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# Comparative microbial community composition from secondary carbonate (moonmilk) deposits: implications for the *Cansiliella servadeii* cave hydropetric food web

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**Abstract:** The microbial diversity of moonmilk, a hydrated calcium carbonate speleothem, was evaluated from two Italian caves to provide context for the food web of highly-specialized troglobitic beetles, *Cansiliella* spp. (Leptodirinae), with distinctive carbon and nitrogen isotope values indicative of a novel food source. The moonmilk and associated percolating waters had low to no extractable chlorophyll, with an average organic C:N ratio of 9, indicating limited allochthonous input and a significant contribution from microbial biomass. The biomass from moonmilk was estimated to be  $\sim 10^4$  micro- and meiofaunal individuals per m<sup>2</sup> and  $\sim 10^7$  microbial cells/ml. Proteobacteria dominated the 16S rRNA gene sequences retrieved from the moonmilk from both caves. The distribution of other proteobacterial classes and phyla in the moonmilk were statistically similar to each other, even though the two caves are geographically separated from each other. Comparing the moonmilk gene sequences to sequences from previously described environmental clones or cultured strains revealed the uniqueness of the moonmilk habitat, as  $\sim 15\%$  of all of the moonmilk sequences were more closely related to each other than to sequences retrieved from any other habitat. However, comparative analyses confirmed that as much as  $\sim 34\%$  of the clones sequences were also closely related to environmental clones and cultured strains derived from soil and freshwater habitats, which is likely due to the fact that the putative inoculation source for the moonmilk bacterial communities is from overlying soil and percolating fluids from the surface. Prior to our studies of *Cansiliella* spp., moonmilk has not been considered a food source for cave animals. Our findings provide unique insight into moonmilk microbial diversity that could reveal the underpinnings of the moonmilk carbon and nitrogen cycle that influences the isotopic composition and the morphological adaptations of the troglobitic beetles associated with the moonmilk.

**Keywords:** calcium carbonate; microorganisms; food web; nitrogen cycling; beetles

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## INTRODUCTION

Chemolithoautotrophic microbial processes control metal and nutrient cycling in karst aquifer systems (e.g., Sarbu et al., 1996; Northup et al., 2003; Opsahl & Chanton, 2006; Gray & Engel, 2013) and support rich and biologically diverse ecosystems with complex trophic-level interactions and food webs in

the absence of allochthonous input (e.g., Holmes et al., 2001; Farnleitner et al., 2005; Goldscheider et al., 2006; Macalady et al., 2008; Birdwell & Engel, 2009; Birdwell & Engel, 2010). These findings contradict a long-held tenet that nearly all ecosystems on Earth are dependent on oxidative photosynthesis for energy and organic carbon (e.g., Horner-Devine et al., 2003; Amend & Teske, 2005; Simon et al., 2007;

Smith, 2007). However, microbial food webs from subaerially exposed (i.e. terrestrial) mineral deposits in caves have received relatively limited attention with respect to microbial food web structure, despite their importance for troglobionts (cave-adapted, terrestrial invertebrates) and even the possibility that their association can alter climate records from speleothem deposits (e.g., Lacelle et al., 2004; Blyth et al., 2008).

Moonmilk -- a spongy to powdery assemblage of microcrystalline carbonate minerals that resembles toothpaste when hydrated -- is a type of secondary carbonate speleothem that occurs subaerially in caves, usually on cave walls (Hill & Forti, 1997). Microbial and fungal biomass is prevalent in moonmilk (e.g., Cañaveras et al., 2006; Rooney et al., 2010; Baskar et al., 2011; Braissant et al., 2012; Sanchez-Moral et al., 2012). Although some investigations suggest that microbes mediate moonmilk development (e.g., Mason-Williams, 1959; Northup et al., 2000; Cañaveras et al., 2006; Richter et al., 2008), microbes are not essential for its formation (Borsato et al., 2000). Recent studies of moonmilk examine biogeochemical and geomicrobiological interactions (e.g., Mulec et al., 2002; Cacchio et al., 2004; Van de Kamp, 2004; Portillo & Gonzalez, 2011; Sanchez-Moral et al., 2012), but there still has been scant effort to evaluate the microbial diversity of moonmilk using culture-independent methods or to consider the possibility that moonmilk, because of the abundance of microbial biomass, may serve as a potential food and energy source for troglobitic invertebrates when allochthonous organic carbon input would be limited (Paoletti et al., 2011). Inclusion of moonmilk into terrestrial cave food web structures represents a significant paradigm shift.

Extensive moonmilk deposits in the Italian caves, Grotta della Foos and Bus della Genziana (Fig. 1A), are associated with the occurrence of *Cansiliella*, a group of troglobitic beetles. *Cansiliella servadeii* Paoletti 1972 (Coleoptera: Leptodirinae) browses the interface of percolating waters and moonmilk in Grotta della Foos, and *Cansiliella tonielloi* is found with moonmilk in Bus della Genziana (Beggio, 2007). *Cansiliella* spp., as well as other beetles from the genus *Hadesia* and possibly the recently described genera *Radziella*, *Velevitodromus*, *Croatodirus*, *Nauticella*, *Tartariella*, and *Kircheria*, have body characteristics distinct from the majority of other troglobitic Leptodirinae and have peculiar, semi-aquatic feeding behaviors attributed to inhabiting rocky surfaces, such as cave walls (Jeannel, 1924; Remy, 1940; Paoletti, 1973; 1980; Moldovan et al., 2003; Sket, 2004; Giachino & Vailati, 2006). Their feeding and mouthpart morphologies suggest a microphagous feeding habit associated with filtration of allochthonous organic material from the hydropetric, cave-wall habitat (Sket, 2004). Prior to our investigations (Paoletti et al., 2009; Paoletti et al., 2011; Paoletti et al., 2013), no previous studies directly evaluated the food source for *Cansiliella* spp.

Our observations of *Cansiliella* spp. led to the working hypothesis that microbes associated with the moonmilk serve as food for the beetles. Using stable isotope ratio systematics, we attempted to establish

the food web position of *C. servadeii* and other cave animals (including *Monolistra lavalensis*, *Niphargus* sp., and *Androniscus noduliger*) with respect to moonmilk (Paoletti et al., 2011). The carbon isotope values of *C. servadeii* are consistent with moonmilk potentially serving as a food source. However, potential overgrazing of the moonmilk by the beetle to obtain sufficient nutrition, and unique and even unknown microbial activities in the moonmilk associated with nitrogen cycling, suggest that the trophic level position for *C. servadeii* is incompatible with moonmilk serving as a food source. Therefore, the goal of the current study is to enhance our understanding of moonmilk microbial diversity from Grotta della Foos and Bus della Genziana that could reveal the underpinnings of a moonmilk nitrogen cycle. This research identifies a common community structure for the moonmilk bacterial communities, and also offers an explanation for why some troglobitic invertebrates, such as *C. servadeii*, have evolved unique body morphologies and behaviors to take advantage of a cave hydropetric habitat like moonmilk.

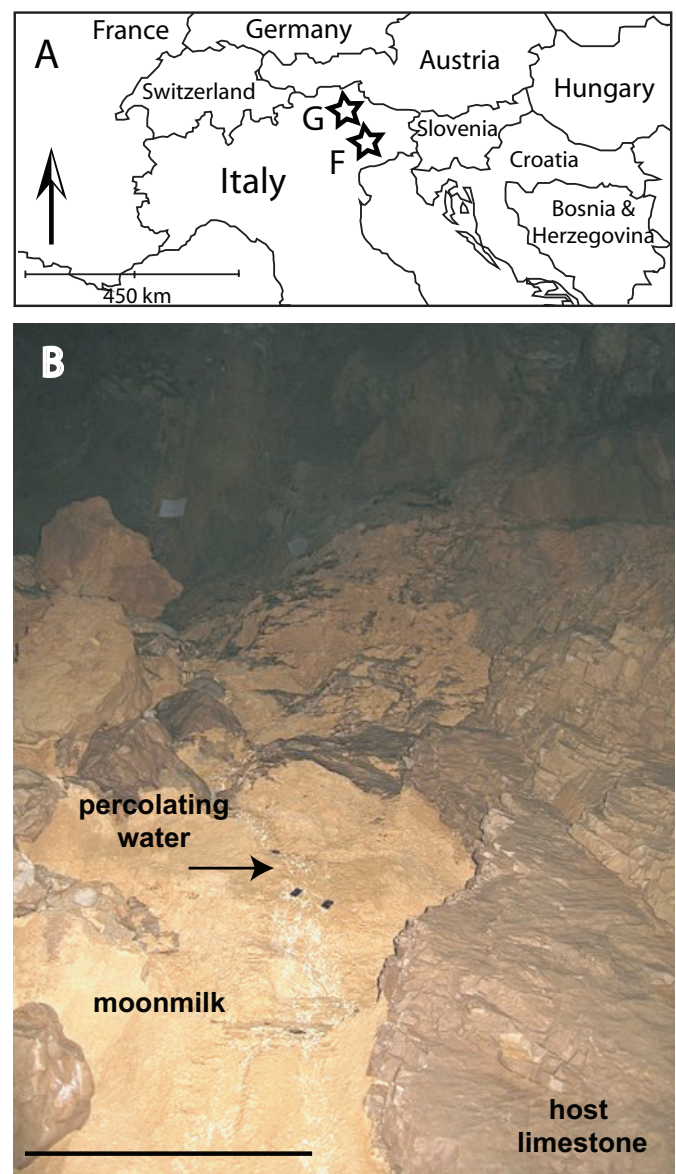


Fig. 1. (A) General location map for Grotta della Foos, Italy (star labeled "F") and Bus della Genziana (star labeled "G") with (B) one of the moonmilk sampling sites in Grotta della Foos, modified from Paoletti et al. (2011). Scale bar = 0.5 m.

## MATERIALS AND METHODS

### Sample collection

Moonmilk was collected from Grotta della Foos in March 2008 and from Bus della Genziana in January 2009 (Beggio, 2007). Both caves formed in Cretaceous limestone, and Grotta della Foos is located at an elevation of 422 m and Bus della Genziana at 1020 m. The primary sample location in Grotta della Foos where *C. servadeii* is found is "Site B" (Fig. 1) (Paoletti et al., 2011). Further sampling location details are withheld because the *Cansiliella* spp. population sizes are not known and the caves are not well-protected, although Bus della Genziana is located in a natural speleological preserve (Paoletti et al., 2011).

### Aqueous geochemistry and moonmilk characterization

Basic geochemical analyses of percolating water samples were done, including pH, temperature, and specific conductance. Water was filtered to 0.2  $\mu\text{m}$ , and anions and cations were measured by single column ion chromatography. The geochemical program PhreeqcI version 2.15.0 was used to determine the saturation indices (SI) of mineral phases according to the logarithm of the quotient of the ion activity product and solubility product constant (Parkhurst & Appelo, 1999). Organic carbon compositions were measured following the removal of inorganic carbonate with a solution of 1N HCl for 16 hr at room temperature while monitoring the reaction and pH (Midwood & Boutton, 1997). Samples for each were rinsed to about pH 5.5 in DI H<sub>2</sub>O, dried at 50 °C, and then ground to a fine powder. Because the acidification procedure could produce a loss of nitrogen-acid soluble organic matter and enhance ingassing of NH<sub>3</sub>, nitrogen content was done on non-acidified samples. Aliquots were flash combusted on an EA 1110 Carlo Erba elemental analyzer.

Moonmilk from Bus della Genziana was thinner and patchier, making collection for additional geochemical and biological characterization difficult. Therefore, examination of chlorophyll content and meiofaunal and microbial cell counts (below) was done only from Grotta della Foos. Chlorophyll and photosynthetic pigment content (*Chl a*) was measured spectrophotometrically to assess the potential presence of photosynthetically-derived (allochthonous) organic matter. For the water, the method of Innamorati (1990) was used. Briefly, 0.5 to 1 L of water was filtered onto a 1  $\mu\text{m}$  glass fiber filter, which was placed into 80% acetone. The filter-acetone mixture was ground, then incubated for 24 hr before centrifugation. For moonmilk, the following modifications were made from Innamorati (1990). Moonmilk was dried for 24 hr at 60° C. The dried material was pulverized and 1 g was used to extract chlorophyll using 1:1 chloroform-methanol. The slurry was incubated for 24 hr, than centrifuged for 25 min at 4000 rpm. Absorbance was measured from the supernatant. Detailed calculations can be found in Beggio (2007).

### Micro- and meiofaunal and microbial cell counts

Twenty glass slides were placed in the percolating water on the surface of moonmilk at Site B in Grotta della Foos (Fig. 1) for ~5 months to capture and enumerate micro- and meiofauna. Slides were transported to the lab in sterilized boxes containing the same percolating water and maintained at 4 °C until analysis. Slides were examined by stereomicroscope and transmission microscope for general identification. For microbial cell abundance, aliquots of the moonmilk with associated percolating waters were fixed in 4% formaldehyde. Briefly, 1 ml of the moonmilk slurry was resuspended in 5 ml of MQ water and vortexed for 5 min. An aliquot of 1ml of suspension or 1ml of water was vacuum filtered on a 0.2  $\mu\text{m}$  pore size polycarbonate filter (Nuclepore) and stained with DAPI ( $\alpha'$ , 6-diamino-2-phenylindole) at a final concentration of 0.2% wt/vol (Porter & Feig, 1980). Cell counts were done with a Zeiss Axioplan epifluorescence microscope, equipped with an HBO 100 W lamp, a Neofluor 100 x objective, 1.25x additional magnification, and filter sets for UV light excitation (Zeiss filter set G365, FT395, LP420). Microscopic fields for counting were grabbed with an image analysis system (Image ProPlus; Media Cybernetics, Silver Spring, MD, USA), equipped with a high sensitivity video camera (CoolSNAP Pro-cf, Media Cybernetics).

### DNA extraction from moonmilk and percolating waters

Two separate samples of moonmilk from Grotta della Foos were collected from Site B (referred to as "Foos7B" and "Foos8B"), and one site from Bus della Genziana (referred to a "GMM"). Total environmental nucleic acids were extracted within 72 hr of collection. Various extraction methods were considered (e.g., Barton et al., 2006), but based on cell count estimates from DAPI, the following method was considered sufficient to retrieve high purity, representative DNA from the microbial community in the moonmilk. Specifically, 2 ml of moonmilk slurry were placed into 10 ml of sterile cell lysis buffer (10 mM Tris and 100mM EDTA, pH 8.0), homogenized with a sterile tissue homogenizer for 2 min, or until large particles and clumps were thoroughly disaggregated to particles no bigger than grains of sand. The suspensions were incubated at 55°C on a shaker overnight at 250 ppm, following addition of 40 ml of proteinase K (20 mg/ml). Protein precipitation was accomplished by combining 1ml of cooled suspension with 1 ml 7.5 M ammonium acetate. The solutions were mixed thoroughly, and then incubated on ice for 15 min prior to centrifugation for 5 min at high speed. The supernatant was transferred to a new tube with an equal volume of 100% isopropanol for nucleic acids precipitation overnight at 4° C. Samples were centrifuged at high speed for 3 min and pellets were washed with cold, 95% ethanol. Nucleic acids were rehydrated in 50 ml TE buffer (10 mM Tris and 0.1 mM EDTA, pH 8.0). DNA purity and concentrations for each extraction were determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, LLC, USA).

### PCR amplification of genomic DNA, cloning, and sequencing

Nearly full length 16S rRNA gene sequences were obtained by PCR amplification using the bacterial primer pair 8f (forward, 5'-AGAGTTTGATCCTGGCTCAG-3') and 1510r (reverse, 5'-GGTTACCTTGTACACTT-3') (Lane, 1991). The archaeal primers UA571F (forward, 5'-GCYTAAGSRICCGTAGC-3') and UA1204R (Reverse, 5'-TTMGGGGCATRCIKACCT-3') (Baker & Cowan, 2004) were also used, but amplifications were unsuccessful. Amplification was performed with a MJ Research Dyad Disciple thermal cycler with 5 U/ml ABGene Taq DNA polymerase (ABGene, Thermo Fisher Scientific, USA). Optimal PCR products for bacteria were achieved for three of the four extractions using the following conditions: an initial hot start at 94° C for 4 min, followed by denaturation at 94° C for 1 min, primer annealing at 47° C for 1 min, chain extension at 72° C for 1 min, repeated for 29 more cycles, followed by a final extension step for 20 min at 72° C.

Amplified PCR products were purified by using a 0.7% TAE low-melt agarose gel with a Wizard PCR prep DNA Purification kit (Promega Corp., USA), following manufacturer recommendations. Concentrations and purity were determined by spectrophotometry. Purified products were cloned using the TOPO Cloning Kit with the pCR2.1-TOPO® vector, according to manufacturer instructions (Invitrogen Corp., USA); ligation reactions were run overnight at 14° C. Clones were screened by PCR with the M13-Forward (5'-GTAAAACGACGGCCAG-3') and M13-Reverse (5'-CAGGAAACAGCTATGAC-3') primers (Invitrogen Corp., USA). Amplified inserts were diluted and sequenced by capillary Sanger sequencing using High-throughput Sequencing Solutions (<http://www.htseq.org/index.html>). Sanger sequencing was chosen over other, more high-throughput methods (e.g., 454 tag pyrosequencing) in order to obtain near-full length 16S rRNA gene sequences for detailed phylogenetic analyses.

### Phylogenetic analysis and estimation of microbial diversity

Resulting 16S rRNA gene sequences were assembled using ContigExpress, a component of Vector NTI Advance 10.3.0 (Invitrogen Corp., USA), and subjected to BLAST searches using GenBank (<http://www.ncbi.nlm.nih.gov/>) to establish gene sequence similarities to culturable and not yet cultured organisms. Sequences were screened for chimera and aligned using greengenes NAST-aligner (<http://greengenes.lbl.gov>) and using the program DECIPHER's Find Chimera tool (<http://decipher.cee.wisc.edu/FindChimeras.html>) (Wright et al., 2011). The Ribosomal Database Project (RDP), release 10 (Cole et al., 2009), Library Compare online analysis tool (Wang et al., 2007) was used to test for statistical differences in the taxonomic representation between the two moonmilk bacterial clone libraries, at alpha = 0.01 and a 95% confidence threshold.

Designation of operational taxonomic units (OTUs) representing the ≥97% sequence similarity predicted

for species-level relationships using 16S rRNA gene sequences (e.g., Stackebrandt & Goebel, 1994) was done in DOTUR using the furthest-neighbor clustering algorithm (<http://www.plantpath.wisc.edu/fac/joh/dotur.html>) (Schloss & Handelsman, 2005). If the DOTUR ≥97% sequence similarity groups were concordant with a monophyletic clade from the reconstructed phylogeny, then those groups were designated as OTUs. Rarefaction curves were generated using DOTUR. More than 400 clone sequences from this study and sequences identified from RDP and BLAST, including outgroup taxa, were used to reconstruct a phylogeny based on a GTR +G model of evolution. Branch support from 500 bootstrap replicates was constructed using the program MEGA (ver. 5.0) (Kumar et al., 2004) based on the Jukes-Cantor model (Tamura et al., 2004). The phylogenetic associations for the moonmilk clones were evaluated from RDP SeqMatch analysis tool, whereby the highest similarity scores (as S<sub>ab</sub>) to environmental clones or cultured strains were compiled and specific habitat types were evaluated (e.g., cave, freshwater, soil, other). Scores of 1.0 would result for identical sequences; the closer the score is to 1.0, the more similar sequences are to each other. Scores >0.96-98 loosely correlate to genus-level associations.

### Nucleotide sequence accession numbers

Representative 16S rRNA gene sequences from Grotta della Foos moonmilk clone libraries were submitted to GenBank under the accession numbers EU431666-EU431834 and KC255251-KC255375 for Bus della Genziana samples.

## RESULTS AND DISCUSSION

### Moonmilk geochemistry

The percolating waters flowing over Grotta della Foos moonmilk (Fig. 1B) had a pH of 8.3 at 8.8 °C, 200 μS/cm specific conductance, and 0.3 mmol/L dissolved oxygen (Table 1). The water associated with the Bus della Genziana moonmilk had a pH of 7.98 at 7.0 °C, 390 mS/cm specific conductance, and 0.24 mmol/L dissolved oxygen (Table 1). For both caves, the water types were characterized as Ca-HCO<sub>3</sub><sup>-</sup> type (Table 1). The waters were supersaturated with respect to calcite (Saturation Index = +0.43) and undersaturated with respect to other mineral phases, confirming the mineralogy of each deposit, although the Bus della Genziana moonmilk appeared to be less hydrated and in a state of erosion rather than deposition.

The moonmilk from Grotta della Foos was soft, whitish to yellowish color, hydrated and of calcite mineralogy. The density of *C. servadeii* on the moonmilk was ~8-12 individuals per m<sup>2</sup> (Fig. 1B). The organic C composition of the moonmilk was 0.06-0.52 %, with 0.01-0.05 % organic N, and no detectable S but minor amounts of Al, Si, and Fe (data not shown). By contrast, the Bus della Genziana moonmilk was markedly thinner and patchier, but also of calcite mineralogy. Traces of chlorophyll and other photosynthetic pigments, as proxies for surface-derived (plant) material, were measured from Grotta

Table 1. Geochemistry of percolating waters. Ions are listed in mmol/L concentrations.

	Grotta della Foos	Bus della Genziana
Air temperature (°C)	8.6	6.2
Water temperature (°C)	8.8	7
pH	8.25	7.89
Conductivity (µS/cm)	225	390
Dissolved oxygen (mmol/L)	0.34	0.24
Na <sup>+</sup>	0.01	1.10
K <sup>+</sup>	<0.001	0.1
NH <sub>4</sub> <sup>+</sup>	0.02	<0.003
Ca <sup>2+</sup>	1.13	1.15
Mg <sup>2+</sup>	<0.002	0.04
Cl <sup>-</sup>	0.03	1.65
HCO <sub>3</sub> <sup>-</sup>	2.45	2.16
NO <sub>2</sub> <sup>-</sup>	0.04	<0.0008
NO <sub>3</sub> <sup>-</sup>	0.07	0.06
PO <sub>4</sub> <sup>3-</sup>	0.002	<0.0005
SO <sub>4</sub> <sup>2-</sup>	0.04	0.03

della Foos percolating waters extractions during the summer ( $0.38 \pm 0.14$  mg/l *Chl a*), but nothing was detected from samples collected in the winter. Only moonmilk collected from the summer had detectable quantities of chlorophyll, with a composition of  $0.14 \pm 0.09$  µg/l *Chl a*. Therefore, it is not possible to rule out that surface-derived organics, even at ultraoligotrophic concentrations, are brought to the area where moonmilk forms in Grotta della Foos. However, the relative contribution of allochthonous material to the overall organic matter composition in the moonmilk does appear to vary seasonally.

#### Microbial biomass from Grotta della Foos moonmilk

Because of comparatively easier access compared to the other caves, the percolating waters from Grotta della Foos were examined on several occasions for micro- and meiofauna, such as protists or annelids (Beggio, 2007). Repeated examination of the percolating waters, and even the moonmilk, resulted in infrequent and low numbers of individuals (mean  $\sim 10^4$  individuals/m<sup>2</sup>) (O. Coppellotti, unpublished results). Of the identified groups, none are known to

be specialized to the subsurface: *Glaucoma scintillans* (a benthic ciliate), flagellates belonging to the genera *Anisonema*, *Bodo*, and *Cercomonas*, Chironomidae larvae (possibly *Eukiefferiella*), and an annelid from the Lumbriculidae. Microbial cells were coccid, bacilliform, or filamentous, and most were a few microns or less in diameter. Rare fungal hyphae were observed, suggesting that fungi do not comprise a significant portion of the moonmilk microbial communities. Cell counts from water were conservatively estimated to be  $10^5 - 10^6$  cells/ml, which is similar to what has been reported previously as  $10^3 - 10^6$  cells/ml for epikarst waters (e.g., Rusterholtz & Mallory, 1994; Laiz et al., 1999). We considered the counts to be a mixture of viable and nonviable cells from the system. We were unable to quantify the number of cells precisely from the moonmilk because high carbonate mineral content (Fig. 2A) interfered with the DAPI signal (Fig. 2B). Nonquantitative estimates revealed that cell numbers in the moonmilk could be 10 to 100 times greater compared to the percolating waters. These microbial biomass estimates would be comparable to those reported by other investigators. Braissant et al. (2012) estimate approximately  $10^5$  colony forming units (CFUs) per gram of moonmilk from Swiss caves using culture-based and microcalorimetric analyses, and Curry et al. (2009) also describe  $10^4 - 10^6$  cells as CFUs per gram of moonmilk from Alaska, depending on the type of moonmilk. Considering the abundance of moonmilk in both caves, there is noteworthy microbial standing stock to cycle organic carbon (e.g., del Giorgio & Cole, 1998; Eiler et al., 2003).

#### Phylogenetic analysis of microbial communities from moonmilk

16S rRNA gene sequences were retrieved from moonmilk to evaluate the microbial diversity, which was expected to be high based on previous research of natural, cave-wall habitats (e.g., Northup et al., 2003; Chelius & Moore, 2004; Van de Kamp, 2004; Barton et al., 2007) and cave-walls with Paleolithic paintings or system having been anthropogenically impacted (e.g., Groth & Saiz-Jimenez, 1999; Schabereiter-

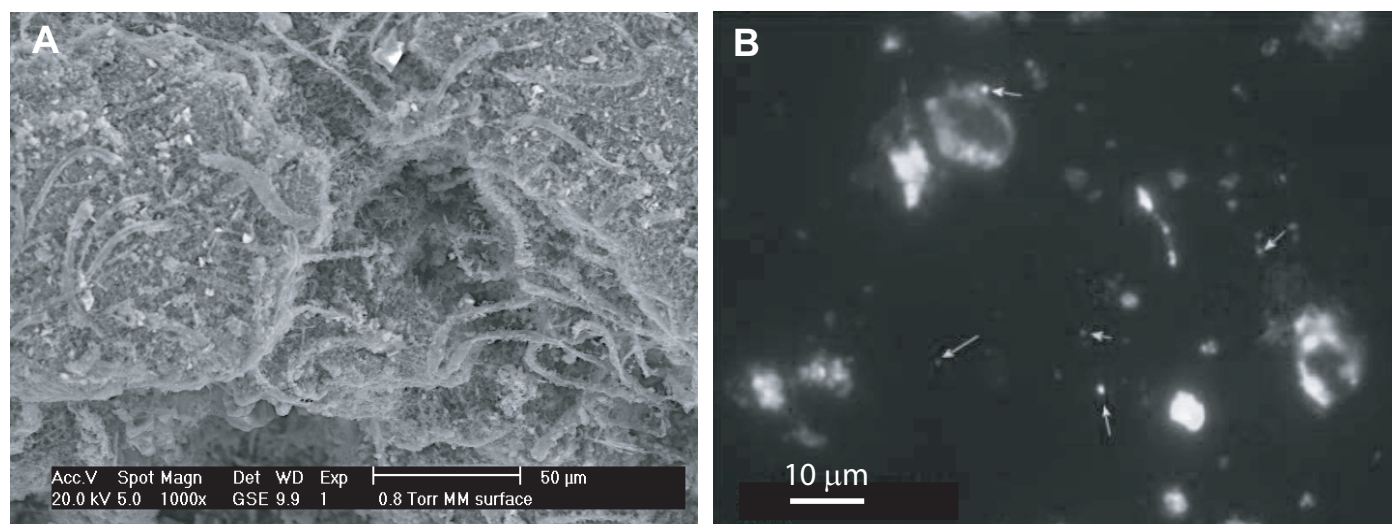


Fig. 2. (A) Environmental scanning electron photomicrograph of moonmilk from Grotta della Foos showing coccid, bacilliform, and filamentous cells intermixed with microcrystalline calcite. Scale bar = 50 µm (B) DAPI-stained cells (arrows) from a slurry of moonmilk. Other, larger bright spots are calcite crystals. Scale bar is 10 µm.

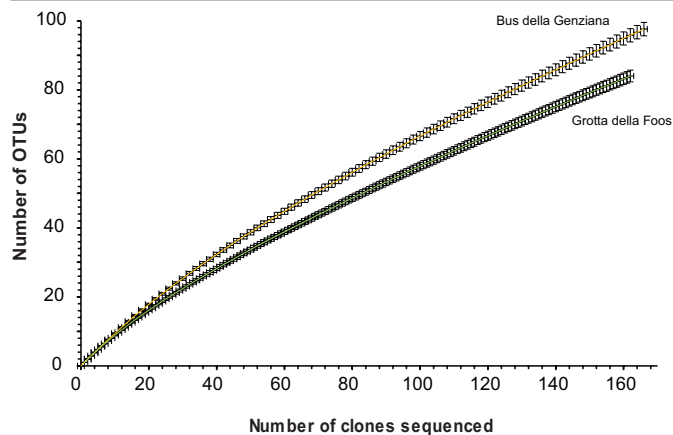


Fig. 3. Rarefaction curves generated at 97% sequence similarities for bacterial 16S rRNA gene sequence clones from Grotta della Foos and Bus della Genziana moonmilk.

Gurtner et al., 2002b; Schabereiter-Gurtner et al., 2002a; Schabereiter-Gurtner et al., 2004; Ikner et al., 2007). At 97% sequence similarity, corresponding loosely to interpreted species-level relationships, 187 novel OTUs were identified from the two moonmilk deposits. Although both samples appeared to be under-sampled based on rarefaction analyses (Fig. 3) and that more diversity could be uncovered with additional sequencing efforts, all of the clones were affiliated with, at most, 12 major taxonomic groups, including 2 candidate divisions (Fig. 4). A comparable level of bacterial diversity has also been noted from moonmilk in Ballynamintra Cave, Ireland (Rooney et al., 2010), and Altamira Cave, Spain (Portillo & Gonzalez, 2011), although near-full length 16S rRNA gene sequences were not produced in these studies so comparison of the communities from the Italian systems and other moonmilk deposits is difficult.

From moonmilk deposits in our study, the majority of clones (>50% of each clone library) were affiliated with the Proteobacteria phylum (Fig. 4A). For Grotta della Foos, more clones were affiliated with the Betaproteobacteria (22.2%) compared to Alphaproteobacteria (16.3%) or Gammaproteobacteria (15.9%). But, for Bus della Genziana, more clones were affiliated with the Alphaproteobacteria (20.4%), compared to the Betaproteobacteria (15%) or Gammaproteobacteria (17.3%). However, the taxonomic representation for the two sites was not statistically different from each other at the phylum, class, or order levels except in one case (Fig. 4B). At the genus-level, 29 of 50 different genera were identified from both sites, representing >45% of each of the clone library sequences. Collectively, these results imply that there may be some commonality among the moonmilk microbial communities even though the caves are geographically separated from each other.

A statistically significant difference between the two sites was only noted for the Oxalobacteraceae family ( $p = 0.003$ ) within the Betaproteobacteria, which represented 22.2% and 15% of the clones for Grotta della Foos and Bus della Genziana, respectively. Representatives from the Burkholderiales order (Betaproteobacteria), and specifically either the Comamonadaceae or the Oxalobacteraceae families,

dominated both moonmilk samples (Fig. 4B; Suppl. Fig. 1). The moonmilk clones were related to other environmental clones and cultured representatives characterized as being chemoorganotrophic, nonfermentative, aerobes common to freshwater and soils and that grow optimally at circumneutral pH (e.g., Grimes et al., 1997; Li et al., 2004; Gillis & De Ley, 2006) (Suppl. Fig. 1). Clones from Grotta della Foos formed a novel clade within the *Acidovorax* genus (Comamonadaceae), which are known to grow chemolithoautotrophically using hydrogen as an energy source, while others are capable of denitrification (Heylen et al., 2008). An isolate belonging to the *Acidovorax* was also retrieved from Ballynamintra Cave moonmilk using minimal medium agar (Rooney et al., 2010), suggesting that there may be some common microbial groups in moonmilk from different caves. From the Italian moonmilk, at least three other novel clades were affiliated with the genera *Janthinobacterium* and *Massilia* spp. (Oxalobacteraceae), at 92-97% sequence similarities. These groups are strict aerobic chemoorganotrophs and are associated with nitrogen cycling, found in aquatic, soil, and metal-contaminated sites, and form plant associations, usually as pathogens (Suppl. Fig. 1). They have also been identified from naturally oligotrophic, but not associated with cave paintings, cave-wall surfaces (e.g., Northup et al., 2003; Barton et al., 2007). The general phylogenetic similarities, even in the absence of high sequence identities, may indicate that the cave habitat, irrespective of geochemical or mineralogic conditions, may be a common habitat for these betaproteobacterial groups.

Other prevalent proteobacterial groups from the moonmilk included the Gammaproteobacteria (Suppl. Fig. 2), Alphaproteobacteria Deltaproteobacteria (Suppl. Fig. 3), and members of the candidate division TM7. Among the Gammaproteobacteria (Fig. 4B; Suppl. Fig. 2), clones were predominately related to various pseudomonad species (Pseudomonadales order). Clones related to *Acinetobacter* spp. were retrieved from Grotta della Foos. Isolates belonging to this genus cause carbonate precipitation in cultures collected from carbonate speleothems from Cervo Cave, Italy (Cacchio et al., 2004). Other notable genera included *Lysobacter* spp., a predatory group (Jurkevitch & Davidov, 2006), and *Perlucidibaca* and *Rhizobacter* spp. Among the Alphaproteobacteria (Fig. 4B; Suppl. Fig. 3), retrieved clones were closely related (93-99% sequence identity) to groups that also cycle nitrogen, including the genera *Sphingomonas*, *Sphingopyxis*, and *Novosphingobium* (Sphingomonadales order) and *Azospirillum* (Rhodospirillales order) (Suppl. Fig. 3). These organisms are obligate aerobes and have the ability to fix  $N_2$  (Addison et al., 2007). Several clades within the Sphingomonadales were comprised of clones from both moonmilk sites, and were more closely related to each other than to previously retrieved environmental clones or cultured strains. Schabereiter-Gurtner et al. (2004) retrieved similar alphaproteobacterial groups from cave-wall rock and Paleolithic paintings in Spain, and Northup et al. (2003) found these genera from caves in New Mexico.

These results suggest that the moonmilk and similar cave and karst habitats likely contain additional novel diversity. Deltaproteobacteria associated with the genera *Bdellovibrio*, *Pedobacter*, and *Haliangium* were retrieved from both caves (Suppl. Fig. 3), but had only previously been reported from the Movile Cave in Romania. *Bdellovibrio* spp. are also obligate predatory bacteria (Jurkevitch & Davidov, 2006).

The Bacteroidetes phylum represented the second most abundant group at the phylum-level (Fig. 4A), and comprised 16.7% and 14.9% of all clone sequences from Grotta della Foes and Bus della Genziana, respectively. Clones were affiliated with predominately the Sphingobacteria class, but representation among

the Flavobacteria was also relatively high (Suppl. Fig. 4). Some of the retrieved clones affiliated with the *Flexibacter* spp. were related to strains previously identified as aerobic chemoorganotrophs from freshwater settings, including a novel clade of Bus della Genziana clones with a sequence from the Frasassi Caves, Italy, and another clade of Grotta della Foes clones and a karst soil clone. Several clones from both caves formed clades within the *Flavobacterium* genus and were related to known chemoorganotrophs, facultative aerobes, as well as N<sub>2</sub> fixers.

Three OTUs (at 97% sequence identity) belonged to the candidate division TM7, with one large novel clade of Grotta della Foes sequences and other clades with

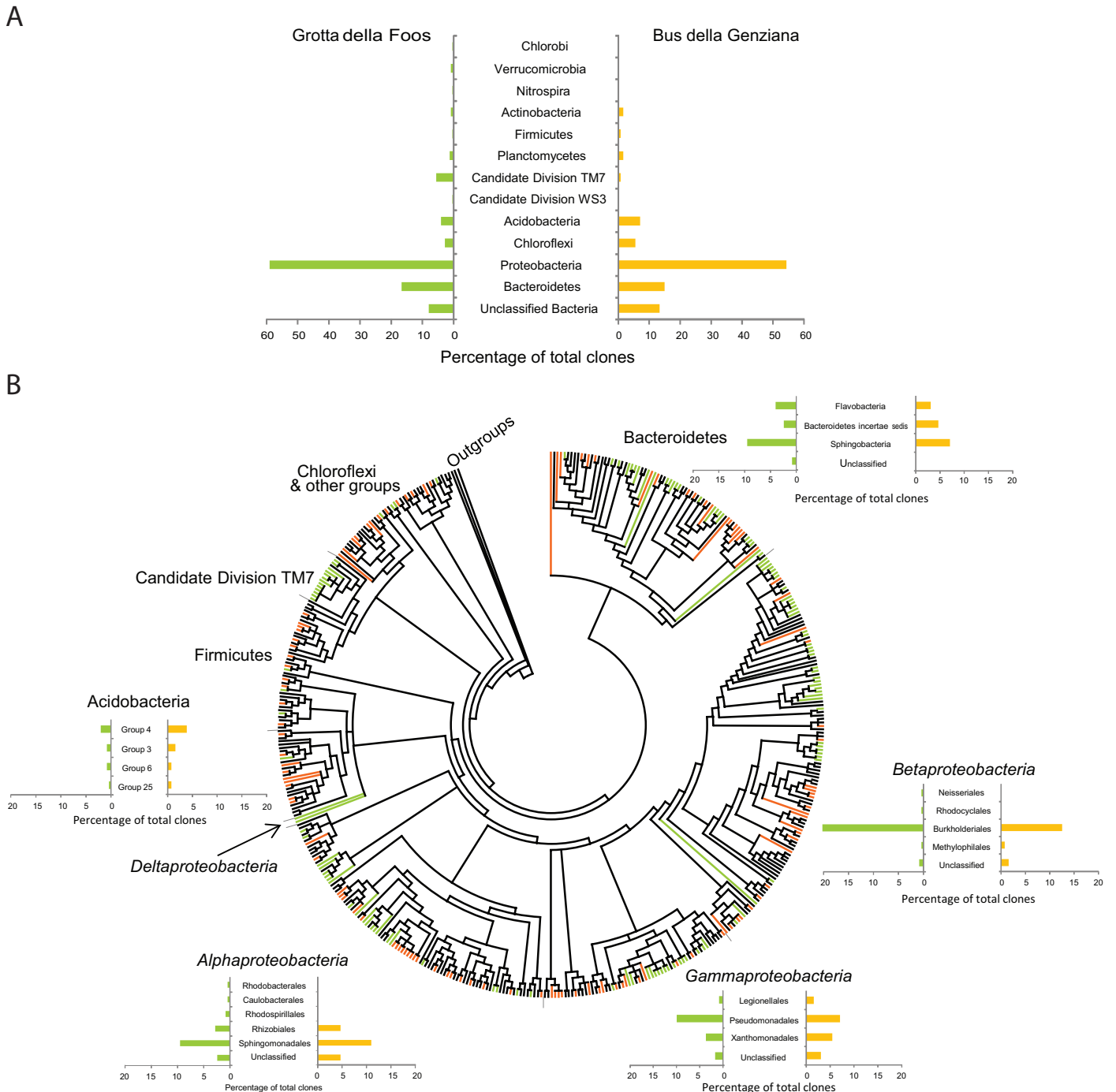


Fig. 4. (A) Summary of the percentage of total 16S rRNA gene sequence clones from Grotta della Foes (colored in green) and Bus della Genziana (colored in orange). (B) Phylogenetic reconstruction using neighbor joining topology for 16S rRNA gene sequences of representative bacterial clones retrieved from Grotta della Foes and Bus della Genziana moonmilk samples. Branches linked to sequences retrieved from this study are colored in either green or orange, corresponding to each of the caves. The bar graphs located around the tree show the total percentage of clones affiliated with the specific taxonomic groups to the class level for Bacteroidetes and to the order level for the proteobacterial groups.

Bus della Genziana clones and other environmental sequences (Fig. 4B). The ecophysiology of the TM7 candidate division remains a mystery (Podar et al., 2007), but finding that these microbes occur in the moonmilk may add to the known metabolic capabilities and physiological requirements for members of this group. To our knowledge, sequences affiliated with the TM7 candidate division have not been retrieved from caves, suggesting that the presence in moonmilk from both caves requires further investigation.

Several culture-independent studies from cave and karst habitats have retrieved sequences belonging to the Verrucomicrobia, Planctomycetes, and Acidobacteria (e.g., Chelius & Moore, 2004; Ikner et al., 2007). Collectively, these groups were represented by <2% each of all of the clones from both Grotta della Foos and Bus della Genziana (Fig. 4A). Verrucomicrobia were only retrieved from Grotta della Foos. Among the Planctomycetes from both sites, none of the clones had high sequence similarities to cultured representatives, but one clone was related to an environmental clone from an 'anammox' reactor, suggesting that anaerobic oxidation of ammonium may be occurring in the moonmilk community. If anammox is occurring, although based on the measured dissolved oxygen concentrations of 0.3 mmol/L, this is unlikely (e.g., Strous et al., 1998), then this metabolism could impact nitrogen isotope systematics of the moonmilk because anammox is associated with large isotopic fractionation (Paoletti et al., 2011). Retrieving four different groups of Acidobacteria in both moonmilk sites is interesting because this group has also been found to have high abundance on cave-walls (Chelius & Moore, 2004; Ikner et al., 2007), as well as cave surfaces associated with paintings (Schabereiter-Gurtner et al., 2002a).

Other moonmilk clones were associated with the Actinobacteria, Firmicutes, Nitrospirae, Chloroflexi, Chlorobi, and candidate division WS3. Actinobacteria and Firmicutes have been identified from numerous cave settings (e.g., Groth & Saiz-Jimenez, 1999; Laiz, 1999; Schabereiter-Gurtner et al., 2002a; Northup et al., 2003; Chelius & Moore, 2004; Gerič et al., 2004; Schabereiter-Gurtner et al., 2004; Van de Kamp, 2004; Barton et al., 2007; Ikner et al., 2007), and the candidate division WS3 has been previously identified from caves in Spain (Schabereiter-Gurtner et al., 2004). The significance of why there were relatively low numbers of clones retrieved from the moonmilk for these phyla is unknown, particularly for the Actinobacteria that have been shown to play a role in the formation of moonmilk by Cañaveras et al. (2006) and organic matter degradation (e.g., Laiz et al., 2000).

Although the Grotta della Foos and Bus della Genziana moonmilk bacterial diversity was statistically similar to each other, the bacteria appear to be distinct taxonomically, especially at the genus level, from previously examined natural and human-impacted cave-wall habitats (e.g., Groth & Saiz-Jimenez, 1999; Vlasceanu et al., 2000; Holmes et al., 2001; Northup & Lavoie, 2001; Northup et al., 2003; Barton & Northup, 2007; Barton et al., 2007; Ikner et al., 2007) (Fig. 5). Some of the groups retrieved

from the moonmilk were more closely related to each other than to environmental clones or strains from other habitats. But, the overlying soil and percolation waters for both caves are considered to be the primary inoculation source(s) for the moonmilk communities. As such, there were also a high number of phylogenetic associations (at >93% sequence identity) between moonmilk clones and environmental clones or cultured strains from soil (e.g., permafrost, forest, grassland) or freshwater systems (e.g., ice, lakes, streams, groundwater), at almost equal representation for all sequence similarity levels (Fig. 5). In terms of the relative proportions of major taxonomic divisions, the community composition from the moonmilk more closely resembles the composition reported in several studies of freshwater systems not soils, featuring over 50% Proteobacteria, mostly from the Betaproteobacteria, followed by Bacteroidetes, and by rare Verrucomicrobia and Actinobacteria (e.g., Zwart et al., 2002; Newton et al., 2006). These associations provide a unique perspective on the microbial diversity of moonmilk because moonmilk is a potentially novel habitat type in karst and the relatively low diversity and dominance among certain bacterial groups could be due to the moonmilk being a specialized habitat that would favor a variety of metabolic specialists (e.g., Smith, 2007).

Although 6% of the clones had close affinities to sequences previously retrieved from organic-rich material, such as manure and sludge (Fig. 5), there is no such organic-rich contribution to the moonmilk from the surface. There is potential that the composition and structure of the moonmilk bacterial communities in both caves may be influenced from fecal material or gut flora from the beetles that graze the moonmilk. However, previous research of arthropod gut flora has indicated that guts contain an abundance of fermentative microbes, and lactogenic and acetogenic groups (Egert et al., 2003) that would likely not be sustained in the moonmilk geochemical system. Moreover, our recent examination of the *Cansiliella* gut flora (Paoletti et al., 2013) reveals that Firmicutes represented >58% of all retrieved 16S rRNA gene sequences from clone libraries constructed from guts from three individuals, and that Bacteroidetes represented ~16% of the sequences, Actinobacteria 11.5%, Deltaproteobacteria 8%, and <5% each of the alpha- and betaproteobacterial classes. Consequently, there was no similarity or affinity of the gut clones to sequences retrieved from the moonmilk, suggesting that there is little correspondence between the gut flora and the potential food source for the beetles.

### Conclusions - implications for a moonmilk-based food web

Excluding sulfur-based cave ecosystems where chemolithoautotrophic microbes are primary producers (e.g., Sarbu et al., 1996; Porter et al., 2009), most microbes in cave ecosystems are generally considered to be degraders of allochthonous organic material, depending on the hydrologic connectivity of the cave to the surface, and/or food for higher level organisms. It is unlikely that the micro- and meiofaunal



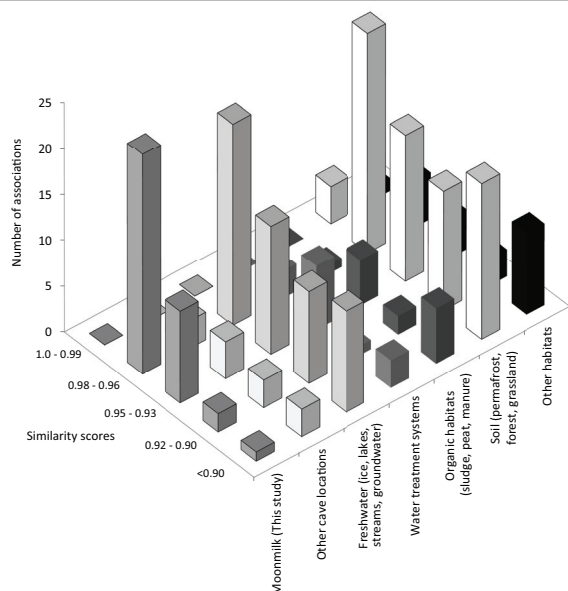


Fig. 5. The number of phylogenetic associations for moonmilk clones to environmental clones and cultured strains from specific habitat types that are described in the literature according to similarity (as  $S_{ab}$ ) scores determined by RDP. Scores of 1.0 are for identical sequences. The closer the score to 1.0, the more similar sequences are to each other. Scores  $>0.96$  loosely correlate to genus-level associations.

densities are high enough to support higher trophic levels (e.g., Simon et al., 2003; Simon et al., 2007). Based on observations of *C. servadeii* foraging on the moonmilk (Paoletti et al., 2011; Paoletti et al., 2013), we consider that the beetles feed directly on the microbial biofilms incorporated within the moonmilk structure. We suspect that the high microbial biomass within the moonmilk alone could serve as the base of a food web, but future work will need to measure the rates of microbial primary productivity specifically from the moonmilk. Further support for our hypothesis may come from the average organic C:N ratio for the moonmilk being 9.4 ( $\pm 1.3$ ) (Paoletti et al., 2011). This ratio implies that the nutritional quality of the moonmilk is potentially greater than plant-derived food, which would be  $\sim 15$  to  $>700$  depending on plant species or soil type (e.g., Snowdon et al., 2005). In general, ratios  $>5$  indicate an abundant carbon supply but a potential reduction in nitrogen availability due to storage of microbial biomass and nitrogen remineralization by microbes, which would have a C:N ratios for the biomass of  $\sim 2$ -8, depending on the cell type (Anderson, 1992).

Acknowledging that phylogeny does not yield unequivocal metabolic information (e.g., Achenbach & Coates, 2000; Rodríguez-Valera, 2002), and the clone libraries were not as exhaustively sampled as possible, there is still some indication from the 16S rRNA gene sequence data that the active metabolism of microbes within the moonmilk influences the geochemistry of the potential moonmilk-based food web. Specifically, the prevalence of microbes associated with the nitrogen cycle, and the detectable concentrations of nitrate, nitrite, and ammonium in the percolating waters, further point to an active nitrogen cycle that would affect how the trophic status is interpreted from stable isotope ratio analyses (Paoletti et al., 2011). Because the C:N ratio is  $>5$ , this would also force the animals to browse and process moonmilk constantly to achieve

sufficient organic N. Browsing behavior by *Cansiliella* spp. would aerate the top of the moonmilk in contact with the percolating waters, and this could not only affect the precipitation dynamics of carbonate minerals within the moonmilk, but also affect the microbial community structure and system geochemistry associated with carbon and nitrogen cycling. These results provide unique insight into the types of food webs that can develop in cave and karst habitats and offer important implications regarding the evolutionary adaptations of animals such as *Cansiliella* that exploit a hydropetric habitat.

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