding constant range of 10^{22} to 10^{32} M⁻¹ (Miethke, 2013) whereas the catecholate-model, enterochelin, had a binding range of 10^{52} M⁻¹ (Saha et al., 2016). Although we identified hydroxamate siderophore production as a majority, we speculate that it is due to the contrasting environments in which catecholate and hydroxamate siderophores are commonly found. Gram-negative bacteria, specifically pathogens like *Escherichia coli*, are well known catecholate siderophores producers (Winkelmann, 2002) and would require extremely high ferric (Fe³⁺) binding affinities if they are to compete with host iron binding proteins for iron (Chu et al., 2010). Thus, combined with the higher likelihood that catecholate siderophores require more energy to make, a luxury not available for bacteria surviving in oligotrophic caves, hydroxamate siderophores would be sufficient to gather needed ferric (Fe³⁺) iron from the surrounding cave environment.

Interestingly, the CAS assay gave an indication that carboxylate-type of siderophores might be produced by mixed cave cultures. Sullivan et al., (2012) reported a range of CAS assay color changes that, along with orange and purple that indicate hydroxamate and catechol, respectively, a color change to clear indicated a carboxylate-type. Although we didn't find any blue color change to clear results, we did observe at least three mixed cultures to have a color change to white and nine mixed cultures that were siderophore positive, but tested negative for the hydroxamate and catechol tests (data not shown). We also identified several *Rhizobium* spp. in the siderophore-strong group including *Rhizobium meliloti* strain DM4, which are known carboxylate siderophore producer (Ali et al., 2013). Determining whether there are carboxylate siderophores from the mixed category would be the focus of a future study.

Mixed cultures (two or more organisms in a culture) can allow for a full suite of interactions among microorganisms in the environment, but interactions can be unstimulated if pure cultures were obtained (Nai and Meyer, 2018). Because oligotrophic cave environments may select for potential microbial consortial microorganisms (Barton and Jurado, 2007) and siderophores have potential to promote nutrient exchange (D'Onofrio et al., 2010), mixed cultures were included to determine siderophore presence in a low nutrient cave environment. Approximately half of the siderophore-producing cultures were mixed cultures with 2–6 genera/culture. Hershey et al., (2004) observed siderophore cross feeding that allowed non-growing Lechuguilla bacteria to grow but didn't take into consideration whether the timing of the production of siderophores is affected by pure or mixed cultures. A future study would determine whether mixed cultures are obligate consortial microorganisms and whether the timing and production of siderophores by pure or mixed cultures are shared among the bacterial community.

How representative our study of CCNP caves are to other cave systems is not known in detail, but Lechuguilla and Spider Caves were chosen as our study sites because of their abundant levels of ferric (Fe³⁺) iron in visible deposits on cave walls (Northup et al., 2003; Spilde et al., 2005). Such deposits are not rare but are also not abundant in carbonate caves. We hypothesized that these ferromanganese deposits (FMD) that line the cave walls would be a valuable source of ferric iron needed for cellular processes. Another key factor that makes these caves different from some other caves is that they occur in a semi-arid environment and, as a result, are more oligotrophic than caves in areas with more

surface precipitation, which could limit the amount of energy available for siderophore production. In addition to limited surface precipitation, the surface soils above carbonate caves are highly oligotrophic due to low carbon levels (Ortiz et al., 2014), resulting in less infiltration of nutrients to fuel metabolic processes. We also hypothesize that surface soil siderophore producers either do not infiltrate into the cave, or if they do, rarely survive. Lavoie et al., (2017) showed that in a comparison of semi-arid lava tube cave microbial mats to surface soils overlying the cave that there was only an 11.2 % OTU overlap in the microbial diversity. These conditions suggest that semi-arid caves are likely very different from caves in regions with more rainfall.

Particularly relevant to siderophore production in our study caves, is the study by Levy (2007), who showed that the amount of Fe in drip water entering Lechuguilla Cave pools was below detectable limits ($<0.06 \text{ mg L}^{-1}$). Thus, in areas without FMD in these caves, iron is likely more limited. Taken together, siderophore usage by microorganisms is preferred in oxidized environments with available ferric (Fe^{3+}) iron sources, both of which Lechuguilla and Spider caves have. To understand how representative the microorganisms from these caves are in terms of siderophore production, caves that occur in areas with more rainfall and hence more nutrients and that lack substantial iron-rich secondary deposits would need to be investigated and compared.

CONCLUSIONS

Our study has identified potential novel siderophore producers that may lead to the identification of new compounds or the roles that siderophores have within mixed microbial relationships. In addition to chelating ferric (Fe^{3+}) iron from the environment, siderophores can enhance growth or protect plants from pathogens, are recognized and used by both fungi and bacteria, serve in soil mineral weathering, and are involved directly or indirectly in bioremediation of environmental pollutants, including heavy metals and hydrocarbons (Ahmed and Holmström, 2014). By understanding the specific role of siderophores in the natural cave environment, which lacks plants and contains minerals, we can enhance our understanding of the context in which siderophores are produced and how cave bacteria can live in this oligotrophic environment.

The identification of siderophore producers from mixed cultures collected from the cave environment revealed the rich diversity of cave bacteria. Understanding the intimate interactions among bacteria in caves can offer clues to understanding how these organisms survive for the long term in this otherwise low-nutrient environment. This rich diversity of bacteria helps promote an ongoing critical search for new antibiotics, potentially sideromycins, for treatment of antibiotic-resistant microorganisms and novel siderophores for bioremediation.

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MICROBIOME OF GRAND CANYON CAVERNS, A DRY SULFURIC KARST CAVE IN ARIZONA, SUPPORTS DIVERSE EXTREMOPHILIC BACTERIAL AND ARCHAEOAL COMMUNITIES

Raymond Keeler¹ and Bradley Lusk^{2,C}

Abstract

We analyzed the microbial community of multicolored speleosol deposits found in Grand Canyon Caverns, a dry sulfuric karst cave in northwest Arizona, USA. Underground cave and karst systems harbor a great range of microbial diversity; however, the inhabitants of dry sulfuric karst caves, including extremophiles, remain poorly understood. Understanding the microbial communities inhabiting cave and karst systems is essential to provide information on the multidirectional feedback between biology and geology, to elucidate the role of microbial biogeochemical processes on cave formation, and potentially aid in the development of biotechnology and pharmaceuticals. Based on the V4 region of the 16S rRNA gene, the microbial community was determined to consist of 2207 operational taxonomic units (OTUs) using species-level annotations, representing 55 phyla. The five most abundant Bacteria were *Actinobacteria* 51.3 ± 35.4 %, *Proteobacteria* 12.6 ± 9.5 %, *Firmicutes* 9.8 ± 7.3 %, *Bacteroidetes* 8.3 ± 5.9 %, and *Cyanobacteria* 7.1 ± 7.3 %. The relative abundance of Archaea represented 1.1 ± 0.9 % of all samples and 0.2 ± 0.04 % of samples were unassigned. Elemental analysis found that the composition of the rock varied by sample and that calcium (6200 ± 3494 ppm), iron (1141 ± 1066 ppm), magnesium (25 ± 17 ppm), and phosphorous (37 ± 33 ppm) were the most prevalent elements detected across all samples. Furthermore, carbon, hydrogen, and nitrogen were found to compose 4.7 ± 4.9 %, 0.3 ± 0.4 %, and 0.1 ± 0.1 % of samples, respectively. Finally, Raman spectra compared to the RRUFF Project database using CrystalSleuth found that the mineral composition of the speleosol consisted of calcite, hematite, paraspurrite, quartz, and trantnerite. These data suggest that dry sulfuric karst caves can harbor robust microbial communities under oligotrophic, endolithic, and troglomorphic conditions.

INTRODUCTION

Microorganisms are the principle components of the formation of the Earth's atmosphere, hydrosphere, and surface, and play a central role in karst cave formation and the cycling of organic and inorganic nutrients. For example, hydrogen sulfide-producing microbes can contribute to the formation of sulfuric karst caves in aqueous environments in a process called sulfuric acid speleogenesis (Engel et al., 2004). During sulfuric acid speleogenesis, limestone dissolves and hypogenic karst emerges as a result of artesian flow (Klimchouk et al., 2000). (An overview of this process is provided in Equations S1–S3.) As the limestone loses mass, microbial and chemical processes collectively drive a pedogenic erosion process that forms a bedrock-facing punk rock layer (Hill, 1987) and a cave-passage-facing oxide layer (Spilde et al., 2006). This process results in the formation of corrosion residues, or ferromanganese deposits (FMD), called speleosol (Spilde et al., 2009). The iron and manganese-rich composition of the oxide layers in speleosol is the result of microbial interactions with the minerals at the interface of the bedrock, punk rock, and oxide layer (Spilde et al., 2006; 2009).

Extremophiles are microorganisms that survive under conditions that are inhospitable to most life on Earth and are expected to be discovered in dry sulfuric karst caves given that the conditions are oligotrophic (limited access to essential nutrients), endolithic (ability to grow within rock or mineral pores) (Wierzchos et al., 2011), xerophilic (limited access to water) (Forbes, 1998; Lebre et al., 2017), and troglomorphic (ability to grow within caves). Elucidating microbial life inhabiting extremophilic subterranean zones, including cave and karst systems, has many benefits. For example, it expands our concept of hospitable zones when searching for astrobiological life on other planets (Northup et al., 2011; Wierzchos et al., 2011; Lusk et al., 2019a). Next, the discovery of new microorganisms and genes from caves has led to the development of antibiotics and antifungals that may suppress the spread of diseases, including white-nose syndrome (WNS) in bats (Bleher et al., 2009; Hamm et al., 2017). Lastly, finding microbes that interact with metals and ores will assist in the discovery of biocatalysts, which can be used to interface living cells with materials for the production of biotechnology, including microbial electrochemical cells (Eddie et al., 2016; Lusk et al., 2018a; Sun et al., 2018).

Understanding the fundamental interactions of abiotic materials with biotic life, including microorganisms that are able to reduce and oxidize materials to produce or dissolve solid metals and ores, will enhance our understanding of the biogeochemical processes that shape the Earth (Eddie et al., 2016; Lusk et al., 2018a; 2019a; Sun et al., 2018). Spe-

¹Central Arizona Grotto of the National Speleological Society, Phoenix, AZ, United States of America.

²ScienceTheEarth, Mesa, AZ, United States of America.

^CCorresponding author: ScienceTheEarth@gmail.com, ORCID: 0000-0002-3094-805X.

leptosols in caves, for example, provide an ideal habitat for investigating robust microbial communities associated with geomicrobially-mediated sulfuric acid speleogenesis. Given the variety of regions and conditions in which geomicrobially-mediated sulfuric acid speleogenesis occurs (Engel et al., 2004), a robust and systematic study of microorganisms residing in caves is essential to develop a comprehensive explanation of this phenomenon.

Grand Canyon Caverns (GCC) is a commercial dry cave in northwest Arizona, USA that was formed via sulfuric acid speleogenesis. In February 2013, approximately 100 meters of steeply up-trending new passages and rooms were discovered containing multicolored corrosion residues composed of punk rock (Hill, 1987) and containing an oxide layer, also called speleosol (Spilde et al., 2006; 2009). Similar deposits in Lechuguilla and Spider Caves located in the Guadalupe Mountains of New Mexico were previously observed to harbor microbial life (Northup et al., 2003; Spilde et al., 2006). The formations discovered in GCC were kept isolated from tourists for the purpose of conducting research on their microbial inhabitants. To assess the diversity of microorganisms, 14 dry samples from three separate areas were collected from sections throughout the cave that had minimal perturbation from human activity (sampling locations shown in Fig. 1). Samples were analyzed for their elemental, mineralogical, and microbial composition.

MATERIALS AND METHODS

Cave Description and Overview

GCC is a commercial dry sulfuric karst cave in northwest Arizona, USA ($35^{\circ}31'41.9''$ N $113^{\circ}13'52.7''$ W) that is located about 100 kilometers south of the southern rim of Grand Canyon National Park. The cave is positioned in the upper part of the Redwall limestone from the Late Devonian / Early Mississippian periods (Huddle and Dobrovolsky, 1952) with a small, inactive volcano located five kilometers northwest. The Redwall limestone dates back to approximately 350 Ma and is composed of 99.5 % pure limestone (Gootee, 2014; 2019). During this period, the region was part of a shallow tropical sea in the equatorial continent of Laurentia (Price, 1999; Gootee, 2019). Presently, the cave has no known freestanding bodies of water and has an average relative humidity of 79.5 % and temperature of 15°C . There are no indications of the presence of non-microbial life in the cave; however, there have not been surveys for non-microbial life in GCC.

A map of the cave, including sample collection locations, is shown in Figure 1. Annually, thousands of tourists visit GCC. At the time of sample collection, cave tours were limited to the main sections of GCC including the Chapel of Angels, the Halls of Gold, Snowball Palace, and the Mystery Chamber, and ceased at gates 1 and 2. Gates 1 and 2 were installed to limit access to newly-discovered areas of the cave for the purpose of mapping and conducting research. Due to the observation of multicolored speleosol deposits on the cave walls on top of the underlying limestone (Fig. 2), 14 samples were collected from Sugar Hill, below the ropes to Disappointment Dome, and Disappointment Dome. Since collecting samples, tourism has now extended to Sugar Hill and below the ropes to Disappointment Dome.

Sample Collection

Sterile Falcon tubes (15 mL) were used to store 14 samples (~500 mg each) that were collected in the cave. Each sample was collected by scraping the Falcon tube directly against the cave surface to collect solid surface deposits of

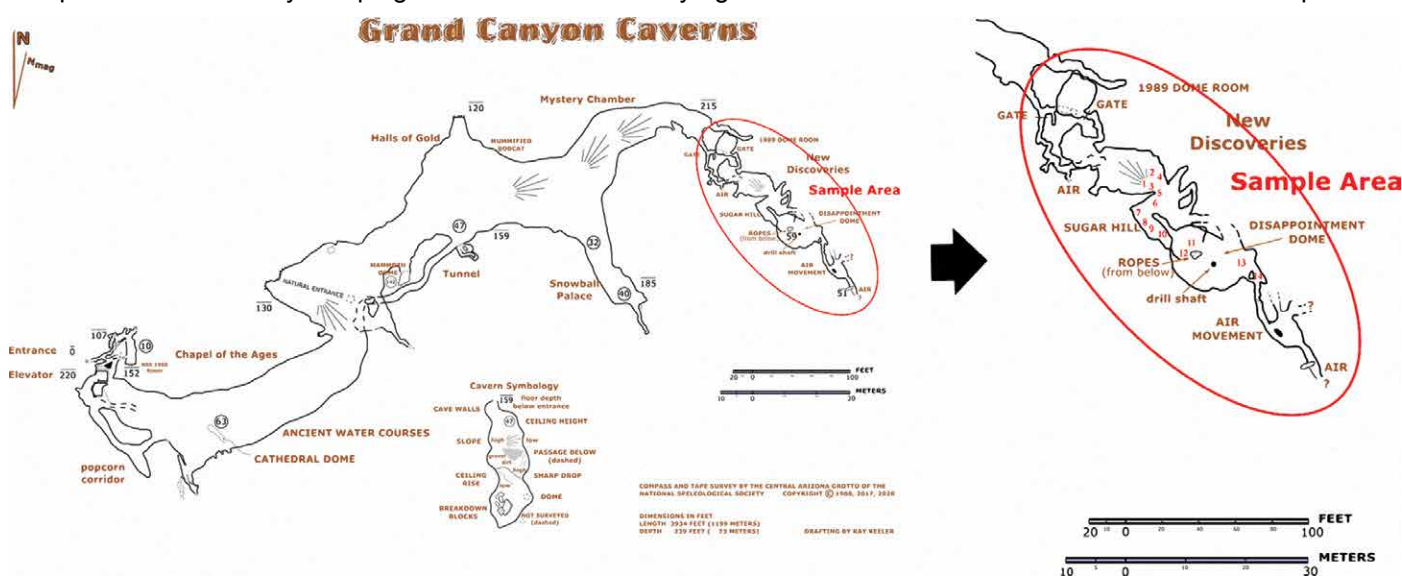


Figure 1: Cave map for Grand Canyon Caverns. Inset on right shows collection locations for 14 samples. The three major sampling locations were Sugar Hill, below the ropes to Disappointment Dome, and Disappointment Dome. At the time of collection, active tourism in the cave ceased at gates 1 and 2.

speleosol from the cave walls. After placing the lid on the Falcon tube, each tube was wrapped in aluminum foil, placed on icepacks, stored in an ice chest, and transported to a -80°C freezer. All samples were collected in the deep zone of the cave, at least 200 m from the nearest entrance with no surface visible light. Sample collection locations varied from a meter to several meters apart and are shown in the inset to Figure 1.

Sample Identification via Raman Spectroscopy and CrystalSleuth

The mineral composition of each rock sample was identified using a Renishaw via Reflex Micro-Raman confocal Raman Microscope (Renishaw, West Dundee, Ill., U.S.A.) equipped with a Leica 566066 N PLAN EPI 20X/0.4 (Leica, Wetzlar, Germany) objective lens and two laser excitation sources: a near infrared diode laser source (300 mW) with an excitation wavelength of 785 nm and an Argon ion laser source (25 mW) with an excitation wavelength of 514 nm. For spectra captured at 785 nm, laser power was varied from 1–50 %, exposure time set to 1–3 seconds, and spectra were averaged over five accumulations. For spectra captured at 514 nm, laser power was set to 100 %, exposure time set to one second, and spectra were averaged over twelve accumulations. For each sample, spectra at 785 nm and 514 nm were taken

at three different locations. After acquiring Raman spectra, light images were acquired under white light using the same objective lens. Spectra were compared to the RRUF Project database using the CrystalSleuth Application Version: May 19, 2008 (Laetsch and Downs, 2006). For identifying samples, background was subtracted, cosmic ray events (CRE) were removed, and a cutoff of 90 % similarity was used to determine a match. Filtered spectra with corresponding light images can be found in Figure S2 GCC1-14.

Digestion and Quantification of Elements

Samples were digested (255.3 ± 6.2 mg) using a MARS 5 Microwave Digester (CEM Corporation, Matthews, N.C., U.S.A.). For the microwave process, each sample was weighed and 10

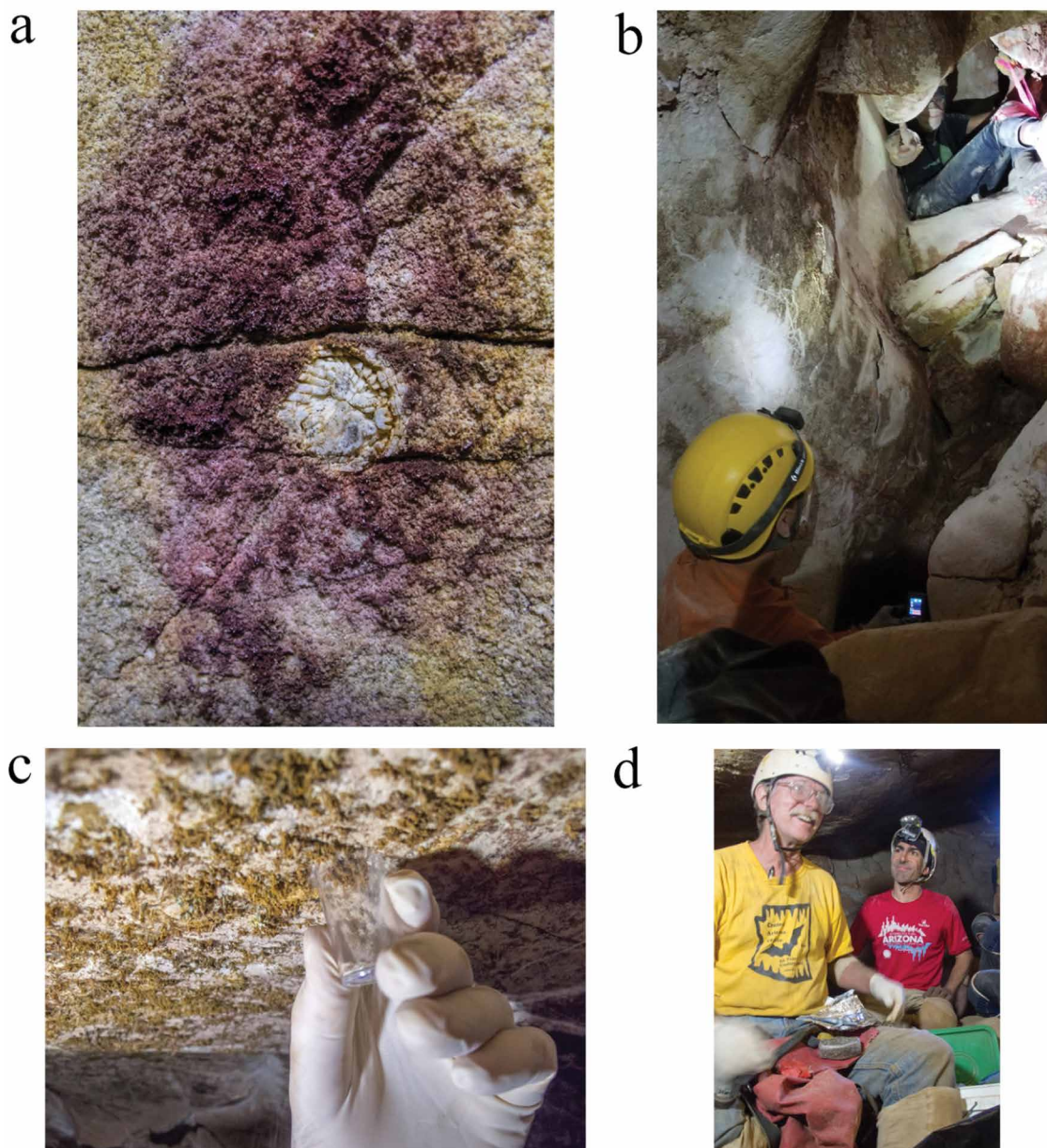


Figure 2: a) Multicolored, powdery speleosol deposit on the cave wall surrounding a Late Devonian/ Early Mississippian period rugose coral fossil (photo credit: Paul Jorgenson). b) Brown colored powdery speleosol deposit on cave wall with cavers shown for scale. c) Collecting a sample from a yellow colored speleosol deposit (photo credit: Paul Jorgenson). d) Co-authors Raymond Keeler and Bradley Lusk wrapping samples in foil and collecting video documentation of cave expedition (photo credit: Paul Jorgenson).

mL of concentrated trace metal grade nitric acid was added. All sample tubes were heated to 180 °C in 5 minutes and held at 180 °C for 10 minutes in a 1600 W microwave. After heating, samples were cooled for 5 minutes. The elemental composition of samples was analyzed using a Thermo iCap 6300 inductively coupled plasma optical emission spectrometer (ICP-OES) (Thermo Fisher Scientific, Waltham, Mass., U.S.A.) with a duo plasma source. Samples were diluted at 1:10 with nano-pure water to acquire accurate measurements for the following metals: Ag, Al, As, B, Ba, Be, Bi, Cd, Co, Cr, Cu, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Si, Sr, Ti, V, and Zn. Due to the high concentration of Ca and Fe, separate samples for these elements were diluted at 1:1000 with nano-pure water. Sample 5 did not contain enough mass for analysis using the ICP-OES.

For quantification of total C, H, and N, a Perkin Elmer PE 2400 Elemental Analyzer was used with 5.4 ± 0.6 mg per sample (<https://cores.research.asu.edu/metals-environmental-and-terrestrial-analytical-laboratory/equipment/analytical-chn-elemental>). Samples were flash combusted at 1760 °C. Resulting gases were chemically scrubbed of the halogens (and of sulfur in the CHN mode) and were separated in a GC column. Detection was conducted by a thermal conductivity detector (TCD).

Scanning Electron Microscopy (SEM) and Color Images

Approximately 5 mg from each of the 14 samples was fixed with 4 % glutaraldehyde for 12 h at 4 °C and then washed and stored in 10 mM PBS (pH 7) solution for ~1 h. Next, the samples were treated with 1% osmium tetroxide for 15 min, followed by graded ethanol series dehydration (50%, 70%, 95%, and 100% for 5 min each), then critical point dried, and ultimately mounted on an aluminum stub. Samples were sputter-coated with Au/Pd alloy with a Technics Hummer II sputter coater. Imaging was conducted using a JEOL JSM6300 SEM at 15X – 95kX with an accelerating voltage of 15 kV. Color images of samples (Fig. 3) were taken with a Samsung Galaxy S7 under white light conditions.

DNA Extraction and Library Preparation for 16S rRNA Sequencing

DNA from the 14 samples was extracted using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, Calif., U.S.A.) per the manufacturer's manual. DNA was released from cells via bead beating using PowerBead tubes (MO BIO, Carlsbad, Calif., U.S.A.). Amplicon sequencing of the V4 region of the 16S rRNA gene was performed with the barcoded primer set 515f/806r designed by Caporaso et al. (2012), following the protocol proposed by the Earth Microbiome Project (EMP <https://earthmicrobiome.org/protocols-and-standards/16s/>) for the library preparation. PCR amplifications for each sample were done in triplicate, then pooled and quantified by using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, ThermoFisher Scientific, Waltham, Mass., U.S.A.). A total of 240 ng of DNA per sample was pooled and then cleaned using QIA quick PCR purification kit (QIAGEN, Valencia, CA, USA). The PCR pool was quantified by Illumina Library Quantification Kit ABI Prism (Kapa Biosystems, Wilmington, MA, USA). The DNA pool was determined and diluted to a final concentration of 4 nM, then denatured and diluted to a final concentration of 4 pM with 30 % PhiX. Finally, the DNA library was loaded in the MiSeq Illumina Sequencer (Illumina, San Diego, CA, USA) using the chemistry version 2 (2 × 150 paired-end) and following manufacturer directions. All samples were sequenced in the Microbiome Analysis Laboratory at Arizona State University (Tempe, Ariz., U.S.A.).



Figure 3: Macroscopic images of samples 1–14.

Determination of Microbial Taxonomy

The analysis of the 16S rRNA gene sequences was performed using the Quantitative Insights into Microbial Ecology 2 software package (QIIME2, version 2018.4) (Bolyen et al., 2019). Sequences were open reference clustered into Operational Taxonomic Units (OTUs) against the Silva-132

database (Quast et al., 2013) using an identity threshold of 97 % with the vsearch algorithm (Rideout et al., 2014). After the alignment of the sequences, the OTU table was constructed and then filtered for the removal of singletons, and chimeras and borderline chimeras using vsearch uchime-denovo (Edgar et al., 2011). Eukaryotic homologues were also removed. The OTU table was rarefied to the minimum number of sequences obtained among the samples (GCC 12 with 44,297 sequences) and an alpha diversity rarefaction table was constructed using 10 iterations for each sample data point. Finally, the taxonomy was assigned to OTUs using a Scikit-learn naïve-Bayes taxonomy classifier against the Silva_132_97_16s OTUs reference sequences (Pedregosa et al., 2018), with counts for classified OTUs at all phylogenetic levels (Table S1), and an Excel file with all observed OTUs (Table S2).

RESULTS AND DISCUSSION

This research study assesses the mineralogical and microbial diversity of multicolored speleol deposits found in GCC. These deposits ranged from several centimeters to several meters spanning entire walls of the cave. Similar formations have been observed in Lechuguilla and Spider Caves in New Mexico, United States (Northup et al., 2003), the Cave of Altamira in Spain (Jurado et al., 2007), Roraima Sur Cave in Venezuela (Barton et al., 2014), and Asperge Cave in France (Tisato et al., 2015), Figure 2a-d shows examples of formations from which samples were collected and the acquisition of samples within the cave. As shown in Figure 2a, some multicolored formations were found surrounding fossils of rugose coral from the Late Devonian / Early Mississippian period (Huddle and Dobrovolny, 1952; Pedder and Murphy, 2004; Denayer et al., 2012). Figure 2b shows a deposit spanning approximately three meters of a wall with surveying cavers to add scale. Figure 2c shows sample collection directly from the cave walls and Figure 2d shows cavers wrapping samples into foil and documenting the sample locations.

Speleol samples collected at 14 sites in the cave varied in color and consistency (Fig. 3). Raman spectroscopy was used to determine the mineral contents of each sample (Table 1) by comparing spectra to the RRUFF™ Project database using the CrystalSleuth Application Version: May 19, 2008 (Laetsch and Downs, 2006). The samples were primarily composed of calcite, hematite, paraspurrite, quartz, and trattenite. A detailed description of each sample can be found in the supplemental information and filtered spectra with light microscopy images in Figure S2 GCC1-14.

An elemental analysis was done on GCC samples (Tables 2a-b). The composition of the speleols varied by sample with calcium (6200 ± 3494 ppm), iron (1141 ± 1066 ppm), magnesium (25 ± 17 ppm), and phosphorous (37 ± 33 ppm) the most prevalent elements detect-

Table 1. Identity of compounds observed within samples collected from 14 collection sites in Grand Canyon Caverns as revealed by comparing Raman spectra collected using either a 514 nm or 785 nm laser source to the RRUFF Project database. Only spectra which shared at least 90 % similarity are shown with the exception of Sample 9, as indicated by *. The * indicates that no match values higher than 90 % were observed under this condition. Order of percentages coincides with the order of RRUFF ID.

Sample	Wavelength = 514 nm			Wavelength = 785 nm		
	Compound	% Match	RRUFF ID	Compound	% Match	RRUFF ID
GCC 1	Calcite	99, 98, 97	R050127, R050127, R050127	Calcite	92	R040170
GCC 2	Calcite	99, 98	R050127, R050127	Calcite, Paraspurrite	94, 90, 93	R040070, R050009, R060707
GCC 3	Calcite	98	R050127	*	*	*
GCC 4	Calcite	97, 95	R050307, R050127	*	*	*
GCC 5	Calcite	99	R050127	Calcite	96	R040170
GCC 6	Quartz	99, 99	R050125, R040031	Trattenite	91, 90	R060623, R060623
GCC 7	Quartz	99	R040031	*	*	*
GCC 8	Calcite	97	R050127	*	*	*
GCC 9	Gypsum	[85]	R040029	*	*	*
GCC 10	Calcite	96	R050127	*	*	*
GCC 11	Calcite	96, 93	R050128, R050307	*	*	*
GCC 12	Calcite	98, 98, 95	R050127, R050307, R050166	Calcite	98, 95	R040070, R050009
GCC 13	Calcite	96, 94	R040170, R050128	Paraspurrite, Calcite	92, 91	R060707, R050009
GCC 14	Hematite	92	R050300	*	*	*

ed across all samples. Aluminum (49 ± 57 ppm) was observed in seven samples, manganese (43 ± 58 ppm) in 12 samples, potassium (93 ± 88 ppm) in 13 samples, and silicon (5 ± 2), sodium (5 ± 5), and zinc (23 ± 62) in all samples. Total carbon, hydrogen, and nitrogen made up $4.7 \pm 4.9\%$, $0.3 \pm 0.4\%$, and $0.1 \pm 0.1\%$ of samples, respectively (Table 3).

Due to the mixed nature of each sample, it is possible that minerals present in small quantities are difficult to observe using Raman spectroscopy since they are masked by more ubiquitous compounds. Nevertheless, the prevalence of Ca, C, Fe, Mg, Na, P, Si, and Zn are likely the result of the hematite, paraspurrite, trantnerite, and limestone that contributes to the composition of the punk rock and underlying cave walls. The high Fe content likely is due to the accumulation of iron oxides resulting from microbial-induced enrichment in the oxide layer of the speleosol (Spilde et al., 2009), although auto-oxidation of ferrous iron at neutral pH may also play a role (Emerson and Moyer, 1997). The presence of Al, Ca, Fe, K, Mg, Mn, and Si corroborates previous literature documenting microbial interactions with minerals in ferromanganese deposits (FMD), or speleosol, including todorokite, lepidocrocite, goethite, illite, and hematite (Northup et al., 2003; Spilde et al., 2005).

Scanning electron microscopy (SEM) on each of the samples (Fig. 4) revealed the presence of diverse microbial morphologies associated with the speleosol. Diverse biological communities have also been observed and associated with cave mineralogy in other cave sites (Cunningham et al., 1995; Boston et al., 2009; Dhami et al., 2018). Although SEM cannot be used to identify specific bacteria, GCC 3, 4, 7, 10, and 14 show the presence of cells with similar morphology to those observed in extremophilic and mesophilic dissimilatory metal reducing bacteria that metabolize metal oxides (Spilde et al., 2005; El-Naggar et al., 2010; Parameswaran et al., 2013; Lusk et al., 2015a,b; 2016; 2018a; 2019a; Wang et al., 2019).

The GCC microbial community consisted of 2207 operational taxonomic units (OTUs) according to species-level annotations, representing 55 phyla. (See Table S1 for sample overview and Table S2 for taxonomy assignments of all OTUs.) As shown in Figure 5, of the 55 phyla found among all samples, the five most abundant bacterial phyla were *Actinobacteria* $51.3 \pm 35.4\%$, *Proteobacteria* $12.6 \pm 9.5\%$, *Firmicutes* $9.8 \pm 7.3\%$, *Bacteroidetes* $8.3 \pm 5.9\%$, and *Cyanobacteria* $7.1 \pm 7.3\%$, while the relative abundance of Archaea represented $1.1 \pm 0.9\%$ of all samples and $0.2 \pm 0.04\%$ of sequences were unassigned. The rarefaction curve for α -diversity in 14 samples (Fig. S1) shows a slope nearing zero for all samples except GCC2, suggesting the data shown represent the true diversity of the samples with the exception of GCC2, which may have greater diversity.

Gram-positive bacteria from the phylum *Actinobacteria*, the most prevalent phylum found in GCC, have been reported to generate several bioactive compounds, produce two-thirds of all clinically used naturally-derived antibiotics, and have been indicated as likely candidates for the development of new antibiotics and antifungals (Nimaichand et al., 2012; Barka et al., 2016; Maciejewska et al., 2016; Rangseekaew and Pathom-Aree, 2019). For example, the genera *Amycolatopsis* ($1.6 \pm 2.3\%$) and *Pseudonocardia* ($16.2 \pm 17.0\%$) were present in all samples and previous studies have indicated that these genera may have antifungal properties (Sen et al., 2009). Furthermore, the *Streptomyces* genus, a common in-

Table 2a. Total amount in mg/L of each measured element as a function of sample number. GCC5 is not shown due to insufficient sample mass for analysis.

Sample	Digest, mg	Ag	Al	As	B	Ba	Be	Bi	Ca	Cd	Co	Cr	Cu	Fe	K	Li
GCC1	258.6	0.00	23.00	2.47	1.15	2.95	0.04	0.00	9229	0.16	0.13	1.12	0.16	414.30	9.92	0.63
GCC2	262.6	0.01	6.02	2.73	0.62	1.56	0.02	0.00	9458	0.02	0.02	0.14	0.08	217.80	3.42	0.16
GCC3	251.6	0.00	104.50	16.57	0.44	4.72	0.22	0.11	5882	0.21	0.34	5.63	1.06	2218	27.56	0.92
GCC4	250.9	0.00	0.00	2.46	0.45	5.12	0.07	0.00	9338	0.41	0.32	5.67	0.37	636.80	110.30	2.83
GCC6	264.4	0.00	0.00	0.20	0.36	2.17	0.08	0.13	182.4	0.04	0.41	2.25	0.00	2368	198.40	0.32
GCC7	260.0	0.14	0.00	15.33	0.21	39.27	0.36	0.11	1110	0.98	1.87	17.48	3.07	2663	148.50	20.71
GCC8	241.5	0.00	0.00	3.64	0.30	21.68	0.08	0.00	6734	0.08	0.49	12.24	0.28	445.80	165.50	1.15
GCC9	258.3	0.00	148.00	9.70	0.02	0.46	0.05	0.00	6570	0.04	0.11	0.49	0.31	935.60	71.32	0.15
GCC10	258.2	0.00	0.00	11.31	0.18	0.63	0.08	0.05	2813	0.04	0.45	1.08	0.52	1360	221.20	0.68
GCC11	255.2	0.00	56.13	3.20	0.07	0.31	0.03	0.00	7041	0.02	0.20	0.68	0.15	314.90	29.22	0.16
GCC12	257.1	0.00	1.58	0.16	0.00	0.16	0.00	0.00	10750	0.01	0.00	0.06	0.01	63.90	0.00	0.04
GCC13	251.1	0.00	6.62	0.70	0.00	2.22	0.01	0.00	8979	0.02	0.04	0.21	0.09	154.10	2.38	0.02
GCC14	249.8	0.00	0.00	16.16	0.50	3.77	0.11	0.00	2512	0.09	0.38	1.82	0.50	3046	218.90	0.16

Table 2b. Total amount in mg/L of each measured element as a function of sample number. GCC5 is not shown due to insufficient sample mass for analysis.

Sample	Digest, mg	Mg	Mn	Mo	Na	Ni	P	Pb	S	Sb	Se	Si	Sr	Ti	V	Zn
GCC1	258.6	7.62	131.50	0.09	5.46	0.64	25.06	2.43	0.83	0.09	0.00	5.62	0.98	1.38	1.26	6.29
GCC2	262.6	4.68	16.90	0.02	2.68	0.09	1.35	0.22	0.00	0.06	0.00	3.99	0.63	0.00	0.48	1.20
GCC3	251.6	25.25	73.18	0.34	2.51	1.71	15.43	6.56	0.82	0.14	0.17	4.61	1.91	0.00	3.43	14.31
GCC4	250.9	26.83	0.00	0.47	3.18	1.62	30.45	10.89	2.33	0.09	0.00	5.64	2.37	3.34	3.15	24.37
GCC6	264.4	40.55	6.45	0.84	21.50	0.51	37.08	1.88	1.62	0.08	0.31	5.76	1.38	0.00	4.19	1.44
GCC7	260.0	35.26	0.00	2.59	5.38	11.14	95.57	73.86	6.42	0.21	0.00	6.37	1.88	17.11	14.41	227.90
GCC8	241.5	31.87	188.50	0.72	2.43	0.93	11.39	3.44	1.80	0.13	0.41	5.31	3.15	1.73	1.79	8.72
GCC9	258.3	27.58	7.10	0.00	5.98	0.29	22.15	1.13	29.12	0.05	0.06	4.99	1.62	0.00	1.42	1.96
GCC10	258.2	63.62	27.94	0.89	7.93	1.50	79.59	1.70	51.05	0.06	0.02	4.49	1.42	0.00	1.86	4.09
GCC11	255.2	16.72	8.53	0.00	2.62	0.27	24.13	0.54	0.96	0.06	0.01	5.54	1.34	0.00	0.56	1.77
GCC12	257.1	4.26	3.31	0.00	0.29	0.02	5.70	0.02	0.00	0.03	0.00	0.45	1.88	0.09	0.07	0.30
GCC13	251.1	8.99	33.27	0.00	1.80	0.44	38.91	0.11	0.42	0.03	0.00	2.61	0.92	0.39	0.58	0.53
GCC14	249.8	32.92	66.64	0.86	2.40	1.77	98.34	3.13	1.68	0.12	0.00	5.36	0.70	0.00	3.22	2.73

habitant of limestone caves (Nimaichand et al., 2012; Také et al., 2018) and responsible for most of the commercially-available naturally-derived antibiotics on the market (Bérdy, 2005; Barka et al., 2016), composed 2.4 ± 3.0 % of sequences observed across all samples. Novel *Streptomyces* species discovered in other caves located in the western United States have shown antifungal effects on *Pseudogymnoascus destructans*, a fungus that causes white-nose syndrome (WNS) in bats (Bleher et al., 2009; Hamm et al., 2017). Lastly, xerophilic members of the *Actinobacteria* phylum including the genera *Geodermatophilus* and *Modestobacter* from the *Geodermatophilaceae* family (Montero-Calasanz et al., 2012; 2013), the *Rubrobacteridae* family (Bull and Asenjo, 2013), and the *Streptomyces* genus (Kurapova et al., 2012; Mohammadipanah and Wink, 2016) account for a relative abundance of 2.7 ± 2.8 % across all samples observed from the cave.

Cyanobacteria of the family *Phormidiaceae* (0.5 ± 0.7 %) were observed in all GCC samples. They were previously observed dwelling in troglomorphic conditions within caves, including Carlsbad Cavern (Behrendt et al., 2019), and are capable of surviving for prolonged periods of time in complete darkness (Hader and Poff, 1982; Montechiaro and Giordano, 2006). Photosynthetic bacteria may be able to persist in the deep zones of the cave that harbor minimal or no surface visible light by having chlorophylls that absorb near infrared radiation (NIR) that is reflected across the limestone walls of the cave (Behrendt et al., 2019).

In addition, bacteria from the *Porphyromonadaceae* family in the phylum *Bacteroidetes* are commonly associated with acid mine drainage and sulfur reducing microbial communities (Gaidos et al., 2009; Bijmans et al., 2010) were present in nine of the 14 samples (0.14 ± 0.14 %). Finally, bacteria from the *Arcobacter* genus in the class *Epsilonproteobacteria* were observed in all samples (1.0 ± 0.8 %); previous research has indicated that these microorganisms are autotrophic and produce globular or filamentous sulfur as a metabolic end product (Wirsén et al., 2002).

Nitrogen, an essential component of amino acids and nucleotides, was found in concentrations of 0.1 ± 0.1 %, and was not de-

Table 3. Total amount, in % of carbon, hydrogen, and nitrogen as a function of sample number.				
Sample	Digest, mg	Carbon, %	Hydrogen, %	Nitrogen, %
GCC1	5.06	11.50	0.03	0.09
GCC2	5.03	11.89	0.00	0.05
GCC3	4.96	7.49	1.33	0.08
GCC4	5.00	8.68	0.04	0.08
GCC5	4.95	2.36	0.32	0.05
GCC6	6.63	0.25	0.35	0.05
GCC7	6.03	0.96	0.79	0.08
GCC8	5.95	9.19	0.05	0.15
GCC9	5.88	9.19	0.05	0.15
GCC10	6.25	0.59	0.58	0.05
GCC11	5.35	0.21	0.12	0.00
GCC12	5.45	0.14	0.02	0.00
GCC13	4.65	0.21	0.00	0.00
GCC14	4.44	0.34	0.09	0.00

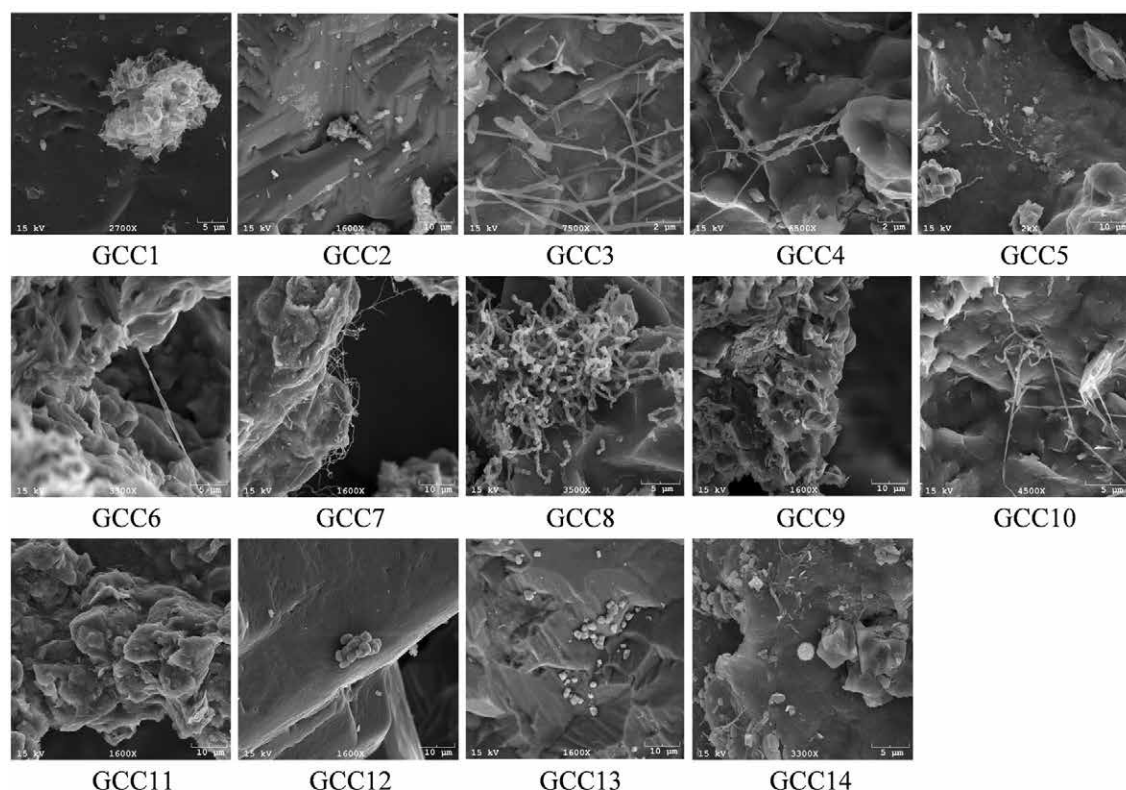


Figure 4: Scanning electron images of Samples 1-14. SEM images contain examples of multiple morphologies from diverse microorganisms. Images are at following magnifications: GCC1 2700x, GCC2 1600x, GCC3 7500x, GCC4 6500x, GCC5 2000x, GCC6 3300x, GCC7 1600x, GCC8 3500x, GCC9 1600x, GCC10 4500x, GCC11 1600x, GCC12 1600x, GCC13 1600x, GCC14 1600x.

et al., 2018) and *Burkholderiales* (Rosenblueth et al., 2018) orders suggests that nitrogen fixation may play a role in microbial communities occupying GCC (Northup et al., 2003; Wagner, 2011); however, this hypothesis requires further investigation.

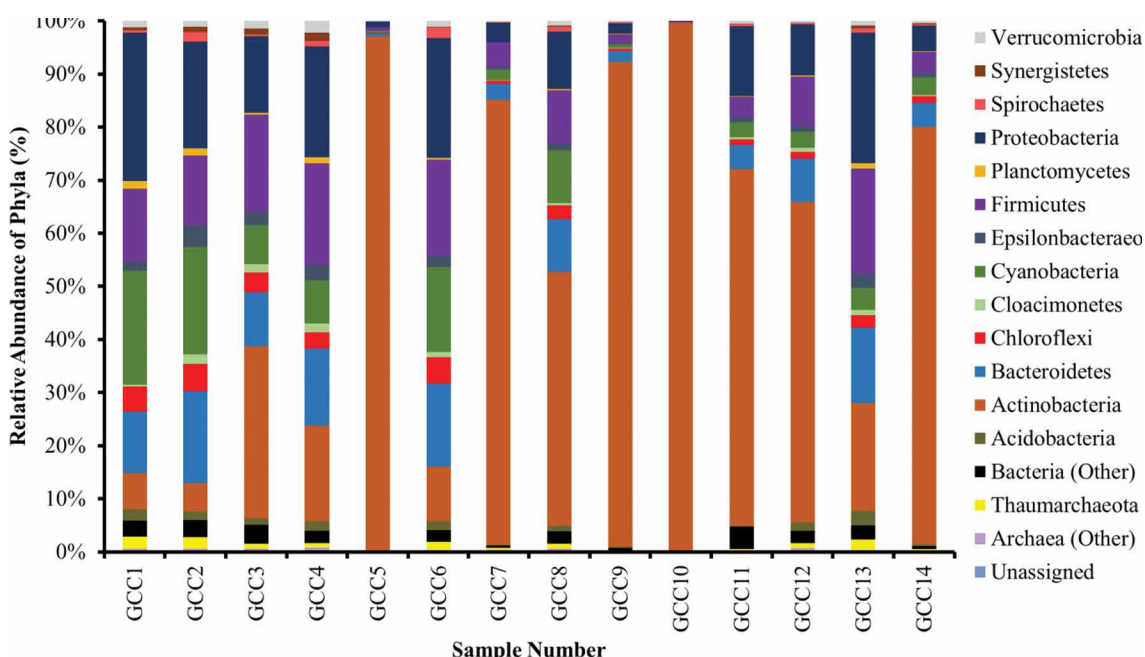


Figure 5: Relative abundance of bacterial and archaeal taxonomy at phylum level. All phyla with at least ~1% relative abundance in one or more samples are listed. Remaining phyla within the Bacteria kingdom are represented in the Bacteria (Other) category. Remaining phyla within the Archaeal kingdom are represented in the Archaea (Other) category. Unassigned sequences ($0.2 \pm 0.04\%$) are also represented.

ected in samples GCC 11, 12, 13, or 14 (Table 3). This concentration of N is comparable to those observed in other caves containing nitrogen-fixing bacteria (Northup et al., 2003). The prevalence of diazotrophs ($2.1 \pm 1.7\%$) including methanogenic *Euryarchaeota* (Murray and Zinder, 1984; Leigh, 2000; Bae et al., 2018), bacteria of the *Clostridium* genus (Chen et al., 2001), *Frankiaceae* from the *Actinobacteria* phylum (Santi et al., 2013), and *Rhizobia* from the *Rhizobiales* (Diaz-Herraiz et al., 2014; Garrido-Oter

In addition, previously characterized microorganisms that are able to reduce and oxidize materials to produce or dissolve solid metals and ores were present in all samples ($3.8 \pm 2.9\%$). Observed genera include: *Geothrix* (Coates et al., 1999), *Pedomicrobium* (Northup et al., 2003), *Thiobacillus* (Provenzio et al., 2001), *Geobacter* (Bond and Lovley, 2002), *Arcobacter* (Wirsén et al., 2002), *Marinobacter* (Bo-

nis and Gralnik, 2015), *Desulfovibrio* (Payne et al., 2002), *Desulfuromonas* (Roden and Lovley 1993), *Pseudomonas* (Caspi et al., 1998), and *Shewanella* (El-Naggar et al., 2010), and the families *Desulfobulbaceae* (Pfeffer et al., 2012) and *Rhodocyclaceae* (Zhou et al., 2016). These dissimilatory metal-reducing and/or oxidizing bacteria are able to use Fe (observed in all samples at 1141 ± 1066 ppm) or Mn (observed in samples GCC 1–3, 6, and 8–14 at 43 ± 58 ppm) as an electron source or as a terminal electron acceptor, as shown in Equations S4–S7.

Conclusion and Future Research

The formation of caves and karst systems relies on a complex interplay between abiotic and biotic geochemical activity (Hose et al., 2000; Kappler et al., 2005; Barton and Northup, 2007; Boston et al., 2009). In many cases, microbial communities contain the metabolic capacity to oxidize and reduce surrounding metallic ores, which greatly influences the structure and mineralogy of the cave environment (Lovley, 1993; Northup et al., 2003; Spilde et al., 2006, 2009). The microbial community data presented in this study suggests that Grand Canyon Caverns (GCC), a dry sulfuric karst cave, harbors robust extremophilic, oligotrophic, endolithic, xerotolerant, and troglomorphic microbial life within multicolored speleosol deposits found overlying its limestone walls. However, while sequencing results indicate that most of the microbial diversity within the cave speleosol has been accounted for (Fig. S1), Archaeal diversity may not be fully represented given the bias the 515f-806r bacterial/archaeal primer pair has against *Crenarchaeota/Thaumarchaeota* (Hugherth, 2014), environmental Archaea, and certain clades of Bacteria (Morris et al., 2002).

Based on the roles microbes observed in GCC play in similar caves, they are hypothesized to utilize a large repertoire of metabolic pathways including nitrogen fixation (Northup et al., 2003; Wagner, 2011), chemolithoautotrophic carbon fixation (Ramos, 2003), and dissimilatory metal reduction and oxidation (Angert et al., 1998; Povencio and Polyak, 2001; Northup et al., 2003; Macalady et al., 2006) that enables them to survive using a diverse set of electron donors and acceptors, including solid ores. Microbial reduction and oxidation of surrounding minerals and the corresponding shift in the elemental composition and oxidation states of these minerals may explain the diversity of colors observed in the cave speleosol and in the collected samples (Fig. 2 and 3). However, future studies to observe the proteomic, metagenomic, and transcriptomic profiles of the communities will offer more insight into their functional and metabolic roles.

Furthermore, finding correlations between microbial communities, surrounding cave formations, and metabolic functions may elucidate communal interactions between phyla and metabolic interactions with abiotic materials as shown in previous studies (Torres et al., 2009; Jones and Bennet, 2017). Next, a systematic study of the microbial communities collected in the cave via lab culturing will provide greater insight into the roles of these microbes in the cave ecosystem including determining the presence of carbon fixation, nitrogen fixation, or dissimilatory metal reduction/oxidation.

In addition, the cave is currently undergoing active exploration and since collecting samples for this study, new passages containing large multicolored speleosol deposits have been discovered, with a breakthrough in 2017 contributing to a ~5 % expansion in the size of the cave. The location of this discovery can be seen in Figure 1, southeast of Disappointment Dome, where the map is labelled too tight. As uncharted areas of the cave continue to be unveiled, analyzing the microbial communities inhabiting these pristine locations may lead to discoveries of novel microorganisms (Jurado et al., 2006, 2007; Bonis and Gralnik, 2015; Hamm et al., 2017) or further elucidate the role of microorganisms in the biogeochemistry of cave and karst systems (Kappler et al., 2005). The presence of microbes previously associated with the production of antifungals for WNS (Hamm et al., 2017) and the development of next generation biofuels (Bond and Lovley, 2002; Rittmann, 2008) and biotechnology (Zhou et al., 2016; Lusk et al., 2018b) also emphasizes the importance of further research to evaluate these possibilities and continued exploration of dry sulfuric cave and karst systems.

Although there are no indications of the presence of non-microbial life in the cave, future studies should investigate the presence of eukaryotic life including invertebrates, yeasts, and other fungi. In addition, human traffic in rooms where samples were collected for this study, including Sugar Hill and below the ropes to Disappointment Dome, has increased so that visitors can observe the speleosols and sampling sites. A longitudinal study can be administered to assess the impact of human activities on the cave microbiome (Leuko et al., 2017) compared to pristine areas. Future investigations may elucidate how human activity indirectly impacts the development of cave and karst systems by influencing the resident microbial communities.

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