

# Freeze–thaw revival of rotifers and algae in a desiccated, high-elevation (5500 meters) microbial mat, high Andes, Perú

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**Abstract** This is the first study of the highest elevation cyanobacteria-dominated microbial mat yet described. The desiccated mat was sampled in 2010 from an ephemeral rock pool at 5500 m above sea level in the Cordillera Vilcanota of southern Perú. After being frozen for 6 years at  $-20^{\circ}\text{C}$  in the lab, pieces of the mat were sequenced to fully characterize both the 16 and 18S microbial communities and experiments were conducted to determine if organisms in the mat could revive and become active under the extreme freeze–thaw conditions that these mats experience in the field. Sequencing revealed an unexpectedly diverse, multi-trophic microbial community with 16S OTU richness comparable to similar, seasonally desiccated mats from the Dry Valleys of Antarctica and low elevation sites in the Atacama Desert region. The bacterial community of the mat was dominated by phototrophs in the Cyanobacteria (*Nostoc*) and the Rhodospirillales, whereas the eukaryotic

community was dominated by predators such as bdelloid rotifers (Philodinidae). Microcosm experiments showed that bdelloid rotifers in the mat were able to come out of dormancy and actively forage even under realistic field conditions (diurnal temperature fluctuations of  $-12^{\circ}\text{C}$  at night to  $+27^{\circ}\text{C}$  during the day), and after being frozen for 6 years. Our results broaden our understanding of the diversity of life in periodically desiccated, high-elevation habitats and demonstrate that extreme freeze–thaw cycles *per se* are not a major factor limiting the development of at least some members of these unique microbial mat systems.

**Keywords** *Nostoc* · Glacial retreat · Exobiology · Polar deserts · Cryobiology · Aeolian zone · Rhodospirillales · Philodinidae

## Introduction

Global warming is causing the rapid retreat of glaciers in most high-mountain areas of the world (Marshall 2014;

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Marzeion et al. 2014; Rabatel et al. 2013). One such site is an intensively studied high-elevation (5000–6000 m) watershed that drains into Laguna Sibinacocha in the Cordillera Vilcanota of Perú (Nemergut et al. 2007; Seimon et al. 2009). This watershed encompasses both a large valley glacier (the Puca Glacier) and ice caps on the surrounding mountains. The new lands exposed as a result of the rapid retreat of the Puca Glacier are the focus of ongoing studies of microbial and plant succession that have revealed new insights into the mechanisms of microbial community assembly and ecosystem formation following glacial retreat (Knelman et al. 2014; Nemergut et al. 2007; Schmidt et al. 2012).

Less studied than the Puca Glacier, are lands left behind by the retreating ice caps that are prominent at higher elevations on the north and west sides of the Sibinacocha Valley. Due to their higher elevation, these ice caps are not retreating as rapidly as the Puca Glacier, but they are none-the-less retreating and leaving behind land that has not been ice-free for at least 5000 years (*cf* Buffen et al. 2009; Thompson et al. 2013). Newly exposed lands near ice caps are generally less disturbed than lands left behind by large valley glaciers, such as the Puca Glacier, because ice caps are not being driven downhill by the mass of ice above them. As a result, ice caps lack much of the grinding action of a glacier moving down a valley. Thus, it is possible to find remnants of former ecosystems (even intact plants) following retreat of some ice caps (Buffen et al. 2009).

The present study builds on previous microbiological studies of the terrain just below an ice-capped ridge on the western side of the Sibinacocha watershed (Schmidt et al. 2008, 2009), and focuses on the highest elevation ice-free site yet studied from a microbiological perspective in the Andes of Peru. The site is a rocky outcrop (5500 m.a.s.l.) that forms a jumble of rock peaks that are surrounded on two sides by ice cliffs. Environmental conditions at this site include a thin atmosphere and extreme diurnal temperature fluctuations (Schmidt et al. 2009). During an expedition to this area in 2003, we discovered a small rock pool at the high point of this outcrop (Suppl Figs. 1 and 2). During a subsequent expedition in 2010, small pieces of the desiccated microbial mat were sampled (Suppl Fig. 3).

The microbial mat described here is significant because it morphologically resembles seasonally desiccated mats found in the Dry Valleys of Antarctica and the high mountains of Central Asia (Hawes et al. 1992; McKnight et al. 1999; Schmidt et al. 2011; Zeglin et al. 2011), and is the highest elevation cyanobacterial mat yet studied. Here we describe the first microbial community analysis of this mat and experiments to determine if the organisms in the desiccated mat could be revived from frozen pieces of the mat collected during the dry season. This work reveals unexpectedly high diversity of both bacterial and eukaryotic

communities in the mat, and that some of the organisms in the mat could be revived even during extreme freeze–thaw cycles and after being desiccated and frozen for 6 years.

## Materials and methods

### Field site and sampling

The study site lies on the western side of the Sibinacocha watershed in the Cordillera Vilcanota, Perú, an area that has been the focus of long-term climate change monitoring (Seimon et al. 2009; Halloy et al. 2006; Schmidt et al. 2009). The site is a rocky peak (5500 m.a.s.l., Latitude,  $-13.767$ , Longitude,  $-71.088$ ) surrounded on two sides by ice cliffs. The small pool containing the microbial mat was first photographed in 2003 (Suppl Figs. 1 and 2) but was not sampled until the dry season in 2010 (August 5, 2010, Suppl Fig. 3). The pool was completely dried out (Suppl Fig. 3) when mat samples were taken in 2010; water content of the samples was 3.97% ( $SE=0.07$ ,  $N=3$ ). Samples were frozen in the field at  $-10^{\circ}\text{C}$  and then transported in a cooler to the lab where they were frozen at  $-20^{\circ}\text{C}$  until they were processed as described below.

Environmental conditions at this site are extreme and include a thin atmosphere and diurnal temperature fluctuations across the freezing point; day-time surface temperatures can reach at least  $+30^{\circ}\text{C}$  and night-time temperatures can plunge below  $-10^{\circ}\text{C}$  on the same day (Schmidt et al. 2009). Plants are sparse on this ridge and the site was recently designated a GLORIA (Gottfried et al. 2012) site to monitor the possible migration of plants into this recently de-glaciated area due to global change. This GLORIA site is called “Yurak” and was officially established in 2012. Yurak is the highest elevation GLORIA site in the world (Cuesta et al. 2016; Halloy et al. 2010; CONDESAN 2012), at 5498 m above sea level, and is just 5 m from the glacier forefront (see Suppl Fig. 2).

### Sequencing

Samples of the desiccated microbial mat were frozen in the field and shipped to the University of Colorado at Boulder and kept at  $-20^{\circ}\text{C}$  until processing. DNA was extracted by using a MO BIO PowerSoil DNA extraction kit (MO BIO Laboratories). The 16S rRNA and 18S genes were PCR-amplified using standard bar-coded primer sets (Caporaso et al. 2012), and samples were multiplexed for sequencing on the Illumina MiSeq platform at the University of Colorado, Boulder (BioFrontiers Institute) using paired-end  $2\times 150$  bp chemistry. A 30% phiX spike was added to the run to compensate for otherwise limited amplicon variability (Caporaso et al. 2012), and paired-end reads were

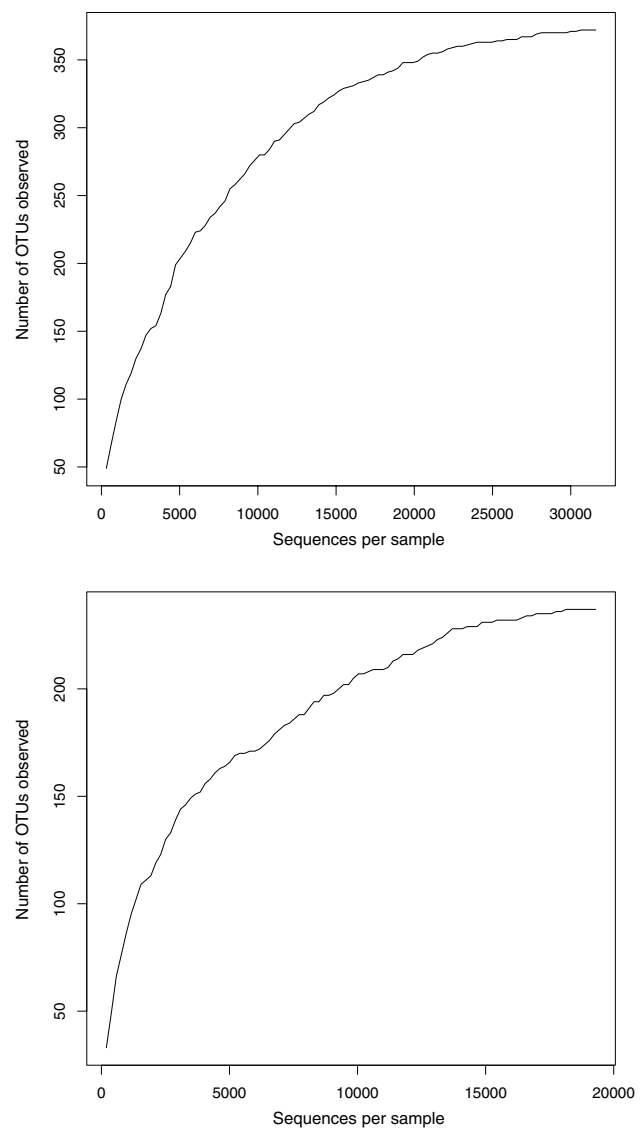
demultiplexed and joined, and OTUs were picked at 97% identity and assigned taxonomy using default parameters in QIIME (Caporaso et al. 2010). Taxonomy for 16S OTUs was assigned using the GreenGenes database and taxonomy for 18S OTUs was assigned using the SILVA Ref NR 99 database version 119 (available at <http://www.arb-silva.de/download/arb-files/>).

### Microcosm studies

Given that high-elevation sites are subject to some of the most extreme freeze–thaw cycles on Earth (Lynch et al. 2012; Yang et al. 2002), we carried out an incubation experiment to determine if organisms present in the mat could revive and become active during freeze–thaw cycles similar to those observed in the Sibiracocha watershed (Schmidt et al. 2009). Pieces of mats that had been frozen for 6 years at  $-20^{\circ}\text{C}$  (since their collection in 2010) were rehydrated and subjected to freeze–thaw cycles (experimental group) or left at room temperature (control group). Specifically, 0.03 g mat fragments were homogenized in 20 mL autoclaved Nice brand spring water for 3 s and 200  $\mu\text{L}$  aliquots of that slurry were distributed to 20 wells in each of 4 Costar 24-well tissue culture plates. Four wells in each plate received only 200  $\mu\text{L}$  autoclaved water instead of mat slurry as a negative control. Each well, including negative controls, also received 800  $\mu\text{L}$  of one of 8 factorial concentrations (Suppl Table 1) of Alga-Gro Freshwater Medium (Carolina Biological Supply) and inulin (MP Biomedicals, LLC), sterilized by autoclaving for 45 min at  $220^{\circ}\text{C}$ . Two of the tissue culture plates were placed in a growth chamber (Model TH024-LT, Darwin Chamber Co., St. Louis, USA) with a light–dark cycle of 12 h each, and a freeze–thaw cycle that mimics field conditions at the site (Suppl. Figure 5, Vimercati et al. 2016), and the other two were placed on a lab bench, where temperatures remained between 20 and  $24^{\circ}\text{C}$  throughout the incubation period (Suppl. Figure 5). All culture plates were observed daily using a dissecting microscope at 45x magnification.

### Results and discussion

Deep Illumina MiSeq sequencing of this mat resulted in almost saturated collector's curves indicating more than adequate sampling to describe the community. Figure 1 shows that there are at least 350 16S OTUs (at the 97% identity level) represented in more than 30,000 sequences obtained compared to just over 200 18S OTUs out of the almost 20,000 eukaryotic sequences obtained. This level of OTU richness for 16S OTUs is comparable to seasonally desiccated mats in the Dry Valleys of Antarctica, where Stanish et al. (2013) estimated from 168 to 371 bacterial

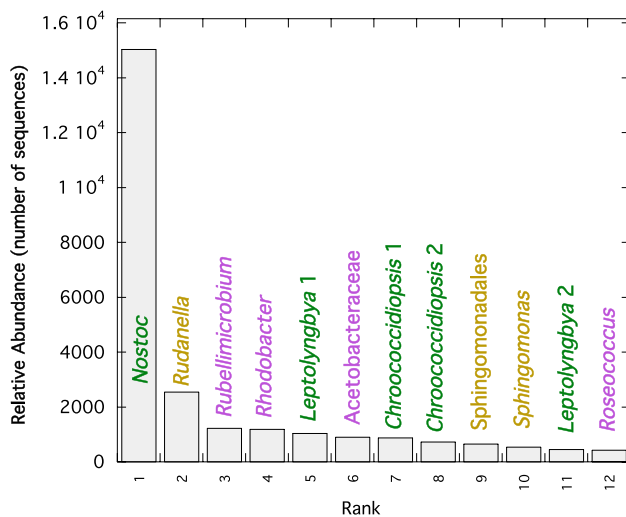


**Fig. 1** Collector's curves or alpha-rarefaction plots for the 16S community (a), and the 18S community (b) in the microbial mat, defined at 97% SSU rDNA sequence identity. Curves represent the richness estimate at any given sampling intensity (sequences per sample). Richness estimates plateau for both 16 and 18S communities, indicating that sampling was sufficient to capture richness of the mat

OTUs per sample based on much lower sequencing depth (3563 sequences per sample) than in the present study. To more fairly compare our results to those of Stanish et al. (2013), we randomly down-sampled our sequences (100 times per sample) to obtain the same sequencing depth used by Stanish et al. (2013). This resulted in a mean of 266 (SE, 7.5) OTUs for the mat studied here compared to a mean of 256 (SE=16.2) for the mats studied by Stanish et al. (2013). These results are also similar to the studies of hypersaline mats of the Atacama Desert region (Fariás et al. 2013; Rasuk et al. 2016; Fernandez et al. 2016). For example, Fernandez et al. (2016) reported between 121

and 316 16S OTUs in mats from the hypersaline Laguna Tebenquiche, Salar de Atacama, Chile. These findings demonstrate that the high-elevation mat described here is as diverse as aquatic mats from the Antarctic Dry Valley and the Atacama region, at least in regard to 16S phylotypes. Stanish et al. (2013) and Hernandez et al. (2016) did not do 18S sequencing but Esposito et al. (2006) have noted that microbial mats in seasonally desiccated streams harbor very diverse assemblages of eukaryotes, particularly diatoms, but estimates of 18S alpha diversity for seasonally desiccated and hypersaline microbial mats (using sequencing approaches) are presently not available in the literature for comparison to the present study.

The dominant OTU among 16S sequences from the mat correspond to the cyanobacterial genus *Nostoc*, with a single *Nostoc* OTU making up over half of all sequences obtained (15,000 of the 30,000 16S sequences; Fig. 2). This corroborates microscopic images which show a predominance of *Nostoc*-like filaments forming the mat (Supplemental Fig. 3). Members of the genus *Nostoc* are well known for being able to form macroscopic structures like mats; in fact, mats formed by *Nostoc flagelliforme* in arid, high-elevation regions of China and Mongolia have long been used as a food source for humans (Gao and Ye 2003) as have macroscopic spheres of *N. commune* in the Andes (Johnson et al. 2008). Mats of *N. flagelliforme* are resistant to UV radiation and actually require cycles of desiccation and rewetting to form mat-like structures (Feng et al. 2012). Cycles of desiccation and rewetting are common at the site, studied here, where there are distinct wet and dry seasons (Seimon et al. 2009).

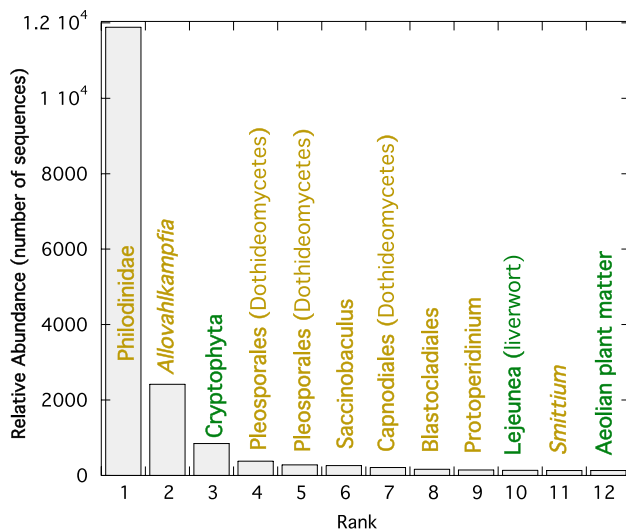


**Fig. 2** Rank-abundance plot of the twelve most abundant 16S OTUs (97% SSU rDNA sequence identity). Colors indicate which broad functional group of organisms each OTU belonged to: *Green* = cyanobacteria, *purple* = purple photosynthetic bacteria, and *brown* = heterotrophic bacteria

Other photosynthetic 16S phylotypes with high relative abundances in the mat include three other cyanobacteria (green in Fig. 2a) and four members of the Rhodospirillales order (Alphaproteobacteria) that are likely photoheterotrophs (Fig. 2). Photosynthetic members of the Rhodospirillales are informally classified as “purple photosynthetic bacteria” (PPB) which, in Fig. 2, include *Rhodobacter*, *Rubellimicrobium*, and members of the Acetobacteraceae, including *Roseococcus*. Previous studies have implicated PPB (e.g., the Rhodospirillales) as playing important roles in high-elevation soils and sediments in the Rockies, Andes, and Himalayas (Demergasso et al. 2010; King et al. 2010a, b; Rasuk et al. 2016; Schmidt et al. 2011), including some of the highest elevation (>6000 m.a.s.l.) sites on Volcán Llullaillaco in the Atacama Desert (King et al. 2010a, b). However, the current study is the first report of PPB in high-elevation mat communities. Many PPB can utilize light in the infrared range (e.g., 875 nm, Ragatz et al. 1995) and are frequently found underneath layers of cyanobacteria (that can filter out visible light) in laminated microbial mats (Madigan 1988). It is likely that PPB inhabit a similar niche in the microbial mat described here, but further work would be needed to confirm this hypothesis.

It is also possible that the two members of the Sphingomonadales order (Alphaproteobacteria) shown in Fig. 2a are also photosynthetic. Recent work has shown that some members of the genus *Sphingomonas* are capable of aerobic anoxygenic photosynthesis (AAP) and occur in high-light biological soils crusts (BSCs) where they, like the PPB discussed above, can use near-IR radiation not used by the dominant cyanobacteria in BSCs (Csotonyi et al. 2010). However, it is impossible to determine if the sphingomonads found in the mat are photosynthetic based just on 16S data, because even very closely related *Sphingomonas* species can be non-photosynthetic (Csotonyi et al. 2010; Reddy and Garcia-Pichel 2007). Nonetheless, our results show that organisms with the evolutionary potential to carry out alternative modes of photosynthesis are among the dominant organisms in this high-elevation mat community.

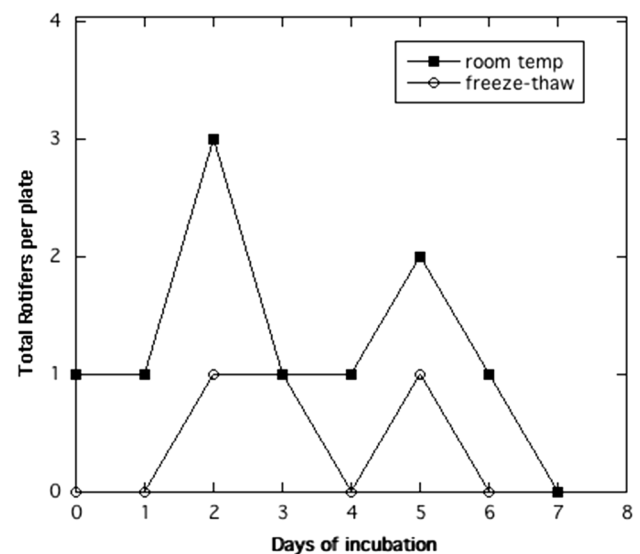
In contrast to the 16S bacterial community, the 18S (eukaryotic) community was underrepresented by photosynthetic members (Fig. 3). This low relative abundance of phototrophic phylotypes is unusual for a proglacial, high-elevation ecosystem (*cf.* Schmidt and Darcy 2015) and may reflect the competitive advantage of mat-forming, N-fixing, cyanobacteria, and PPBs in this system. The most abundant 18S phylotype in a putative photosynthetic group (Cryptophyta, Fig. 3) is related to an environmental sequence (TAGIRI-28, AB191436) isolated from deep-sea sediments near a fumarole (Takishita et al. 2005). The Cryptophyta are transitional forms that have secondarily acquired eukaryotic endosymbionts (Curtis et al. 2012), and may not always be photosynthetic. Curiously, the next



**Fig. 3** Rank-abundance plot of the twelve most abundant 18S OTUs (97% SSU rDNA sequence identity). Colors indicate which broad group of organisms each OTU belonged to: *Green* = photosynthetic, and *brown* = heterotrophic

most abundant 18S photosynthetic phylotypes in the mat are related to a liverwort (*Lejeunea*) and a plant (Fig. 3). Given that there are no liverworts or plants associated with the mat, it is possible that these sequences are from aeolian deposited debris or from lower-elevation plants. Similar detection of alien DNA has been reported in other high-elevation environments, that is, Vimercati et al. (2016) found similar levels of plant DNA in volcanic deposits at 6030 m.a.s.l. on Volcán Lullailloco, despite the fact that plants cannot grow above 5100 m.a.s.l. on that mountain.

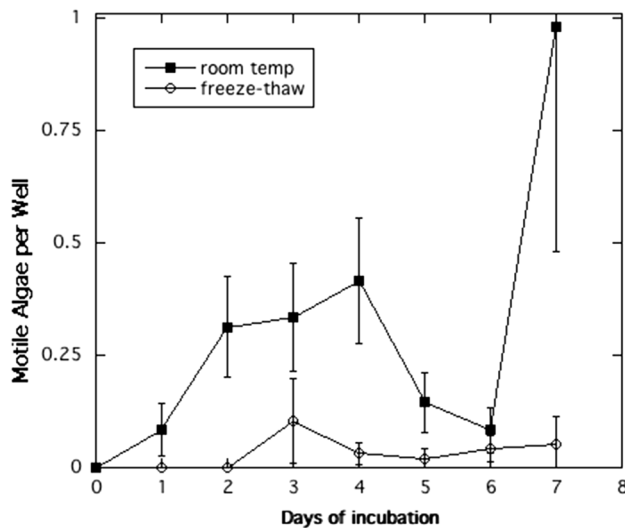
Our 18S data point towards Eukaryotes being important consumers and predators in the mat. The dominant 18S OTU corresponds to the Philodinidae family of the Bdelloid Rotifers, with a single Philodinidae OTU (closest BLAST match, GQ922300, Robeson et al. 2009) making up over 57% of all sequences obtained (11,883 of the 20,737 18S sequences; Fig. 3). Members of the Philodinidae are common in microbial communities in extreme environments (Porazinska et al. 2004; Robeson et al. 2011), especially environments that go through periods of desiccation (Alpert 2006), such as the mat in the present study. Bdelloid rotifers are predators of other microbes and likely subsist on free living bacteria and small eukaryotes during periods when the mat is fully hydrated. All of the other dominant eukaryotic phylotypes shown in Fig. 3 are members of groups that are either predators/parasites of other microbes, or decomposers. For example, the second most abundant 18S OTU is related to *Allovahlkampfia spelaea*, an amoeba that has been isolated from carbonate precipitating microhabitats of karst caves (Walochnik and Mulec 2009; Brown et al. 2012). Other potential parasitic



**Fig. 4** Active Bdelloid Rotifers observed in pieces of the mat incubated at room temperature (*filled squares*) or under extreme freeze–thaw (*open circles*) conditions (Supplemental Fig. 4). There are no error bars due to the fact that rotifers were not present on every replicate piece of mat used in the study. Nonetheless, these data do show that rotifers were able to become active during extreme freeze–thaw cycles

eukaryotes include the Blastocladiiales group of zoosporic fungi, although some members of that group can be decomposers (James et al. 2006; Naff et al. 2013). Other decomposers include three OTUs from the Dothideomycetes (Fig. 3) related to similar melanized “black yeasts” found in extreme high-elevation and high-latitude sites (Friedmann 1982; Lynch et al. 2009; Onofri et al. 2008) and deserts (Staley et al. 1982). Several of the eukaryotic OTUs may be from aeolian deposition or from insects visiting the mat from lower elevations, that is, *Smittium* and *Saccinobaculus* (Fig. 3) are groups normally found in insect guts. It is also possible that small insect could persist at these mats during the wet season, but none were present during the dry season when the mat was sampled.

In the microcosm experiment, rotifers were observed moving under both freeze–thaw and control conditions after only 2 days of incubation (Fig. 4). Because rotifers, ciliates, and motile algae were sparse and not meaningfully different across the various media, the numbers presented are summed over all treatments in each temperature regime. Likewise, mobile algae were observed in both freeze–thaw and controls, whereas ciliated protozoa only revived in the control plates (Fig. 5). These results highlight that even complex organisms such as rotifers can rapidly become active during severe freeze–thaw cycles, and that they can easily revive despite being in a frozen, desiccated state for 6 years, as Antarctic rotifers have been reported to do as well (Newsham et al. 2006). It is interesting that smaller

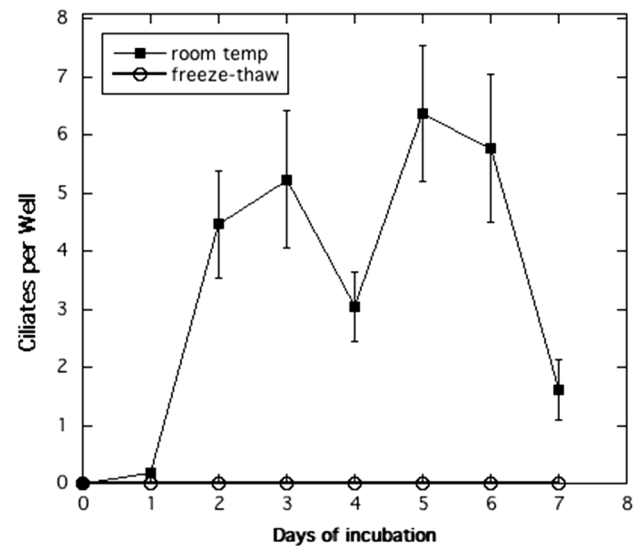


**Fig. 5** Swimming algae observed in pieces of the mat incubated at room temperature or under extreme freeze–thaw conditions, showing that algae became active after 1 day at room temperature and after 3 days under freeze–thaw conditions

organisms such as the ciliated protozoa were apparently not able to become active during freeze–thaw cycles, but were quite common in the control incubations. This is similar to the observations of Smith et al. (1990) who showed that the Antarctic ciliate *Colpoda* required at least 48 h of continuous temperatures above 4°C for excystment to occur and may indicate that the mat studied here probably experiences extended periods (>48 h) of temperatures above freezing in the field in order to explain the abundance of active ciliates in the mats at room temperature and their complete absence under freeze–thaw conditions (Fig. 6). Such extended periods of warmth may occur during the wet season at this site, but currently no data exist to test this hypothesis.

## Conclusion

Overall, this preliminary study of the highest elevation microbial mat yet studied gives insights into the inhabitants and possible trophic structure of this unique ecosystem. Both from visual observations and sequencing data, the dominant primary producers in this mat are cyanobacteria and PPBs (Fig. 2 and supplemental Fig. 3). It is likely that the eukaryotic inhabitants of the mat are mostly playing the role of consumers and decomposers as indicated by the predominance of Bdelloid rotifers and amoeba related to *Allovahlkampfia* (Fig. 3). These organisms are likely feeding directly on the primary producers and the abundant heterotrophic bacteria associated with the mat. In addition to this ecosystem being supported by indigenous photosynthesis, there is also evidence of some



**Fig. 6** Swimming ciliated protozoa observed in pieces of the mat incubated at room temperature or under extreme freeze–thaw conditions, showing that ciliates became active after 2 days at room temperature, but remained encysted during freeze–thaw cycles

aeolian support in the form of biomass input from lower elevations; that is, the mat contained DNA from plants, liverworts, and insect gut symbionts. Aeolian support of micro-ecosystems has been well documented in other high-elevation ecosystems (Mladenov et al. 2012; Swan 1992; Vimercati et al. 2016). Finally, this study provides the first evidence for the ability of complex predators such as bdelloid rotifers to come out of dormancy during freeze–thaw cycles. This finding broadens our understanding of how life can persist in an environment where daily freeze–thaw cycles can occur on a year-round basis. Future work is needed to understand the seasonal dynamics of this unique system and to compare it from a biogeographical perspective to similar mats in Antarctica and elsewhere.

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