A Comparison of Winter and Summer Microbial Culture Libraries from a High-elevation Mountainous Soil Ecosystem

Hans Singh

ABSTRACT

Microbes play a key role in regulating the flow of nutrients through high-elevation, mountainous ecosystems. Although there is a well-established microbial biomass crash along the winter-spring transition period in mountainous ecosystems, the extent to which this is coupled to the plant growing period is still being determined – especially in the face of climate change acceleration. Model isolates can be used to characterize the pre-snowmelt and post-snowmelt soil communities and analyze how they differ physiologically to determine the potential impacts of climate change on high-elevation systems. In this study, I examined what microbial isolates could be cultured from the winter and summer montane soil communities under various growth parameters. In all, 423 microbial isolates were cultured and saved as glycerol stocks, with 363 appearing to be bacterial and 60 fungal. Of these, a representative subset of 87 bacterial isolates was chosen for sequencing and physiological assays. Summer isolates showed slightly greater diversity and appeared significantly quicker on plates than winter isolates. The nutrient levels in the media and the temperatures the isolates were grown at did not change the taxa that could be cultured, but the presence of Streptomycin was key in generating Proteobacterial isolates. In all, Actinobacteria was the most commonly phyla seen of those sequenced (57 isolates), while Proteobacteria (21 isolates), Bacteriodetes (8 isolates) and Firmicutes (1 isolate) were also seen. Isolates saved as glycerol stocks can be used in further laboratory-controlled experiments to simulate the effects of various growth conditions on select high-elevation soil microbes.

KEYWORDS

Microbial diversity, culture-dependent, isolates, winter-spring transition, montane, optimal growth conditions

INTRODUCTION

If current trends continue, projections estimate that within 50 years, the snow season could be shortened by almost two and a half months in mountainous regions (Magnusson et al. 2010). Hydrologically, montane regions are projected to receive less precipitation in the future due to climate change (Ernakovich et al. 2014). Snow melt can also affect the hydrological cycles of these regions, as runoff contributes to the creation of water reservoirs that sustain life in the non-winter months (Magnusson et al. 2010). With climate change, increased and earlier melting will first lead to greater buildup of water reservoirs – but over long timescales, as glaciers recede, water reservoirs could partially or completely dry up (Xu et al. 2009). In alpine and mountainous regions, while snow melt will begin to occur earlier in the year due to climate change, the spring growing season will largely be constrained by photoperiod (Ernakovich et al. 2014). With the timing of snowmelt shifting earlier and plant growing season staying put, this asynchronous timing could lead to decreased water availability for plants, which could have ecosystem-wide impacts such as decreased plant production and increased nutrient loss. Freezing and thawing of ice in montane regions can also lead to elevated levels of microbial nitrogen release from soils, which can thus alter the global nitrogen cycle. (Matzner and Borken 2008).

Microbes play a large role in controlling the flow of nutrients (Hodson et al. 2008) through high-elevation ecosystems and are subject to high turnover rates (Schmidt et al. 2007). In particular, montane systems are characterized by a winter-spring transition period that occurs at snowmelt. Prior to snowmelt, microbial biomass increases under the winter snowpack as Nitrogen is immobilized by the soil microbial community (Xia et al. 2014). With snowmelt comes a crash in the microbial biomass, and this crash allows for a pulse of nutrients (in particular dissolved Nitrogen as Nitrogen is mineralized) that is timed to aid the spring plant growing period. Much like the asynchronous water availability from earlier snow melts, there is a concern that earlier snow melts could lead to microbes releasing nutrients at times before plants have historically become active in mountainous regions (Tan et al. 2014). As such, the microbial nutrient pulse would occur too early, and this mistiming could cause cascading effects such as decreased plant productivity and nutrient leaching. Although the microbial biomass crash at the winter-spring transition period is well-established, the extent to which this microbial release of nutrients is coupled to plant productivity is not well understood, and could perhaps be analyzed through model

isolates. With the ever accelerating rate of climate change, it is crucial to consider how pre-snowmelt and post-snowmelt montane microbial communities are characterized – both taxonomically and physiologically - to project the impacts of possible ecosystem shifts due to climate change.

Recent work has shown four ecological strategies governing bacterial response across the winter-spring transition (Sorensen et al. 2018). Taxa that decrease from winter to summer fall into (Snowmelt Tolerant – Winter Adapted), taxa that increase from winter to summer fall into (Snowmelt Tolerant – Spring Adapted) and taxa that stay the same from winter to summer are either (Snowmelt Sensitive – Winter Adapted) or (Snowmelt Sensitive – Spring Adapted). Notably, taxa that decrease from winter to summer are not phylogenetically clustered, while those that increase from winter to summer are phylogenetically clustered. As such, perhaps there are traits that characterize the microbial community that thrive post-snowmelt. The exact factors that determine which microbes survive the snowmelt transition period have not been elucidated. However, it has been suggested that osmotic tolerance (Jefferies et al. 2010) or substrate utilization (Lipson et al. 1999) could be traits that separate pre-snowmelt and post-snowmelt soil microbial communities.

There is an importance in culturing bacterial isolates that has been overwhelmed in the rise of metagenomics. Although it has been well established that culturing bacteria is relatively inefficient – only around 1% of bacterial strains have been able to be cultured - it is still incredibly important in building our knowledge of microbial communities (Vartoukian et al. 2010, Pham et al. 2012, Overmann et al. 2017). Cultured isolates allow for the analysis of bacterial physiology and gives model bacterial isolates that can be used in controlled laboratory conditions to simulate how site-specific bacterial isolates can respond to various manipulated conditions (Overmann et al. 2017). Transcriptomic and proteomic studies have also been conducted to look at bacterial gene expression under changing conditions using cultured bacterial isolates (Dang et al. 2016). Previous studies have shown that long incubation times of up to 50 days and usage of dilute nutrient concentrations can lead to increased diversity in culturing isolates (Nunes et al. 2015). With montane microbial isolates, one can run physiological tests to figure out the traits that determine which taxa are able to survive the microbial biomass crash at snowmelt. High-elevation microbial isolates can also be used for a broad range of other laboratory-controlled experiments to see how microbes respond to manipulated variables – for example, one could study the microbial

competition and symbioses under various conditions using co-cultures or the genes expressed at simulated climatic variations using transcriptomics.

In this study, I will undertake an examination of what isolates can be cultured from the winter and summer soil communities at the high-elevation East River site. To determine how temperature affects what can be cultured, I will grow bacterial isolates under 2 temperature conditions. Microbial diversity is expected to be greatest at room temperature, because higher temperatures makes it easier for microbes to grow. Media will be tailored to culture both bacteria and fungi. In addition, I will examine if dilute nutrient concentrations have any effect on which bacterial isolates can be cultured, because dilute nutrient plates have allowed for the isolation of a different subset of bacteria (Nunes et al. 2015). The time scales upon which bacterial isolates can be cultured will also be noted and compared across the phyla level, with the majority of isolates expected to be seen within the first two weeks. Once isolates have been stored as a glycerol stock, their taxonomic breakdown, growth rates, freeze-thaw tolerance and substrate preferences can be examined.

METHODS

Study Site

The East River watershed near the Rocky Mountain Biological Laboratory is the site for many ongoing research investigations into how mountainous ecosystems are responding to change. The location of this site is directly northeast of the town of Crested Butte, Colorado. Ten soil samples were collected for my project using a standard soil core at depths of between 5-15 meters. While a portion of the site is devoted to an ongoing project simulating advanced warming during the winter months, the soil samples that were used for my project from the lower montane floodplain were not from soil that has experienced accelerated warming. Site characteristics include an elevation of 3266 meters, average minimum and maximum temperatures of -9.2 ^{o}C and 9.8 ^{o}C and roughly 600 mm of precipitation per year, most of which falls as snow (LBNL).

Collection of Bacterial Cultures

To culture bacterial isolates from the East river site, soil samples were first taken during both summer and winter time points and transported on ice back to LBNL. Three grams of soil collected from the experimentally warmed study site were diluted and plated onto agar media. For both summer and winter months, two replicates were used to give a total of four samples. I carried out dilutions in a 50g Falcon tube to get 30-300 bacterial isolates per plate, using an estimation of 10^9 cells/gram of soil and using PBS buffer. Gelzan agar media was used at two different nutrient concentrations, and Streptomycin was used as an antibiotic to try to create a media more proficient in isolating fungi. In total, I used four types of agar media – 1/10 R2G, 1/10 R2GF, 1/100 R2G and 1/100 R2GF. Plates were stored at room temperature (18 ^oC) and in a fridge (4 ^oC). As colonies appeared, they were transferred to a new plate and restreaked until pure. Plates were dated to help determine the timescales upon which colonies appeared. Pure isolates were then grown as liquid cultures and replated to double check that they were pure cultures. If they were indeed pure cultures, the liquid culture was then permanently stored as a glycerol stocks in a -80 ^oC freezer (using 25% glycerol).

Taxonomic Characterization

A representative subset of 96 isolates was then chosen for taxonomic characterization. Care was taken such that this subset both maximized the morphological diversity and represented the full spectrum of growth conditions (seasonal soil community, growth temperature, nutrient level, Streptomycin resistance and time to appearance). A colony PCR was then carried out on a pure culture of each isolate using the 27F and 1492R primers, and DNA was visualized using gel electrophoresis. Each PCR sample that produced bands of DNA was then put through PCR cleanup and Sanger sequencing was used to get sequence reads. The Geneious program was used to assemble the consensus sequence for each read, and these sequences were compared to the BLAST database to assign their taxonomic identity. The corresponding FASTA sequences were then inputted into ITOL to create phylogenetic trees for my isolates.

RESULTS

Cultured isolates and their time to appearance

In all, 423 microbial isolates were cultured and saved as glycerol stocks (table 1). As expected, way more microbes were able to be cultured at room temperature (319 isolates) than when refrigerated (104 isolates). Culture totals were roughly the same for both winter (204 isolates) and summer (219 isolates) soil microbial communities. Based on morphological characterizations, 363 of my isolates were bacterial and 60 were fungal. The amount of bacterial and fungal colonies were proportionally similar amongst my four treatment groups (table 2). When using a Chi-square test to analyze the frequency of fungal isolates, the p-value was 0.35, indicating that there was not a significant difference in the rate of isolating fungi in any of my various treatments. Isolates from the summer soil community were more successful at being grown on plates with Streptomycin (table 3). When using a Chi-squared test to evaluate the amount of Streptomycin-resistant and non-Streptomycin resistant colonies from the winter and summer soil communities, the p-value was 0.0005. Thus, at the 5% significance level, we can conclude that summer isolates grew significantly better on plates with Streptomycin. Additionally, we see that significantly more isolates from the summer soil community were able to be grown in the fridge than from the winter soil community (p-value = 0.0006).

	Summer	Winter	Total
Room temperature (18 ºC)	150	169	319
Fridge (4 ºC)	69	35	104
Total	219	204	423

Table 1 – All isolates grouped by soil community (winter or summer) and temperature grown at (4 ºC or 18 ºC).

	Summer, RT	Winter, RT	Summer, Fridge	Winter, Fridge	Total
Bacterial	126	151	58	28	363
Fungal	24	18	11	7	60
Total	150	169	69	35	423

Table 2 – All isolates grouped by whether they were morphologically characterized as bacteria or fungi. Colonies with hyphae were morphologically characterized as fungi, while colonies without hyphae were characterized as bacteria. Isolates are grouped by the four treatment groups they belong to (summer soil community –room temperature grown, summer soil community – fridge grown, winter soil community – room temperature grown and winter soil community – fridge grown).

	Summer	Winter	Total
Room temperature (18 ºC)	20	5	25
Fridge (4 ºC)	6	1	7
Total	26	6	32

Table 3 – All isolates grown on plates with Streptomycin. Grouped by soil community (winter or summer) and temperature grown at (4 ºC or 18 ºC).

For isolates grown at room temperature, there was a difference in how long it took colonies to appear from winter and summer soil communities, as the median time to appearance for winter isolates was 18 days, while the median time to appearance for summer isolates was 7 days (figure 1). When using an independent samples t-test to compare the mean time to appearance between summer and winter isolates, a t-value of -5.33 was obtained, which gives a p-value of less than 0.00001. Thus, at the 5% significance level, we conclude that summer colonies appeared significantly more quickly on plates than winter colonies. In all, colonies appearance time ranged from as short as 2 days to as long as 77 days.

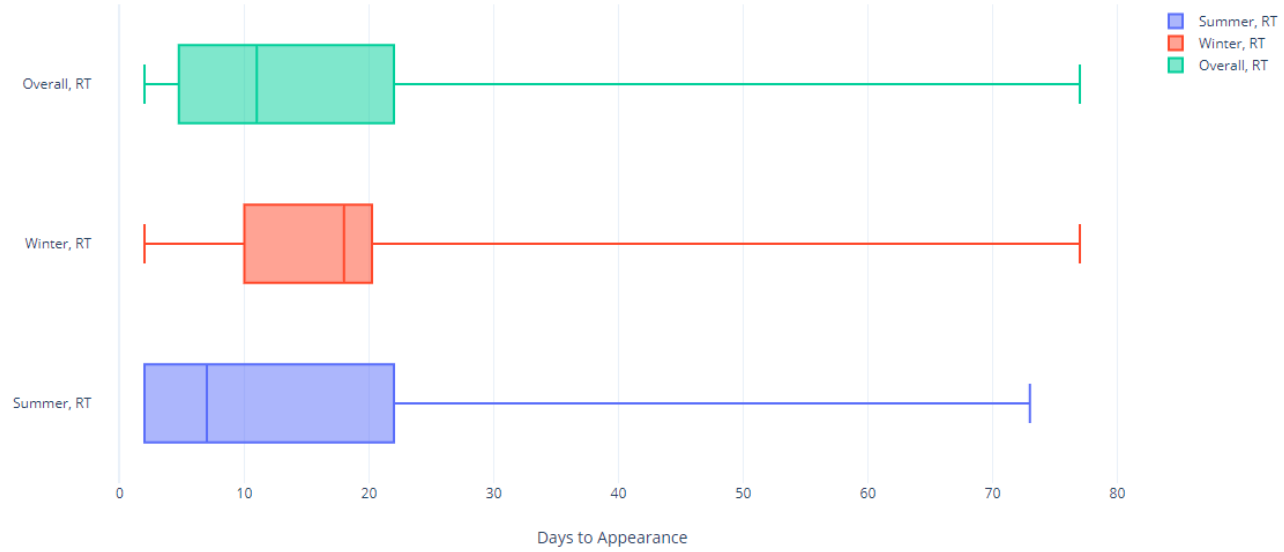

Figure 1 – Box and whisker plot showing time to appearance for isolates grown at room temperature. The top bar shows all room temperature grown isolates (median of 11 days to appearance), the middle bar shows all room temperature grown isolates from the winter soil community (median of 18 days to appearance), and the lowest bar shows all room temperature grown isolates from the summer soil community (median of 11 days to appearance).

For isolates grown in the fridge, no isolates appeared within the first ten days. As such, it was assumed that perhaps no isolates would appear at all when grown in the fridge. However,

when I checked back in on October 20th (at 22 days in for summer fridge isolates and 27 days in for winter fridge isolates), 54 colonies from the summer soil community and 30 colonies for winter soil community had appeared (figure 2). Thus, since the mean time to appearance for winter isolates cannot be safely determined, one cannot compare the mean time to appearance for fridge-developed and room-temperature developed colonies. Instead, we see that amongst room-temperature developed colonies 49% (156/319) appeared within the first ten days. Comparatively, when grown in the fridge, no colonies appeared within the first ten days. Upon using a Chi-square test to compare the amount of isolates that grew within the first ten days, the p-value was less than 0.000001, indicating that there was a significant difference in the frequency of colony appearance within the first ten days between fridge-grown and room temperature-grown isolates.

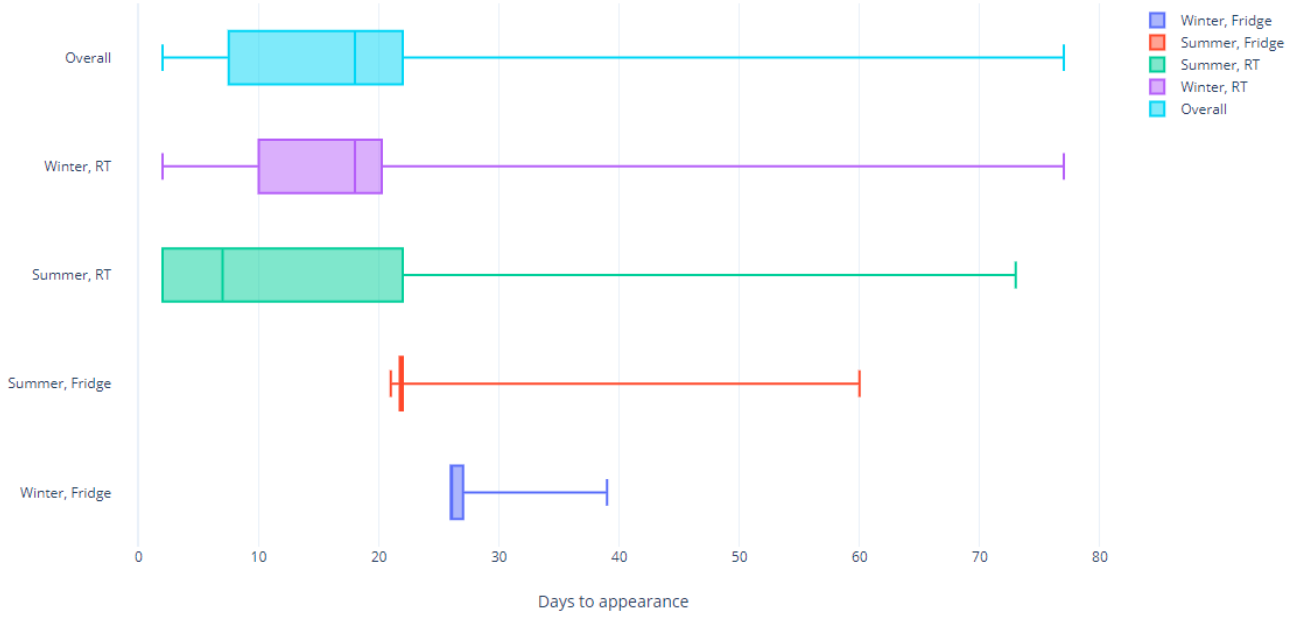

Figure 2 – Box and whisker plot showing time to appearance for all isolates across our four treatment groups. The top bar shows all all isolates (median of 18 days to appearance), the second bar shows all room temperature grown isolates from the winter soil community (median of 18 days to appearance), the third bar shows all room temperature grown isolates from the summer soil community (median of 11 days to appearance), the fourth bar shows fridge grown isolates from the summer soil community (median of 22 days to appearance), and the final bar shows all fridge grown isolates from the winter soil community (median of 11 days to appearance). For isolates grown in the fridge, no isolates appeared within the first ten days. As such, it was assumed that perhaps no isolates would appear at all when grown in the fridge. However, when I checked back in on October 20th (at 22 days in for summer fridge isolates and 27 days in for winter fridge isolates), 54 colonies from the summer soil community and 30 colonies for winter soil community had appeared (figure 2). Thus, the median times for the fridge-grown colonies are inexact.

Taxonomic data

Of the subset of 96 representative isolates (table 4) that were chosen to undergo sequencing, 87 returned high quality sequence reads (table 5). In all, 4 different phyla were recovered (figure 3), with Actinobacteria being the most common (57 isolates), followed by Proteobacteria (21 isolates), Bacteriodetes (8 isolates) and Firmicutes (1 isolate). Bacterial diversity increased steadily at each taxonomic level (figure 4), and in total, 41 different genera were sequenced.

	Fast growers	**Slow growers**	**Total**
Fridge, winter, 1/10	**0**	**7**	**7**
Fridge, winter, 1/100	**0**	**7**	**7**
Fridge, summer, 1/10	**0**	**10**	**10**
Fridge, summer, 1/100	**0**	**10**	**10**
Room temperature, winter, 1/10	**8**	**7**	**15**
Room temperature, winter, 1/100	**7**	**8**	**15**
Room temperature, summer, 1/10	**8**	**8**	**16**
Room temperature, summer, 1/100	**8**	**8**	**16**
Total	**31**	**65**	**96**

Table 4 – Subset of isolates chosen for taxonomic evaluation. Care was taken to get a proportional amount of isolates for each of the various parameters (growth temperature, initial soil community, nutrient levels). Furthermore, we aimed to get roughly half of our room temperature isolates to be fast growers (quicker than the median time to appearance) and half of our isolates to be slow growers (slower than the median time to appearance. All of our fridge-developed isolates were slow growers (slower than the median time to appearance).

	Summer	Winter	Total
Room temperature	30	26	56
Fridge	18	13	31
Total	48	39	87

Table 5 – Subset of isolates that were sequenced and returned high quality reads grouped by soil community (winter or summer) and temperature grown at (4 ºC or 18 ºC).

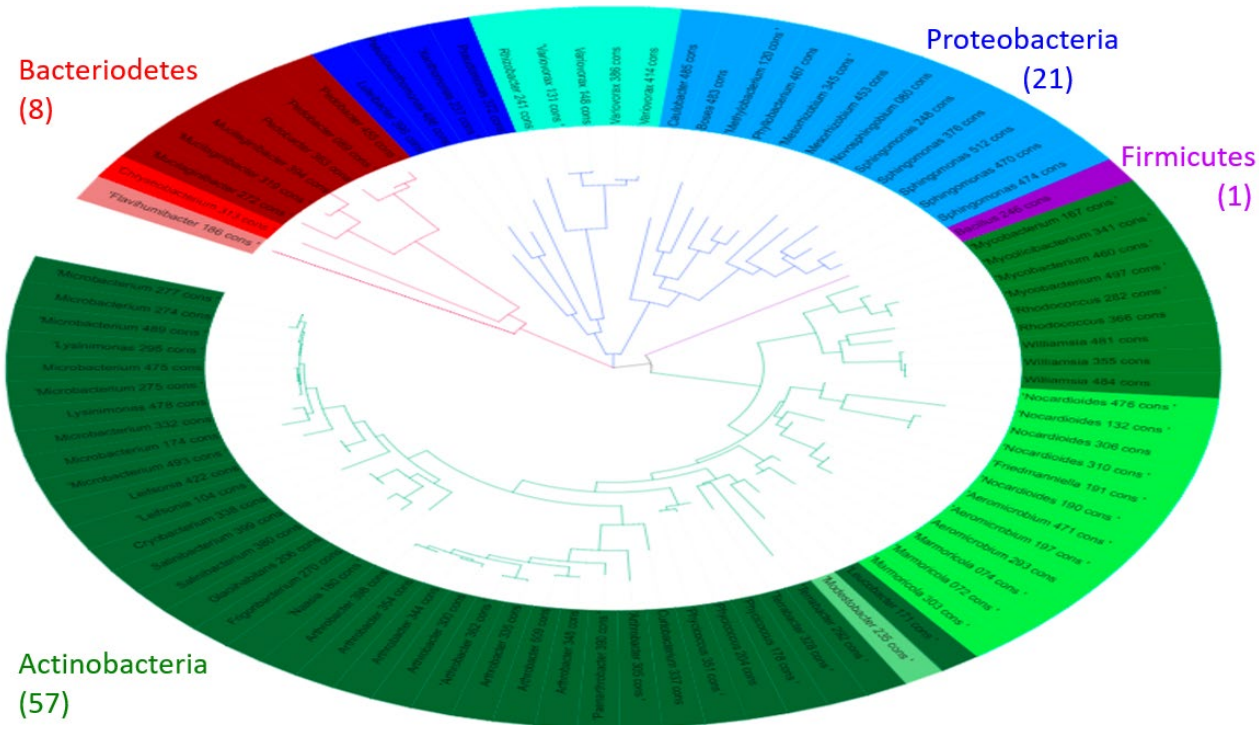

Figure 3 – Phylogenetic tree showing the phyla of the sequenced subset of isolates. The Bacteriodetes isolates (denoted in red, 8 isolates) fell into three different classes – Chitinophagia (1 isolate), Flavobacteriia (1 isolate) and Sphingobacteria (6 isolates). The Proteobacteria isolates (denoted in blue, 21 isolates) fell into three different classes – Gammaproteobacteria (4 isolates), Betaproteobacteria (5 isolates) and Alphaproteobacteria (9 isolates). There was one Firmicutes isolate (denoted in purple). Lastly, the Actinobacteria isolates (denoted in green, 57 isolates) fell into four different orders – Corynebacteriales (9 isolates), Propionibacteriales (12 isolates), Geodermatophilales (1 isolate) and Micrococcales (35 isolates).

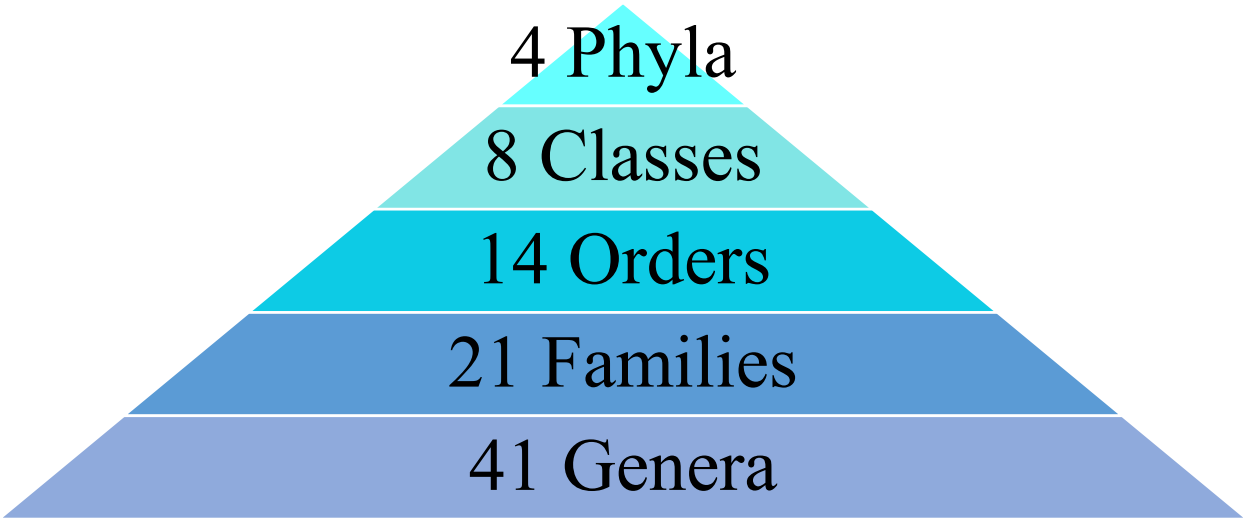

Figure 4 – Image showing the results from the sequenced subset of isolates. 4 phyla, 8 classes, 14 orders, 21 families and 41 genera were able to be isolated.

The 57 Actinobacteria isolates that were cultured belonged to four different orders – Micrococcales (35 isolates), Propionibacteriales (12 isolates), Corynebacteriales (9 isolates) and Geodermatophiles (1 isolate). Microbacteriaceae was the most common family isolated (18 isolates), and Arthrobacter (9 isolates) just edged out Microbacterium (8 isolates) as the most common genus isolated. One notable trend here is that 10 of the 12 Propionibacteriales isolated were from the winter soil community. Using a Chi-squared test to look at the difference in Propionibacteriales isolated from the winter and summer soil communities, we get a p-value of 0.004. Thus, we conclude that at the 5% significance level, significantly more Propionibacteriales isolates were able to be cultured from the winter soil community.

While the vast majority of our isolates were Actinobacteria, 34.5% (30/87) belonged to other phyla. Of these 30 isolates, 29 were gram-negative and fell into either the Bacteriodetes or Proteobacteria phylas. The Bacteriodetes (figure 3) isolates fell into three different classes – Chitinophagia (1 isolate), Flavobacteria (1 isolate) and Sphingobacteria (6 isolates). Of the 8 Bacteriodetes isolates, 6 were recovered from the summer soil community. Similarly, the Proteobacteria isolates (figure 3) also fell into three different classes – Alphaproteobacteria (12 isolates), Betaproteobacteria (5 isolates) and Gammaproteobacteria (4 isolates). Interestingly, all four of the Gammaproteobacteria isolates were from the summer soil community. Also, 5 of the 6 isolates in the Sphingomonadales order (under the Alphaproteobacteria class) were developed on plates with high nutrient levels. Finally, our only recovered gram-positive isolate that didn't belong to the Actinobacteria phyla was a Bacillus isolate (Firmicutes phyla) that was from the summer soil community and grown at room temperature.

When comparing winter and summer isolates, the summer soil community was able to recover slightly greater diversity (tables 6-7). Because the majority of isolates we recovered were Actinobacteria isolates, bacterial diversity can be measured by how many isolates from other phyla we were able to isolate. However, when using a Chi-square test to compare the frequency of recovering phyla other than Actinobacteria between winter and summer isolates, the p-value was 0.117. This indicates that at the 5% significance level, there was not a significant difference between the winter and summer soil communities in isolating the Proteobacteria, Bacteriodetes and Firmicutes phyla. Additionally, the variables of media nutrient level (p-value = 0.50) and temperature grown at (p-value = 0.47) did not show differences in ability to isolate phyla other than Actinobacteria.

	Summer	Winter	Total
Actinobacteria	28	29	57
Other phyla	20	10	30
Total	48	39	87

Table 6 – Subset of isolates that were sequenced and returned high quality reads grouped by soil community (winter or summer) and the phyla the isolate belonged to (Actinobacteria or another phyla). If we characterize the ability to recover non-dominant phyla, i.e. non-Actinobacteria, as a measure of increased bacterial diversity, it appears as if the summer soil community was able to return slightly more bacterial diversity. However, when using a Chi-square test to compare the frequency of recovering phyla other than Actinobacteria between winter and summer isolates, the p-value was 0.117 (not significant at significance level 0.05).

Winter	**Summer**
39 Isolates sequenced	**48 Isolates sequenced**
29 Actinobacteria	**28 Actinobacteria**
8 Proteobacteria	**13 Proteobacteria**
2 Bacteriodetes	**6 Bacteriodetes**
0 Firmicutes	**1 Firmicutes**
29/39 G+ (0.74)	**29/48 G+ (0.60)**
3 Phyla isolated	**4 Phyla isolated**
5 Classes isolated	**7 Classes isolated**
10 Orders isolated	**11 Orders isolated**
13 Families isolated	**18 Families isolated**
23 Genera isolated	**31 Genera isolated**

Table 7 – A comparison of the isolates sequenced grouped into either the winter or summer soil communities. The summer soil community showed slightly greater diversity across the board – notably there was a greater proportion of gram-negative bacterial isolates from the summer soil community, although it is important to note that more summer isolates were sequenced in general.

The variable that produced the clearest trends was the presence of Streptomycin on the plates (table 8). Of the 8 isolates that were sequenced from Streptomycin-developed media, 7 Proteobacteria isolates and 1 Bacteriodetes isolate were recovered. This represented an important source of Proteobacterial diversity, as 3 of our 4 recovered Gammaproteobacteria isolates and 4 of our 12 recovered Alphaproteobacteria isolates were recovered from Streptomycin plates. Upon using a chi-squared test for the difference in isolating Proteobacteria isolates from Streptomycin and non-Streptomycin plates, the p-value was 0.00001, indicating that plates with Streptomycin were significantly better at isolating Proteobacteria.

Streptomycin	No Streptomycin
8 Isolates sequenced	79 Isolates sequenced
0 Actinobacteria	28 Actinobacteria
7 Proteobacteria	14 Proteobacteria
1 Bacteriodetes	7 Bacteriodetes
0 Firmicutes	1 Firmicutes
0/8 G+ (0.00)	58/79 G+ (0.73)
3 Gammaproteobacteria isolated	1 Gammaproteobacteria isolated
4 Alphaproteobacteria isolated	8 Alphaproteobacteria isolated

Table 8 – A comparison of the isolates sequenced grouped into either growing on plates with Streptomycin or growing on plates without Streptomycin. The isolates grown on plates with Streptomycin were a significant source of gram-negative, and Proteobacterial bacteria.

DISCUSSION

Hypotheses / Key Findings

In this study, I undertook an examination of what variables would affect the microbial isolates that could be cultured from a mountainous soil community. In terms of comparing the winter and summer soil communities, our hypothesis that isolates from the summer soil community (characterized by high turnover times) would adapt to the media more rapidly was supported (Schmidt et al. 2007), as the time to appearance was significantly lower ($p = 0.00001$) for summer isolates than winter isolates. Our initial hypothesis that the warmer, less stressful environment of the summer soil community would be better suited to support microbial diversity (Lipson et al. 2008) showed some signs of support - the summer soil community was able to culture more non-dominant species (i.e. non-Actinobacteria) than the winter soil community, although this trend was not quite significantly different at the 5% level ($p = 0.117$). One surprising outcome was that summer isolates grew better in the fridge ($p = 0.0006$), as we had expected more isolates from the winter soil community to be able to grow in the fridge. However, as we expected, colonies took longer to appear (Pham et al. 2012) on plates in the fridge than at room temperature ($p = 0.00001$). The variable that had the least effect on microbial culturability was the nutrient level (dilute vs nondilute levels), as there were similar times to appearance ($p = 0.11$) and overall amount of microbes cultured (166 to 153) for both treatments. Additionally, while we expected different taxa to be able to take advantage of the varying nutrient levels (Nunes et al. 2015), similar taxa were seen in both treatments (table 4). Finally, in contrast to previous studies (Shade et al. 2013)

the few isolates that were able to be developed on plates with Streptomycin (n = 32) were a significant source of Proteobacterial diversity (p = 0.00001).

Microbial Culturability and Time to Appearance

Microbes from both the winter and summer soil communities grew best when grown at room temperature - 319 colonies were grown at room temperature and only 104 colonies were grown in the fridge. Additionally, microbes grew best without the presence of the Streptomycin antibiotic, as only 32 of our 423 isolates were developed from plates with Streptomycin. When these variables were shifted to suboptimal growth conditions (i.e. in the fridge, or with Streptomycin), the summer soil community was better at adapting to these changing variables. While previous studies have found fungi to dominate microbial biomass in the winter (Schmidt et al. 2007), the vast majority of our winter isolates were bacterial.

When grown at room temperature, colonies from the summer soil community appeared significantly quicker (p = 0.0001) than those from the winter soil community, with a lower median time to appearance by 11 days. This supports the idea that mountainous microbes from the summer soil community are better suited towards growing in new environments (Schmidt et al. 2007) and adapting to changing conditions than those from the winter soil community. Also supporting this conclusion is that significantly more microbes from the summer soil community were able to grow on plates with Streptomycin (p = 0.0005), which reinforces that perhaps microbes from the winter community are less adept at responding and growing under conditions of stress. Additionally, it was hypothesized that perhaps isolates from the winter soil community would be better adapted to growing in the fridge, but in fact colonies from the summer soil community grew at a significantly higher rate in the fridge (p=0.0006). As such, it appears as if the summer microbial community was better at adapting and growing under suboptimal conditions. One possible explanation for this is that perhaps the microbes that survive the microbial biomass crash at the winter-spring snowmelt transition (Sorensen et al. 2018) and make it to summer have traits that allow for better adaptation to stressful conditions.

Taxonomy of Mountainous Microbes

In all, a fairly wide range of isolates were able to be recovered, reflecting the large soil microbial diversity seen in high-elevation ecosystems. Mountainous microbes isolated from the summer soil community showed slightly greater diversity than winter isolates at the phyla level (table 4), albeit not quite at the 5% significance level ($p = 0.117$). This slight difference in diversity could be because a larger subset of microbes are able to survive in the warmer, less stressful summer soil communities. Another study from a high-elevation system (Lipson et al. 2008) found the Janthinobacterium (a fast-growing Proteobacterial isolate) to be cultured most frequently from the winter soil community, while the *Burkholderia* and *Variovorax* genera (both slow-growing Proteobacterial isolates) were cultured most frequently from the summer soil community. In contrast, our study found the most culturable isolates from high-elevation systems to be Actinobacteria, and we didn't find any significantly dominant genera for the winter or summer montane soil communities. While we had originally expected a significant difference in the taxa that we were able to isolate from the winter and summer soil communities, microbial dormancy could be a possible reason for why there was not a significant difference. Perhaps certain isolates that are more summer-adapted or more winter-adapted enter a stage of dormancy (Lennon et al. 2011) in suboptimal conditions. Then, when the communities were grown under new conditions (agar media), they were able to end their dormancy period. Thus, if certain isolates were dormant in the initial soil communities, it would explain why winter and summer isolates were similar taxonomically.

The growth temperature ($p = 0.47$) and nutrient levels ($p = 0.50$) both had very little impacts on the overall diversity sequenced at the phyla level, which runs counter to previous studies (Nunes et al. 2015). Even at a more minute level, only a couple smaller trends showed up. For example, 5 of the 6 Sphingomonodales isolates were developed at high nutrient levels and 6 of the 8 Bacteriodetes isolates were developed at room temperature. However, on the whole it appeared as if mountainous microbes from the Actinobacteria, Proteobacteria and Bacteriodetes phyla are able to respond efficiently and grow across varying levels of nutrient levels and growth temperatures.

The presence of Streptomycin had by far the largest impact on what taxa was isolated, with all of the isolates recovered from these plates being gram negative (7 Proteobacteria and 1 Bacteriodetes). This is in contrast to previous studies (Shade et al. 2013), which found the

application of Streptomycin to have little effect on the diversity of taxa cultured. In my study, the Streptomycin plates isolated Proteobacteria at a higher frequency ($p = 0.00001$). In addition, they proved to be an important source of overall Gammaproteobacterial diversity (3/4 isolates from Streptomycin plates) and Alphaproteobacterial diversity (4/12 isolates from Streptomycin plates). Streptomycin is an antibiotic produced by *Streptomyces griseus*, a bacterium from the Actinobacteria phyla. As such, perhaps Streptomycin is adept at targeting other Actinobacteria isolates and less adept at targeting Proteobacteria and Bacteriodetes isolates.

Limitations and further directions / Broader Implications

The main limitation of my project was the scope – I could only taxonomically identify 87 out of my 423 isolates. As such, I had to attempt to maximize the diversity of the samples I chose, while also making the subset as representative as possible. Another limitation was that only two time points of samples were used, one from the summer and one from the winter. With only two samples used from each time point, we are assuming that these four total samples are representative of the entire watershed. Finally, a third limitation was that I did not transfer colonies daily past 14 days, so the actual time to appearance past the first two weeks is liable to be variable.

The work from my thesis is important in that it allows for the characterization of how winter and summer soil isolates differ. In the face of rapidly changing conditions in montane ecosystems, insights into how these shifts can impact microbial populations could prove significant. A wide range of microbial isolates – covering 4 phyla and 41 genera - was also able to be developed. The subset of sequenced bacterial isolates can be compared against metagenomic data to give an indication of which isolates are significant ecologically in mountainous ecosystems. Then, these select isolates can be investigated further using laboratory-controlled experiments with manipulated conditions. This can allow for a better sense of how changes to the system can impact key microbial isolates seen at the East River site. Microbial traits of certain isolates can then perhaps be investigated further using physiological tests such as substrate utilization profiles or genetic approaches such as transposon mutagenesis fitness assays.

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