ABSTRACT

Title of Document:	MICROBIAL ECOLOGY AND ENDOLITH COLONIZATION: SUCCESSION AT A GEOTHERMAL SPRING IN THE HIGH ARCTIC
	Verena Starke, Doctor of Philosophy, 2012
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A critical question in microbial ecology concerns how environmental conditions affect community makeup. Arctic thermal springs enable study of this question due to steep environmental gradients that impose strong selective pressures. I use microscopic and molecular methods to quantify community makeup at Troll Springs on Svalbard in the high arctic. Troll has two ecosystems, aquatic and terrestrial, in proximity, shaped by different environmental factors. Microorganisms exist in warm water as periphyton, in moist granular materials, and in cold, dry rock as endoliths. Environmental conditions modulate community composition. The strongest relationships of environmental parameters to composition are pH and temperature in aquatic samples, and water content in terrestrial samples. Periphyton becomes trapped by calcite precipitation, and is a precursor for endolithic communities.

Microbial succession takes place at Troll in response to incremental environmental disturbances. Photosynthetic organisms are dominantly eukaryotic algae in the wet, high-illumination environments, and Cyanobacteria in the drier, lower-illumination endolithic environments. Periphyton communities vary strongly from pool to pool, with a few dominant taxa. Endolithic communities are more even, with bacterial taxa and cyanobacterial diversity similar to alpine and other Arctic endoliths. Richness and evenness increase with successional age, except in the most mature endolith where they diminish because of sharply reduced resource and niche availability. Evenness is limited in calcite-poor environments by competition with photosynthetic eukaryotes, and in the driest endolith by competition for water. Richness is influenced by availability of physical niches, increasing as calcite grain surfaces become available for colonization, and then decreasing as pore volume decreases.

In most endoliths, rock predates microbial colonization; the reverse is true at Troll. The harsh Arctic environment likely imposes a lifestyle in which microbes survive best in embedded formats, and to preserve live inocula for regrowth.

ARISA is commonly used to assess variations in microbial community structure. Applying a uniform threshold across a sample set, as is normally done, treats samples non-optimally and unequally. I present an algorithm for optimal threshold selection that maximizes similarity between replicate pairs, improving results.

MICROBIAL ECOLOGY AND ENDOLITH COLONIZATION: SUCCESSION AT A GEOTHERMAL SPRING IN THE HIGH ARCTIC

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Preface

And this is how it all began...

To See a World in a Grain of Sand
And a Heaven in a Wild Flower,
Hold Infinity in the Palm of Your Hand
And Eternity in an Hour.

Auguries of Innocence, William Blake

Dedication

I would like to dedicate this dissertation to everybody who believed in me.

Acknowledgements

Although only my name is written on this dissertation, this would have never been possible without many helping hands during the process. And I would like to thank all those many people that supported me and helped me in many different ways. I apologize if I forget somebody and don't thank those listed enough.

I always thought that I would do my dissertation in Germany, but after I worked at the Carnegie Institution of Washington for one year as a technician, I decided to stay. Andrew Steele (Steelie) asked me if I would like to do my Ph.D. in his research group, and so I did. Steelie has supported me as my advisor every step of the way, scientifically and financially. He especially made it possible to get samples from Svalbard for my research, and he financially supported me the whole way. He introduced me to Svalbard and a completely new field of environmental research, which I fell in love with.

Together with Frank Robb from UMD we started the Ph.D. endeavor as co-advised project between UMD and the Carnegie. I had great support from both sides. Frank always had a great sense on how I was doing the process and was always there when I needed advice.

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enough about how important it is to have a supportive and knowledgeable committee as I did. My committee was an amazing team. I am very grateful for all their help, advice and support.

And, of course, there are so many other people at the Carnegie. In particular, I would like to thank Marilyn Fogel and Mihaela Glamoclija. Both listened very patiently, answered my questions and gave me great advice. Marilyn is the voice of reason at GL and you can count on her for any type of advice. She is always ready to go to battle for you if needed. Mihaela is always joyful in what she does, a great office mate and a lot of fun to be around. Also, I would like to thank past and present Steele-lab members. I also would like to acknowledge the Geophysical Laboratory at Carnegie Institution of Washington that has supported me throughout the whole process.

I would have never been able to collect the samples necessary for this research without the help of people on the Arctic Mars Analog Svalbard Expeditions (AMASE). So many people made it possible to get my work done during the expedition. I especially want to acknowledge the “Troll Patrol”. We were a “sampling unit” for several years in a row: Mihaela Glamoclija, Marilyn Fogel, Liane Benning, Lauren Kerr, Dominique Tobler and Steve Squyres. Thanks to Kjell Ove Storvik, our expedition photographer, who took most of the beautiful photographs of Troll Springs and the sampling shown in this thesis.

I am very grateful to Jacques Ravel and his team for the pyrosequencing. He introduced me to 454 sequencing and sequenced the samples for my research.

I have come a long way, and there are several people in the past that have put me on this path. One of them is my biology teacher, Rudolf Richert, from my high school in Germany who sparked my interest in biology and then encouraged me to go to university to follow this interest. During my years at the University in Marburg I met Gerda Horneck who introduced me to Exobiology that led to where I am now. I spent a great time as an intern in her lab. She also invited me to a conference on Astrobiology where I met Steelie and the rest is history. Thank you for encouraging me to go after my interests!

Last, but not least, I want to thank my family and friends, especially my husband, parents and grandparents. Although not everybody knew exactly what I was working on, they always believed that I could do it! Thanks for your confidence and faith.

A special acknowledgement goes to Troll and Trollette (figure below). Both came from Norway and were my PCR workers in the laboratory (figure: right at work on top of the PCR machine).

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I know that I probably have forgotten someone or something. Please forgive me if I didn't mention your name in these acknowledgements.

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CHAPTER 1: INTRODUCTION – A TROLL'S VISION

Ecology is the branch of biology that deals with the relationships of organisms among one another and with their environment. Decades of ecological studies have explored the principles that control the structure of biological communities in environments across the globe. Historically, most of these studies have concentrated on large organisms that can be observed readily in a field setting. In more recent years, however, new molecular techniques have allowed field ecology to be extended to microorganisms.

This dissertation describes a molecular study of microbial ecology at a remote field site in the high Arctic. It is motivated by three basic principles. The first is that the small size and rapid reproduction and adaptation rates of microorganisms lend important advantages to ecological studies. The second is that a small field site with very steep local gradients in environmental conditions provides an ideal setting to explore relationships between microbial community makeup and a manageable number of environmental parameters. The third is that the field site spans two very different types of ecosystems, aquatic and terrestrial, that have previously been studied separately.

I begin this chapter by reviewing the literature of ecology along environmental gradients and of ecological succession, with particular emphasis on microorganisms. Next, I describe the field site for my dissertation, Troll Springs in the Svalbard archipelago north of Norway. After that, I review previous work that has been done in similar settings elsewhere, and identify the new

opportunities that are provided by working at Troll. Finally, I summarize the ecological concepts and principles that I will explore more deeply in the remainder of the dissertation.

1.1 WHAT ARE DIVERSITY, RICHNESS AND ABUNDANCE?

Before embarking on a discussion of microbial ecology, it is useful to define some of the ecological terms that will be used commonly throughout this dissertation. Among the most important terms used in ecology are “diversity”, “richness”, “abundance” and “community makeup” or “composition”. All these terms describe the properties of a community of organisms, but they look at communities differently.

1.1.1 Definitions

Species: Conventionally, a species is a group of organisms consisting of similar individuals that are capable of exchanging genes or interbreeding. The species concept is debatable for microorganisms, however, so the more general term “taxon” will be used here to define a group of organisms with similar genetic makeup.

Richness: Richness is the number of distinct taxa that are present in a community. It does not take into account either which specific taxa are present or how numerous the individual members of the taxa are.

Evenness: Evenness is a measure of how close in number the individuals in different taxa are to one another. A commonly used measure of evenness is the Pielou Index.

Diversity: Diversity is a parameter that combines the concepts of richness and evenness into a single quantity. The most commonly used index to measure diversity is the Shannon-Wiener or Shannon-Weaver Index (H').

Abundance: Abundance refers to the total number or total mass of organisms within a taxon. When abundance is expressed as a mass, it is also referred to as "biomass".

Community makeup: Community makeup describes the identity of taxa in a community, along with some measure of the abundance of each. It is also sometimes referred to as composition.

Figure 1.1 illustrates these ecological terms with simple examples of community makeup. Samples A, B and C represent three different samples with identical diversity (H' = 1.60) but from different environments. Bacteria are most abundant in sample B. Although sample A has higher richness (*i.e.*, more taxa), their abundance and evenness are both low compared to Sample B, with one dominant organism. Sample C has the same abundance as A, and the same

richness (and evenness) as B. This means that going from environments A and C to environment B leads to an increase in the total abundance of organisms present, but not an accumulation of additional taxa. In fact, the number of taxa decreases from A to B – *i.e.*, the richness decreases. The community makeup is unique to each ecosystem. B and C do not share any taxa, showing a complete change in community makeup with environmental conditions, despite maintaining identical diversity and richness. The important point is that just because environments have similar diversity does not mean that their communities are similar to one another.

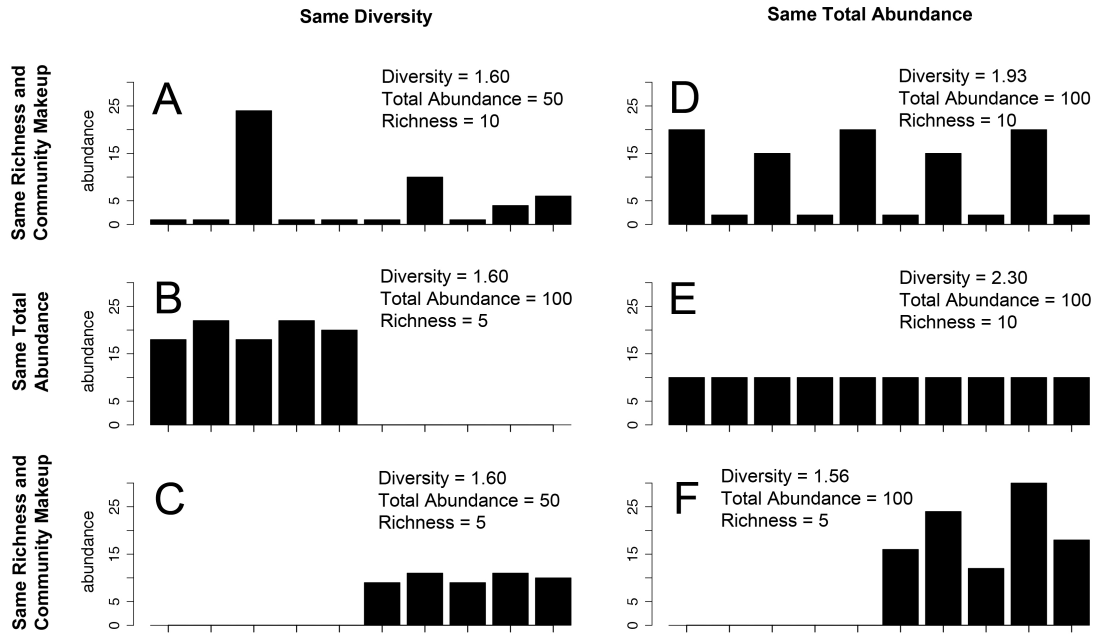


Figure 1.1: Fictive samples with varying diversity, richness and community makeup.

Samples D, E and F provide additional comparisons. For example, samples with the same richness (A, D, E or B, C, F) can have different abundances (A, D, E or C, F) or different community makeup entirely (B and C). Environments can have the same diversity but have no taxa in common or vice versa. It is important to understand that diversity, richness, abundance and community makeup are all dependent on underlying parameters in the surrounding environment. Changing environmental conditions result in a changing community.

1.2 ECOLOGY ALONG ENVIRONMENTAL GRADIENTS

One of the central questions of ecology is how environmental conditions control the properties of communities of organisms. Studies of this problem are aided by investigation of “environmental gradients”: settings where environmental parameters change in a systematic way with geographic location.

Ecologists studying macro-organisms, such as plants and animals, have pioneered investigation of ecology along environmental gradients. Variations of diversity with latitude have been widely studied (37, 234, 286) and have yielded the general observation that species diversity decreases with latitude. Other examples include variation of diversity and richness with elevation (112, 181, 224) and of plant species richness with soil moisture (147) and with water levels on shores (7).

Microorganisms also respond to environmental parameters, and it is therefore appropriate to test ecological models using microbes. In fact, microbial

communities are particularly well suited to application of ecological studies in many respects. Usually, microorganisms have higher reproduction and colonization rates than multi-cellular organisms, enabling them to adapt and come into equilibrium with their environment quickly. High-throughput sequencing of microbial samples enables rapid and detailed quantitative analysis of community makeup. With molecular methods, microbial ecologists can observe patterns of occurrence in much more detail than is typically the case for communities of macro-organisms.

It must be asked, however, whether all ecological relationships observed for plants and animals apply to microorganisms. Microorganisms are present in high abundances, have frequent and long-distance dispersal, and project low extinction rates, perhaps resulting in relatively weak geographic patterns (75). In fact, Hillebrand and Azovsky (116) argue that the strength of the latitudinal gradient in species richness is correlated with organism size. Macro-organisms like trees and vertebrates show the strongest decrease in richness with increasing latitude, whereas protozoa and diatoms show weak or no correlation. I will argue below, however, that these differences can be understood when the specific details of the local environments in which microorganisms live are considered.

Scale is a particularly important consideration when comparing macro-organism ecology to microbial ecology. Microbial habitats can be heterogeneous on very small scales due to microscale biotic and abiotic factors (145). This heterogeneity of samples can lead to many habitat niches, potentially resulting in

higher diversity and coexistence of species. For example, a dense microbial mat has different layers resulting in gradients (*e.g*. redox levels, light, nutrients, etc.), building niches for different type of organisms (280). Soil is another example of an environment that can provide many niches for microorganisms.

A key point that motivates this dissertation is that the small scales of microbial environments potentially offer a distinct advantage for conducting ecological studies. Investigation of environmental gradients can be complicated significantly when many environmental parameters vary simultaneously with geographic position – it becomes difficult to disentangle the effects of the different parameters. But if a study can be carried out in a setting where a few key environmental parameters change over distances of just meters instead of many kilometers, the confounding effects of other changes can be reduced or eliminated.

Many previous studies have investigated how environmental conditions affect the properties of microbial communities. For example, microbial communities have been studied along pH gradients (80, 158, 231), temperature gradients (183, 190, 278), elevation gradients (78), water or moisture gradients (4, 6, 39, 212, 281, 298) and salinity gradients (3, 32, 51, 55, 117, 164, 201, 222, 228, 256). Studies have also been conducted of microbial variations with ocean water depth (62), at geothermal vents along an intertidal gradient (139), in estuarine gradients with varying chemical parameters like salinity and nutrients (114) and of phytoplankton variations with latitude (93).

Ecological patterns of plants and animals show a general decrease in diversity with an increase in latitude and/or a decrease in available water. Spatial patterns of microbial diversity, however, seem to be different. Fierer and Jackson (80) found that bacterial soil diversity is not strongly influenced by gradients in latitude, temperature or moisture. Instead, they found that soil pH is the primary determinant of soil bacterial diversity and richness, with the lowest levels of diversity and richness observed in acid soils.

While pH appears to be the primary determinant of diversity and richness in soils, the specifics of community makeup can vary significantly with other local parameters like the availability of nutrients and moisture. In the work of Fierer and Jackson (80), local bacterial community makeup depended on environmental parameters like soil moisture, pH, soil organic C content and soil C:N ratio. Interestingly, pH was also the strongest predictor of soil microbial community makeup in this study. The degree of similarity in the makeup of soil bacterial communities was largely unrelated to geographic location, depending instead on the specific local environmental conditions. The same phenomenon was seen along an elevation gradient in eastern Peru (78), where soils exhibited no significant variation in diversity with elevation, but different soil types differed in the specifics of their community makeup and abundances.

Bachar *et al*. (6) and Angel *et al*. (4) found similar results. All of the soils they studied had similar pH, and all had similar diversity despite significant variations in precipitation. However community makeup varied with ecosystem type, and bacteria were more abundant (*i.e.,* biomass was greater) in wetter

soils. Of all measured parameters, water content was found to have the strongest influence on community makeup. Bachar *et al.* also found high correlations of environmental factors, such as calcium or magnesium concentrations, to community makeup. So soils appear to have a dominant parameter (pH) that determines diversity, and secondary parameters (like the availability of water) that determine the specifics of community makeup. Stated differently, although sites with similar pH tend to have similar numbers of community members (richness), they can show significant differences in the type of members (different species) depending on other environmental parameters.

Water availability is not a restricting parameter in an aquatic environment, but can become a strong selecting factor in some terrestrial zones, such as deserts. Transitional zones, like a water gradient going from aquatic to terrestrial, can cause variable pressure on communities present and result in a major change in community makeup. Zeglin *et al*. (298) studied parafluvial sediments across moisture gradients in stream–soil transition zones in a cold (Antarctica) and hot (New Mexico) desert. Samples had a similar pH for both deserts. The diversity did not change along the gradients and was similar in both environments, showing that diversity and richness were not related to temperature and water content. However, bacterial community makeup differed between hot and cold desert sediments, and between wet and dry sediments.

Under the most extreme environmental conditions, selective pressures on microbial communities become severe. The Arctic, the Antarctic and deserts are examples. Yergeau *et al*. (297) studied soils of sub-Antarctic (51°S) to Antarctic

sites (78°S), comparing microbial communities beneath vegetation with those inhabiting bare sites. At sub-Antarctic locations where both types of sites were present, they found that bacterial diversity in bare soil decreases with latitude, but no pattern was observed for the corresponding vegetated sites. Furthermore, they found that soils under vegetation are characterized by greater nutrient availability and more favorable physical conditions, giving greater protection compared to bare soils. The soil pH was very similar among all the vegetated sites (ranging from 4.29 to 4.76) whereas the bare soil pH from the same sites varied from 6.14 at lower latitudes to 4.10 at higher latitudes. Like the observations of Fierer and Jackson (80), then, bare soils at sub-Antarctic latitudes showed decreasing diversity with decreasing pH.

At the highest latitudes, where conditions are most extreme and only bare soils were present, diversity was low despite neutral pH. These soils are exposed to extreme changes in temperature and water availability, extremely low inputs of nutrients, and high levels of UV radiation in summer (296). So while it appears that pH can be a good predictor of microbial diversity under favorable conditions, other more limiting parameters can come into play when conditions are most extreme.

High temperatures also constitute extreme environmental conditions. Norris *et al*. (190) studied a nearly linear soil temperature gradient from 35°C to 65°C at Yellowstone National Park. The high temperature resulted in a significant reduction in soil microbial diversity, and community makeup changed along the temperature gradient. The hottest soils were rich in thermophilic organisms,

which were also found in much lower abundance in the cooler soils. The authors suggest that the thermophiles subsist at low levels or as spores in low-temperature environments, and become prominent under favorable conditions for growth. The hottest soil also had lower pH, higher electrical conductivity, and higher nitrate and ammonia concentrations than cooler soils, likely also influencing microbial diversity and community makeup. But the high temperatures observed appear to have the strongest influence on the microbial community, excluding a wide range of non-thermophilic microbial taxa.

High temperatures can also have a limiting effect on microbial diversity in aquatic environments. In high temperature thermal spring environments, only limited microbial taxa, such as the cyanobacterium Synechococcus, and filamentous green nonsulfur-like bacteria, *e.g*. *Chloroflexus* and *Roseiflexus*, have been observed as dominant organisms (280). Miller *et al*. (183) found that diversity decreases with increasing temperature along temperature gradients (39ºC to 70ºC) at the outflow channels of two alkaline silica springs at Yellowstone. Although the two springs differed in their community makeup, community similarity and diversity changed in similar ways along the two channels. The authors suggest that environmental temperature primarily controls the community properties. Unfortunately, pH measurements were not documented for this study, so it is difficult to determine how much pH changes along the outflow channels could also have had an influence.

Microbial community diversity and makeup in water-saturated environments, such as oceans, lakes and hot springs, seem to be controlled by

different environmental parameters than in soil environments. For example, microbial communities of marine surface waters exhibit increasing diversity and richness from the poles toward the equator (93, 216). Richness in warm tropical waters is almost twice as high as in cold, high-latitude waters. Due to only modest equator-to-pole variations of other chemical and physical environmental variables (such as salinity), the authors conclude that surface water microbial communities are controlled primarily by parameters like temperature that vary more strongly with latitude. This observation is in contrast to the pattern for soil bacteria described by Fierer and Jackson (80), who detected no latitudinal pattern and found instead that diversity and richness are primarily correlated with other factors such as pH, soil type, and ecosystem type.

There are at least two reasons why ocean environments and soils have different underlying major parameters selecting for diversity. One is that the availability of a critical resource like water is limited and variable in soils, but effectively infinite (and therefore not limiting) in an aquatic environment like the ocean. Another is that some parameters, like pH, have only a small range in seawater (about 7.5 to 8.5), which is an excellent buffer system, but a much larger range in soil, where it therefore becomes a much more important determinant of microbial diversity. Salinity is also relatively stable in seawater, whereas temperature, dissolved oxygen, and nutrients have higher variability and therefore more influence on microbial diversity. In summary, environmental parameters that have a high influence on microbial diversity in a terrestrial setting do not necessarily have similar influence in an aquatic setting, and vice versa.

In addition to determining diversity, environmental parameters also influence community makeup. Again, whether a resource is unlimited or scarce is important; water is more likely to have a strong influence on community makeup in a desert setting than in an aquatic setting. Lozupone and Knight (164) compared a wide range of environments, including soil, seawater and sediments, with normal and extreme temperatures, salinity, pH and nutrient availability. They found that salinity was the most important determinant of microbial community makeup, and that substrate/water abundance (aqueous *vs*. soil and sediment) was also important. Of course, within given salinity conditions (for example, in freshwater environments with no salinity), other factors like pH or temperature are likely more significant. Extremes of temperature, pH, or other physical and chemical factors, which as discussed above can be important determinants of diversity, had less influence on community makeup in saline settings.

Estuarine and brackish waters can exhibit strong gradients in salinity, temperature and nutrient concentration, created by mixing of freshwater and seawater. These gradients influence aquatic community makeup and provide greater niche availability than seawater or freshwater alone, influencing richness and diversity. Hewson and Fuhrmann (114) found that the richness of estuarine bacterioplankton correlated positively with bacterial abundance, but they found no strong correlations of diversity with salinity, nitrate and phosphate concentrations, or chlorophyll a concentration. Some bacterioplankton taxa were specific to distinct environments while others had a ubiquitous distribution, indicating that environmental parameters dictate community composition. In this

study, bacterioplankton communities varied significantly along the estuarine gradient, whereas diversity appeared to be primarily related to habitat and resource availability.

Andersson *et al*. (3) studied brackish waters in the Baltic Sea with a salinity gradient. They argued that a complex combination of variables likely selects for a bacterioplankton community uniquely adapted to the estuarine/brackish environment, whereas typical marine bacterial populations have higher salinity requirements. The authors found that the community makeup varied by season, mostly correlated with changes in phosphorus concentration and temperature. In summary, salinity does not seem to exert a strong influence on diversity in estuarine and brackish settings, although diversity can be reduced under extreme salinity conditions as the environment reaches salt saturation (228). Instead, salinity primarily dictates microbial community makeup, where certain microorganisms have specific salt requirements and are not found in low- or high-salt environments.

Salinity gradients are also found in some inland water bodies. Particular attention has been paid to lakes and sediments with broad salinity gradients at high altitudes in Tibet (136-138, 292). Jiang *et al*. (138) described changes in microbial community structure across the water-sediment interface. The changes in community makeup were correlated to a decrease in salinity from the lake water to the sediments, and, perhaps, to the change from water to sediment environment itself. The redox state also changed from oxic to anoxic across the interface and may have further contributed to a shift in the microbial community.

Vertical salinity gradients also contributed to systematic changes in microbial community composition within the sediments, but changes were not related to mineralogy (136). Jiang *et al*. (137) argue that the similarities in pH and salinity of marine and Tibetan saline lake sediments lead to presence and dominance of similar organisms in those two widely separated settings.

Wu *et al*. (292) studied the influence of salinity on bacterioplankton community makeup in 16 stagnant inland lakes on the Tibetan Plateau. They found that salinity, not altitude, is the dominant factor controlling bacterioplankton community composition. Bacterioplankton communities only showed minor overlaps between freshwater and hypersaline lakes. Wu *et al*. (292) argued that the overlapping groups have high “ecological plasticity”, meaning that they are able to adapt to the changing environmental conditions. So in these settings, salinity is a major determinant of microbial community makeup, with similar microbial communities found in environments of similar salt concentration, independent of geographic location.

Freshwater systems of course have no salinity gradients, but can be influenced by variations in pH. Fierer *et al.* (81) found that freshwater streams with distinct pH levels had distinct bacterial communities, indicating that environmental factors, not geographic location, appear to be the primary drivers of bacterial community composition. Similarly, community makeup patterns were found to be strongly correlated with pH in lakes in northern Europe, followed in importance by temperature and retention time (161). In a study of 30 lakes in Wisconsin, water clarity and pH were significantly related to variations in bacterial

community makeup. Southern Wisconsin lakes tended to have a higher pH and lower water clarity than northern Wisconsin lakes (294). Southern Wisconsin lakes showed a higher bacterial richness compared to northern lakes.

The examples above show variations in microbial community structure caused by differences in environmental variables. However, Papke *et al*. (200) and Whitaker *et al*. (283) have shown that thermophilic bacteria and archaea, respectively, can also exhibit genetic differences due to dispersal limitation. Both studies were made on single organisms rather than communities. Geographic separations of several thousand kilometers between occurrences of these highly specialized organisms have resulted in genetic drift. It is likely that the mutation rate of genomic markers in these organisms is higher then their dispersal rate, causing this diversification and drift (177). The conclusion is that physical isolation can also drive patterns of microbial biogeography.

It is noteworthy that Whitaker *et al*. (283) surveyed chromosome loci and not solely 16S regions. In fact, the organisms studied showed a high similarity in their 16S sequences. This could indicate that community structure, revealed by study of 16S, is perhaps dependent mostly on environmental parameters, whereas geographic distances can result in genetic drift on different loci of the chromosome. Therefore, physical isolation also can drive patterns of microbial biogeography. In microbial ecology, most molecular studies concentrate on phylotypes (bacterial species) rather then genotypes (strains or subspecies). Nevertheless, it is clear that adaptation and geographical isolation must also be considered as factors potentially leading to ecological drift and influence

microbial communities together with measured and unmeasured environmental parameters.

1.3 ECOLOGICAL SUCCESSION

1.3.1 Principles of succession

Succession in an ecosystem can be defined as changes in community makeup, usually with an increase in community complexity, over time. By community complexity I mean the number, nature, strengths and structural pattern of biotic interactions among species (*e.g*. competition, facilitation, predations, food web, *etc*.) (185). In the microbial world, a transition from monospecies communities to diverse communities is a successional process similar to what is observed for macroscopic ecosystems (45, 46). As conditions change, existing species are replaced by species that are better adapted to the new conditions.

In general, succession is a progressive change in the makeup, structure and function of a community that will not return to the former state unless a disturbance resets the process of succession. Succession can be a continuous process over time, as changes, such as small disturbances, accumulate that lead to different functions and the importance of different taxa in the developing community. Succession does not include regular seasonal changes or changes due to foreseeable climatic events, such as a desert rain.

An important feature of succession is that communities can have a variety

of starting points, but tend to proceed towards a more limited number of end-community states. These “climax” communities are characteristic of the particular ecological setting. For example, endolithic community composition is dictated by environmental parameters, and endoliths at sites with moderate climates have a different community makeup than endoliths from hyperarid environments. However, the endolithic ecosystem itself is very similar, with functions contributed by different taxa.

Study of succession has been pioneered in the field of plant ecology. In that context, a succession is defined by (220):

- An increase in total organic matter
- Nutrients that are increasingly bound up within organisms rather than free
- An increase in nutrient conservation, with slow nutrient loss
- A decomposer community that becomes larger and more important
- A community that becomes more diverse in terms of species number and balance
- A community that becomes more spatially diverse and heterogeneous
- A community that becomes more complex, with biotic interrelationships that diversify
- Flow of material around the community that becomes increasingly slow

The series of communities observed during succession is called a “sere”, where each step in the sere is called a “stage” (49). Stages are not necessarily

distinct from each other, and tend to show a gradual transition. Also, a succession is characterized by a process rather than by specific communities involved. For example, taxa involved in a succession involving calcite precipitation could be different in endolith formation than in stromatolite formation. A sere begins with a “pioneer” stage, and ends in a stable climax stage (42). Biotic and abiotic processes can influence the progression of communities, which are called autogenic and allogenic succession, respectively. In an example of autogenic succession, plants influence the progress by processes such as water and nutrient uptake, light capture or nitrogen fixation (46, 68, 247). These processes are termed “facilitation”, where site factors are improved during succession (*e.g.*, increased organic matter), or “inhibition”, where factors inhibit the establishment of other species (*e.g.*, competition) (46). In allogenic succession, environmental factors cause the process of succession.

While investigation of ecological succession has been conducted most widely for plant communities (99, 127, 262), improvements in molecular techniques have also made it possible to study succession of microorganisms. For example, studies include succession of biofilms in different environments (15, 133, 134, 170, 214), on surfaces in marine waters (57), along transects in a glacier forefront (176, 244), of culturable cells (95) and in grasslands (73). However, it remains to be established in more detail whether microbial succession follows the succession model derived from plant studies.

In plant ecology, succession can take place by colonization of a new and unoccupied habitat (primary succession) or after disruption of a pre-existing

community by a disturbance (secondary succession). Because of the theoretically predictable nature of the plant succession process, such successions will ultimately proceed to the same end point and on the same path whether or not they are interrupted by other subsequent disturbance events.

Fierer *et al*. (79) suggest a different classification scheme for microbial succession that distinguishes among forms of primary succession based on whether the initial colonizers are autotrophs, endogenous heterotrophs, or exogenous heterotrophs. They stress, however, that their classification is restricted to the initial colonization of sterile environments, and does not include temporal changes that occur following disturbances or changes in environmental conditions.

1.3.2 Mechanisms of succession

In one of the earliest theoretical succession models, Clements (42) considered succession to be a predictable process with definite stages, where autogenic processes predominate. In contrast, Cowles (49) saw it as influenced by allogenic processes, leading to different end points depending on circumstances.

Clements formulated the “integrated hypothesis” or “community-unit hypothesis” (also sometimes called the “superorganism hypothesis”) where communities are highly structured, repeatable and identifiable associations. In these communities, species are closely linked and are associated by mandatory

biotic interaction, with the community functioning as an integrated unit. In contrast, Gleason (98) argued that succession is less predictable, and formulated the “individualistic hypothesis” in which individual species are tolerant of local environmental conditions, not necessarily including interspecies interactions. In this model, chance assemblages of species that have similar biotic requirements can be found sharing the same general habitat. Gleason’s hypothesis was expended to the “continuum model” (53) and “gradient analysis” (284), where plant communities change gradually along complex environmental gradients. Collins *et al*. (44) combine the community-unit and continuum models, arguing that individualistic distribution of species gives rise to discrete communities as well as to a continuum. In Chapter 3 I will draw heavily on the idea that small, cumulative disturbances along an environmental gradient drive microbial succession at Troll Springs.

Connell and Slatyer (46) proposed three models, of which the first, called “facilitation”, follows Clements, while the other two are called the “inhibition” and “tolerance” models. The facilitation model is a sequence where one stage of the sere prepares the habitat for the introduction of the next stage. This sequence is accompanied by changes in soil (*e.g*., addition of organic matter) that facilitates colonization by the next group of species.

The inhibition model is based on the “initial floristic composition” model of succession (68), where patterns of succession are primarily a function of life-history patterns. Short-lived species express themselves best in the early stages of succession whereas slower growing and larger plants outcompete smaller

pioneer species in the later stages. Species replacement is not necessarily orderly because resident species attempt to exclude new colonists, so that the succession order depends on which species become established first.

The tolerance model is intermediate between facilitation and inhibition. In this model, different species have different strategies for exploiting resources. Species are replaced by other species that are more tolerant to limiting resources. These new species grow in the presence of the early ones, eventually outcompeting them. As the community dominance shifts, the succession proceeds.

Not all of these models need be mutually exclusive. For example, some studies have shown that succession can be a function of a combination of facilitation, inhibition, life-history traits, and stochastic factors (27, 276).

1.4 TROLL SPRINGS

Many of the studies cited above were carried out over large geographic regions, with numerous environmental parameters changing across the study area and many independent opportunities for disturbances capable of driving succession. In this dissertation, I have chosen instead to concentrate on one small setting in which disturbances are small, and in which a few key environmental parameters change dramatically while many others remain constant. The setting is a geothermal spring in the high arctic that has steep gradients in temperature, moisture, and mobility that place strong selective pressures on microorganisms.

Troll Springs (Figure 1.2), located near 79°23'N, 13°26E in the Svalbard archipelago north of Norway, is one of the northernmost documented thermal springs on land (9). The spring is located near the Sverrefjellet volcano, a stratovolcano that was most recently active between 10 and 6 thousand years ago. The waters of Troll are rich in sodium and bicarbonate, due to seawater interacting with the underlying Hecla Hoek marble (9). Subsurface mixing of geothermal waters with cold groundwater results in the temperature of the spring at its source being just 25°C - 28°C. The geologic context of the thermal activity at Troll is discussed in greater detail in Appendix C.

Precipitation of travertine (calcium carbonate, or calcite, $CaCO_3$) from Troll's carbonate-rich waters has built a complex terrace structure. Travertine precipitation at geothermal springs is thought to result primarily from loss of CO_2 from the water to the atmosphere, increasing pH and hence calcite supersaturation (24, 66, 86). The process is driven by two equations:

$$H_2O + CO_2 \leftrightarrows H_2CO_3 \quad (1)$$

$$CaCO_3 + H_2CO_3 \leftrightarrows Ca^{+2} + 2HCO_3^- \quad (2)$$

Water cools as it flows away from the spring source, reducing the solubility of CO_2. As CO_2 is driven out of solution, the equilibrium in equation (1) shifts to the left, decreasing the concentration of carbonic acid (H_2CO_3) and hence increasing pH. The decrease in carbonic acid also shifts the equilibrium of equation (2) to the left, resulting in precipitation of calcite.

The terrace system at Troll spans 100-120 m north-south and 150-200 m east-west. Individual terraces are typically 2-10 m in size (143). They lie on a hillside, with a vertical extent of 30-50 m from the source to the lowest terraces. Prominent features of the terraces are lips up to 50 cm wide and draped overflow edges that can reach several meters in height (143).

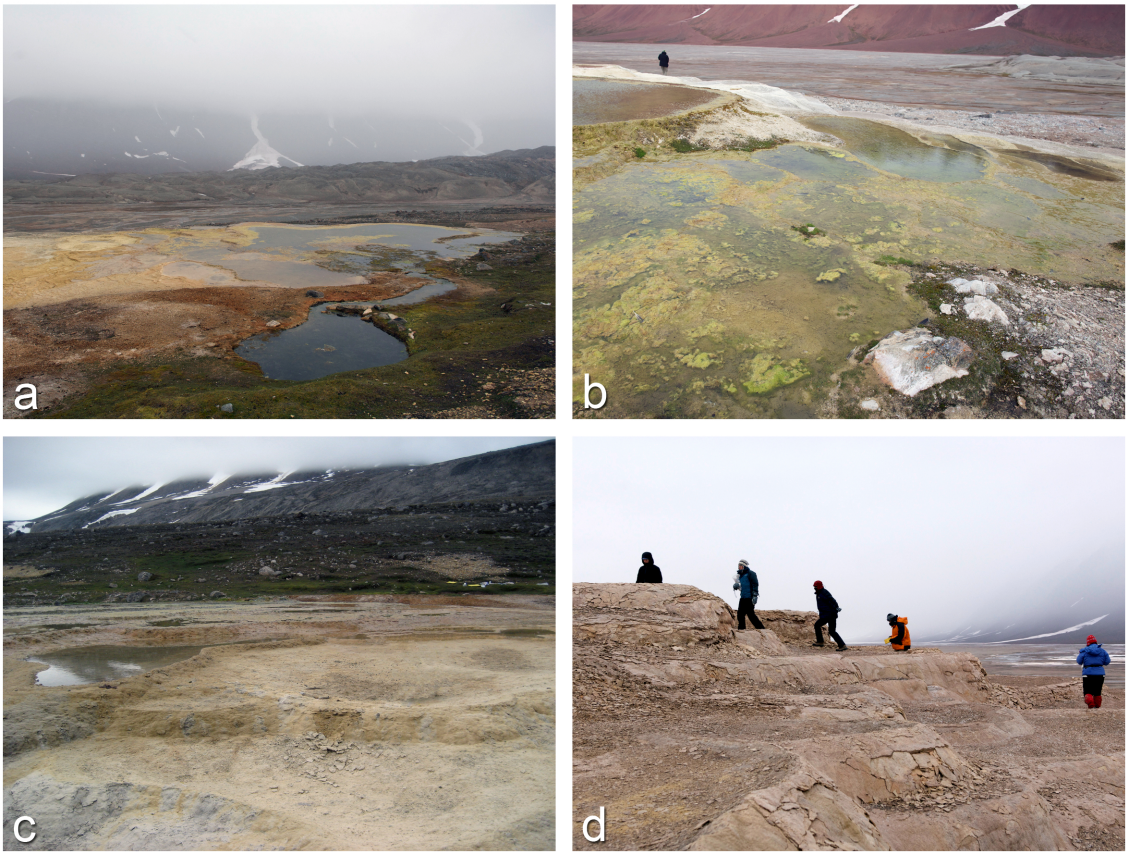


Figure 1.2: Troll Springs during August 2008. a: source and uppermost pools. b: pools downhill from the source. c: drying out pool, water has evaporated. d: dried out terraces.

The pool at the spring's source remains ice-free throughout the winter. The temperature drops, however, in the pools that fill or partially fill some terraces below the source, indicating that that the source pool is the primary fluid

source for the whole spring system (106). More distal pools in terraces below the source can freeze during winter, covered by snow and ice (135). Only the upper terraces contain water, whereas most of the lower terraces are partially dry or completely dried out.

Biological materials are present at all levels of the spring structure. The pool at the spring's source contains mostly clear water with small quantities of periphyton (defined here as a complex cohesive community attached to submerged aquatic surfaces) on top. The outflow of this source goes into the uppermost terraces. These water-filled terraces are warm, with green periphyton on top of the water and around the edges. Biological material also drapes the overflow edges of these terraces. In the lower partially dry terraces, green material is visible on the rock surface. The lowest terraces are completely dry. Microorganisms inside the rocks in those terraces are visible as a 1-3 mm thick green layer 1-3 mm below the rock surface; these are endolithic communities. So progressing downhill from the source pool to the terraces, environmental conditions change from wet to dry, warm to cold, and mobile to immobile (Fig. 1.3).

These steep environmental gradients at Troll Springs provide an ideal setting for studying the complex dynamics between microorganisms and their environment. At Troll I can perform an ecosystem study at the microbial level over a wide range of environmental conditions, investigating whether microbial community makeup shows patterns that reveal information about ecosystem function, and what role succession plays in driving community makeup there.

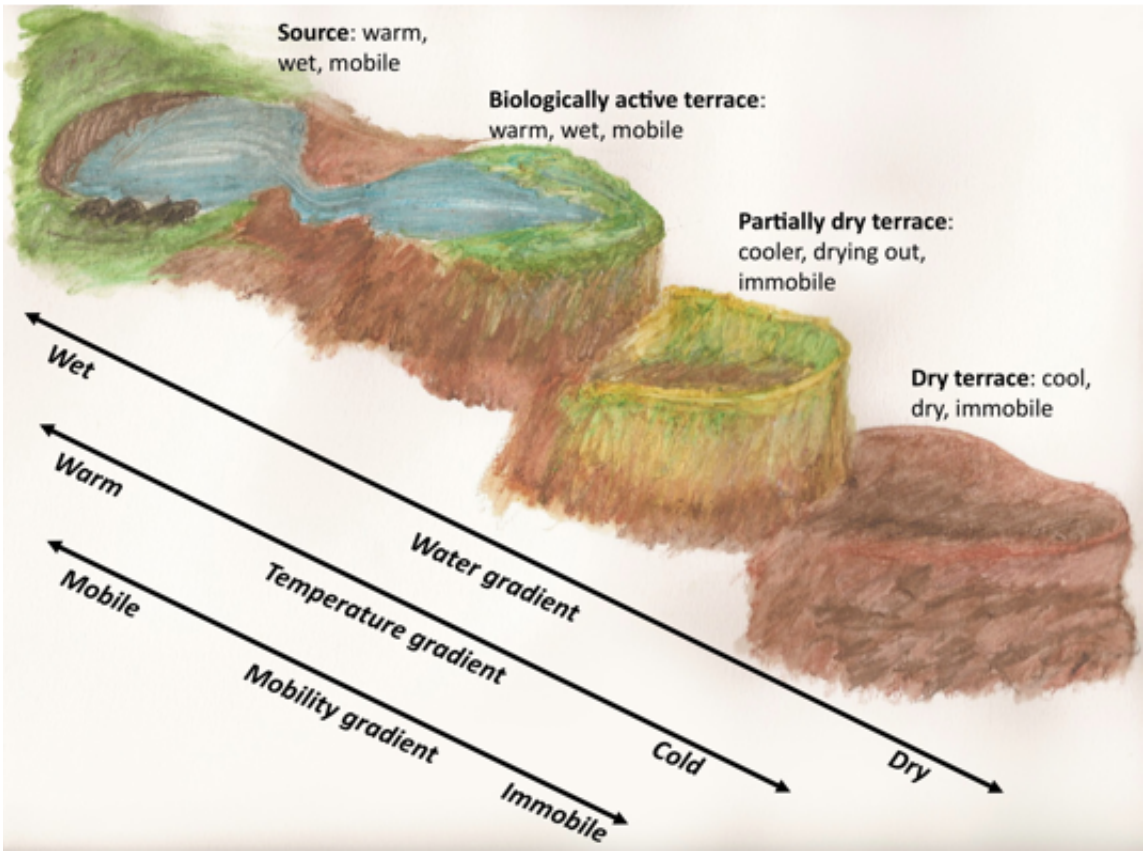


Figure 1.3: Simplified view of the Troll Springs terrace system. The system exhibits several environmental gradients that impose selective pressure on microbial communities.

1.5 OTHER DEPOSITIONAL GEOTHERMAL SPRING ENVIRONMENTS

A limited amount of work has been done on the microbial ecology of other settings with broad similarities to Troll. These include both depositional geothermal springs and endoliths.

Depositional geothermal spring environments involve precipitation of minerals from carbonate-rich or silica-rich waters, creating depositional features that can interact with organic matter. Carbonate-depositing springs usually have an underlying limestone or marble source. Carbon dioxide dissolved in hot water creates weak carbonic acid that percolates through the rock and dissolves

calcium carbonate from it. As the water surfaces, the calcium carbonate re-crystallizes as travertine. Similarly, the waters of silica-depositing springs are saturated with dissolved silica, which typically arises via dissolution of quartz at depth. Hot silica saturated waters cool at the surface and become supersaturated, precipitating siliceous and opaline sinters. Both travertine and silica can be deposited over wide areas as sheets or terraces.

Active travertine-precipitating geothermal springs with some similarities to Troll are found at Mammoth Hot Springs in Yellowstone, Pamukkale in Turkey and Le Zitelle in Italy. Pamukkale is the largest recorded travertine spring on Earth (206), and the one most similar to Troll Springs. At Pamukkale, thermal springs on a hillside ~100 m high feed a deposition area of about 10 km^2 of white travertine from several different sources (1). Prominent features of the spring include active travertine terraces ("bathers basins") 1-6 m high that cover an area of about 240×300 meters. The emerging water at sources above the terraces has a temperature of 35ºC.

As described for Troll Springs in Chapter 2, active depositing springs can incorporate organic matter (*e.g*. bacteria, heterotrophic microbes and eukaryotic algae) into travertine. A similar process has been observed at Pamukkale by Pentecost *et al*. (204). The most visually prominent photosynthetic organisms there are Cyanobacteria (order Oscillatoriales, mostly *Phormidium*), followed by diatoms and eukaryotic green algae. The endolithic community at Pamukkale is more widespread and more species rich than epilithic mats in the spring. Epilithic Cyanobacteria mats show precipitated calcite within the mucilaginous sheath at

the cooler lower travertines, suggesting the same kind of calcite precipitation onto biomaterial seen at Troll. This study, however, did not use molecular techniques to characterize the microbial communities at Pamukkale in detail.

A similar interaction of calcite precipitates with microbes has been observed at Le Zitelle, Italy (207), where endolithic communities are found at the edge of a spring's depositing stream. In contrast to Troll and Pamukkale, the water at Le Zitalle is relatively hot (62ºC to 50ºC). The authors identified three major depositional facies there: aragonite close to the source, followed by aragonite-calcite and then travertine with deep green mats where flow is weaker and the water is shallow. *Spirulina*-dominated mats (order Oscillatoriales) are present at lower parts of the stream, whereas the upper hotter section is apparently inhospitable to epilithic cyanobacteria. The authors suggest that this observation is associated in part with oxygen availability. Other photosynthetic organisms include filamentous Cyanobacteria (order Oscillatoriales, *Phormidium* and *Fischerella*), *Chloroflexus* and diatoms. *Fischerella* and *Phormidium* are dominant endolithic organisms in the aragonite and travertine facies, respectively. The thermophilic cyanobacterium Mastigocladus (Fischerella) is known to thrive in temperatures greater than 45°C (279). Again, molecular techniques were not used to investigate ecological variations at Le Zitelle in detail.

Another high-temperature carbonate spring is Mammoth Spring in Yellowstone, USA. The travertine at Mammoth exhibits five distinct depositional facies, which are based on shape, structure and chemical composition: vent,

apron and channel, pond, proximal slope, and distal slope (86). The travertine of each facies has distinct crystalline growth form and fabric, mineralogy, and elemental chemistry. Carbonate precipitation at Mammoth occurs as radial growths of microfibrous aragonite around 70ºC, and calcite at temperatures below 30ºC (86). This is in contrast to Troll Springs, which precipitates only blocky to prismatic calcite crystals due to the much lower temperatures.

Microbial communities at Mammoth are strongly correlated with depositional facies, implying that changes in the bacterial community composition vary with environmental conditions like pH and temperature along the spring outflow. Fouke *et al*. (87) argue that the presence of microbes seems to have an influence on carbonate structure and chemistry during precipitation. Areas with high temperature and pH have lower microbial diversity than areas farther below the source. Martin *et al*. (179) found that fewer taxa are present at the highest temperatures due to low availability of organic matter and the thermal upper limit for photosynthesis. Additionally, the last three facies (pond, proximal slope, and distal slope) have fewer taxa than expected although the environment could support both autotrophic and heterotrophic lifestyles (87). The authors suggest that variable temperature, pH and water flow (271) influence the environmental stability within each facies, and, consequently, microbial community makeup. The size and complexity of Mammoth therefore leads to distinct intra-facies environmental gradients. Troll Springs is much smaller than Mammoth, and strong gradients within the pools are not found, simplifying analyses and enabling easier application of ecological models.

Silica can also be deposited in a geothermal setting. Siliceous sinters are found around most hot springs and geysers, many of which discharge water at or close to boiling. The fluids are saturated with silica, which is precipitated in amorphous form when cooling and evaporation takes place. Siliceous sinters that support microbial communities have been found in New Zealand (142), Iceland (149, 264, 265), Tibet (157) and Yellowstone (105, 130).

Like the microbial communities at carbonate springs, communities in silica springs tend to be zoned according to temperature. Lau *et al*. (157) studied the diversity of microorganisms involved in early colonization and silicification along a thermal gradient from 46 to 77°C at a spring in Tibet where active silica precipitation occurs. They found a transition of the microbial community from "pioneer biofilm" at higher temperatures to mature mats at lower temperatures. The anoxygenic phototroph *Roseiflexus* was the dominant organism in all assemblages. Only a thin surface cyanobacterial layer existed in the mature community. The authors suggested that *Roseiflexus* and *Synechococcus* resist silicification and therefore protect against mineralization. In another example, Tobler and Benning (264) studied microbial diversity in sinters of five diverse (*e.g*., varying temperature, sinter growth rate, pH, salinity) Icelandic geothermal systems. Each site was characterized by a distinct bacterial community and dominated by one phylogenetic class, such as Aquaficae, Deinococci or gamma-proteobacteria, which comprised between 49 and 95% of the community. The authors suggested that the diversity and microbial composition were dependent on temperature and sinter growth rates because all of the sites were

characterized by very similar water chemistries. Like many other geothermal settings, the hottest study site (83ºC) had the lowest diversity.

At the most extreme temperatures, some phyla are absent altogether. Blank *et al*. (16) studied seven silica-depositing springs at Yellowstone with temperatures close to the boiling point. Streamers in those hot springs are composed of thermophilic organisms with primary production driven by chemoautotrophic hydrogen oxidation. Cyanobacteria and green algae are absent, presumably because those temperatures are above the limit for photosynthetic organisms.

Some silica-precipitating hot springs (*e.g*. Yellowstone, Iceland, New Zealand) have an abundant diversity of alkalithermorphilic (pH 7-9 and temperatures 60-95ºC) microorganisms, which are mostly chemolithoautotrophic. Key organisms in those environments include Aquificales, *Thermus*, Deinococci, *Chloroflexus*, *Sulfolobus* and *Synechococcus*. Cyanobacteria are typically found only where the waters have cooled below 70ºC, again because of the thermal limit of photosynthesis.

1.6 OTHER DRY ENDOLITHIC ENVIRONMENTS

At the cold and dry end of the spectrum of environmental conditions at Troll Springs we find microorganisms living inside rocks as endolithic communities. Endolithic organisms occupy pore spaces several millimeters below the rock surface, protecting themselves from the environment. The

organisms are largely photosynthetic, providing the primary production for the endolithic ecosystem.

At other sites around the world, endolithic organisms colonize a wide variety of host rocks, in a range of climates. In moderate climates, endolithic communities are found in silica (275) and travertine deposits (189) in Yellowstone National Park, in dolomite in the Swiss Alps (119, 245), in travertine deposits in Turkey (204), in limestone, sandstone and granite in the Rocky Mountains (48, 211, 274, 275), and in silica in New Zealand (96). In cold and arid to hyper-arid regions, such as in Antarctica, endoliths are found in sandstone (88, 155, 269), gypsum (126) and granite (60), as well as in limestone at high altitudes in Tibet (290). In the Arctic, which is cold but much wetter than the Antarctic, endoliths have been found in sandstone (195-197) and carbonates (43). Endoliths are also found in hot and hyper-arid environments, such as the Atacama desert in halite (285) and gypsum (65).

Colonization of rock by microorganisms can also be chasmolithic (colonizing fissures and pores in natural rocks), hypolithic (inhabiting areas beneath a rock), and epilithic (colonizing the surface of a rock). Hypolithic communities have been found in a variety of extreme settings, ranging from hot and hyper-arid in the Atacama Desert (156, 282) to cold and hyper-arid in the Antarctic desert (48, 211), as well as four deserts in China that vary in their aridity and mean annual temperatures (212, 281)

1.6.1 Endolith Colonization

In most studies of endolithic communities, organisms colonize pre-existing rock. Walker and Pace (273) have suggested that these endolithic communities may be seeded from a cosmopolitan metacommunity adapted to the endolithic environment; these organisms were likely dispersed by wind (91). In some cases, the void spaces necessary for microbial colonization are already present. For example, De Los Rios *et al*. (60) suggested that colonization of natural rock fissures and cavities in Antarctic granite led to formation of endolithic communities. Hoppert *et al*. (118) investigated colonization of freshly exposed homogenous carbonates with few natural cracks or pores. They found that the rock was subjected to gradual and continuous biogenic chemical dissolution, creating homogeneous cavities that became occupied by endolithic organisms. So in these and many other instances (88, 290), formation of the rock matrix predates microbial colonization.

As shown in Chapter 2, the reverse is true at Troll Springs: the rock develops in the presence of microorganisms. Other examples of this form of endolith colonization include Pamukkale geothermal springs in Turkey, where calcium carbonate (travertine) is being precipitated in the presence of microbial communities (204), and at Bagni di Tivoli hot spring and on the side of a travertine-line aqueduct at Viterbo, Italy (203, 205, 207). In none of these instances, however, has the endolithic community been characterized in detail using molecular techniques.

Calcite precipitation at geothermal springs can interact with already-present microbial material, particularly periphyton, entrapping it in travertine. Grain boundaries and pore spaces enable colonization by microorganisms as endolithic or chasmolithic communities. I argue in Chapter 2 that the entrapped periphyton become a precursor for endolithic communities at Troll, occupying space that is later colonized by other organisms that play minor roles in the periphyton but are better adapted to the cold, dry environment of the endolith.

1.6.2 Geographic distribution and microbial ecology of dry endolithic communities

Walker and Pace (273) have argued that endolithic communities are among the simplest microbial ecosystems known, with low species diversity. Because they experience relatively static environmental conditions, these communities are also resistant to disturbances and probably have low turnover rates. Their relative simplicity and stability make it easier to observe ecological patterns than in more complex systems.

One of the most fundamental characteristics of an endolithic community is the source of primary production. Microbial primary producers include oxygenic photosynthetic organisms such as lichen (symbiosis of algae and fungi) or Cyanobacteria, and in some cases anoxygenic organisms, such as Rhodobacter. All of these organisms compete for the same space and resources, and all fulfill the same function in the ecosystem. Under ideal conditions they can coexist, but

it is more typical for endolithic communities to either be lichen (and algae) or Cyanobacteria dominated.

One environmental parameter that seems to play a major role in determining the importance of Cyanobacteria versus lichen/algae is pH, with low pH favoring lichens and algae, and high pH favoring Cyanobacteria. Friedmann *et al*. (89) collected samples from the Ross desert in Antarctica that were either lichen or Cyanobacteria dominated. All of the algae/lichen-dominated communities were associated with low pH values in the sandstone. The endolithic community in low-pH silica is dominated by red algae in Yellowstone (275) and in Italy (104). Omelon *et al*. (197) showed that Cyanobacteria dominate under higher pH conditions and calcium concentrations in sandstone in the Arctic, whereas algae or fungi dominate under lower calcium and pH conditions in the same sandstone. Similar results have been found in Antarctic sandstones (141).

Illumination can also affect the makeup of primary producers. For example, Wong *et al*. (290) showed that eukaryotic (algae) lichen dominate endolithic communities in granitic rocks with high UV and visible wavelength transmission, whereas Cyanobacteria dominate rock with lower UV and visible transmission. This result could reflect relatively high UV tolerance of lichen, and/or better adaptation of Cyanobacteria to lower light levels.

The makeup of primary producers in endolithic communities is also influenced by the availability of water. Bell *et al*. (12) found that Cyanobacteria and green algae can coexist in near-equal abundances in semi-arid areas. In

contrast, deserts, particularly hot deserts, impose a strong water stress on endolithic microorganisms, leading to dominance of adapted Cyanobacteria (*e.g.* Chroococcidiopsis) and absence of eukaryotic members (199). This effect is less pronounced in cold deserts, where water can become available when temperatures are above freezing.

The composition and diversity of primary producers also change within phyla, depending on environmental factors like temperature and the availability of water. Harsh conditions favor particularly hardy organisms, and therefore tend to lead to low diversity. For example, in some of the driest areas on Earth, including the Ross desert of Antarctica (89), the Dry Valley in Antarctica (211), the Atacama desert (212) and the Tibetan plateau (290), Cyanobacteria diversity is low, with Chroococcidiopsis dominating. Interestingly, richness, diversity and community makeup of hypolithic communities are significantly correlated with the availability of water, but not with temperature (212).

As conditions become wetter, diversity increases. Endolithic Cyanobacteria diversity is comparatively high in moderate climates like the Rocky Mountains (274), and Chroococcidiopsis decrease in abundance or are not observed (120, 155, 245, 274), suggesting that other cyanobacterial phylotypes out-compete them in cold-wet conditions. Specifically, *Leptolyngbya, Gloeocapsa* and eukaryotic algae are prominent in alpine and arctic regions (120, 195, 245).

In relatively diverse cyanobacterial endolithic communities, several Cyanobacteria taxa are often found in common, although in different

combinations. These include Oscillatoria (*e.g*. *Leptolyngbya, Lyngbya, Phormidium, Microcoleus*), Chroococales (*e.g*. *Chroococcidiopsis, Gloeocapsa, Hormathonema*) and Nostocales (*e.g*. *Scytonema, Anabaena*). Significantly, a number of studies have shown that settings with similar environmental parameters, such as nutrient availability, water availability, and UV intensity, have similar community makeup (91, 274), even if they are from different geographic locations.

In addition to primary producers, diverse communities of heterotrophic bacteria that play an important role in decomposition and nutrient cycling are also common in endoliths, as revealed by molecular methods. These include an α-Proteobacteria and a member of the Thermus-Deinococcus, as well as other groups with broad metabolic properties (120, 155).

Interestingly, microbial community makeup does not appear to depend strongly on rock type. For example, Walker *et al*. found that the microbial composition of Rocky Mountain endoliths does not cluster by lithology. Also, Chroococcidiopsis, which is a common in the world's driest deserts, has been found in both quartz sandstone in the Antarctic (211) and in limestone in Tibet (290).

In general, microbial community makeup in endoliths seems to depend on a few key environmental parameters. Endoliths are mostly phototrophic, so light plays a major role. Microorganisms colonize the rock matrix at depths appropriate to their ability to utilize the illumination at that depth. Photosynthetic community makeup, and probably the makeup of heterotrophs as well, is

influenced by variations in substrate pH and water availability (141). This relationship is similar to the influence of water on microbial community makeup in soil mentioned earlier. Temperatures do not seem to influence endolithic diversity and microbial community makeup in the Arctic (196). However, temperature does influence community makeup under the most extreme temperature conditions, such as in the Dry Valleys of Antarctica (90).

1.7 SUMMARY

Microbial communities around the world exhibit distinct patterns that reflect ecosystem functions and the influence of environmental conditions. Environmental parameters and the competition for resources control the presence of organisms, sometimes on a micro-scale. Competition is a complex interaction involving a variety of environmental factors that directly influence survival, growth and reproduction. The outcome of competition may differ markedly under different sets of environmental conditions, and relative competition abilities can change along environmental gradients. Temporal changes in environmental conditions, both abrupt and gradual, can drive succession. The challenge for microbial ecologists is to understand the causal relationships between environmental parameters and the properties of microbial communities as the parameters change.

1.7.1 Key ecological concepts

The review of the ecological literature in the preceding sections illustrates and can be summarized in a number of key ecological concepts. The concepts stated below are a summary of material discussed and referenced above or in the following sections. Here I use the term “community structure” to refer to the combined concepts of diversity, richness, abundance, and community makeup:

- If an environmental parameter shows little variation, then it will have little influence on community structure.

- If an environmental parameter shows a large variation, then it can have a large influence on community structure.

- If a resource is abundant, then variations in that resource do not have a large influence on community structure. Other less abundant resources are more important. (This concept is similar to Liebig’s “Law of the Minimum”, discussed below.)

- If a resource is scarce and important for survival, then variations in the availability of that resource tend to have a large influence on community structure.

- A direct consequence of the preceding concepts is that the effect of environmental gradients on community structure is typically different in aquatic environments (where the critical resource of water is abundant) than in terrestrial environments.

- Environmental conditions can exceed the survivability limit for some organisms and functions (*e.g.*, the upper thermal limit for photosynthesis at very hot springs). This concept is also known as the “Law of Tolerance”, discussed further below.

- If a resource is important to only a subset of a community (*e.g.*, access to light for photosynthetic primary producers), variability in that resource may have a strong influence on only that subset of the community.

- Diversity and richness tend to be lowest under the harshest conditions, because fewer organisms can survive such conditions.

- While specific community makeup in an ecosystem may vary with environmental conditions, certain critical community functions like primary production remain relatively constant, independent of the conditions in the particular ecosystem.

- An environment like soil with many niches can support many coexisting organisms that serve the similar ecological function, resulting in high diversity.

- An environment with few niches can support fewer coexisting organisms, leading to increased competition and lower diversity.

- Ecological succession can take place following major environmental disturbances that cause an abrupt change in community makeup.

- Succession can also be driven by small, cumulative environmental disturbances whose net effect is to cause gradual change in community structure over time.

It is important to ask whether these key ecological principles apply as well to microorganisms as they do to macro-organisms. While the principles sound entirely general, the ecological patterns exhibited by microorganisms and macro-organisms do differ in some respects, and these differences must be explained. For example, macro-organisms show greater dependence of diversity on latitude than do microorganisms (77, 116). However, when comparing “macro-bial” and microbial patterns, it is important to consider the actual environments to which the organisms are exposed. In the case of latitude, plants and animals are typically exposed to much harsher conditions at high latitudes than soil

microorganisms below the surface at the same location. The more protective local environment within the soil can result in survival of more microorganisms, thriving in a microenvironment that is less harsh than the one to which surface plants are exposed. With more stable subsurface conditions (*e.g.*, temperature), other parameters like pH, as shown above, can exert the dominant influence on soil community diversity.

The key point is that the specifics of the environmental conditions must be considered in order to understand community variability, not just a simple geographic descriptor like latitude. While microbial diversity does not vary strongly with latitude in soils, it does in marine surface waters (93, 216). Consideration of the environmental details in light of the key ecological concepts listed above makes the reason for this clear. Near the ocean surface, water is an unlimited resource, and other parameters like salinity and pH show little variation (as discussed above), so none of these factors strongly influence community structure. Surface water temperature, on the other hand, is a parameter that varies significantly with latitude, so it becomes a strong determinant of community structure.

Based on such considerations, I conclude that the key ecological concepts listed above apply to microorganisms as well as they apply to macroorganisms. This is significant, because it means that ecological studies that are based on observations of microorganisms can be used to test ecological models that were originally developed for communities of macroorganisms as long as the actual environmental conditions to which the microorganisms are exposed are

appropriately considered. It also means that all of the desirable properties of microorganisms – high reproduction and colonization rates, easy identification via high-throughput sequencing, *etc*. – can be used to full advantage in performing ecological studies of broad relevance.

1.7.2 Environmental parameters

The environmental parameters that govern microbial communities include physical parameters (illumination, temperature, water flow velocity, water depth, *etc*.) and chemical parameters (pH, salinity, dissolved oxygen, nitrate concentration, phosphate concentration, *etc*.). These and other parameters influence the distribution, abundance and activity of microorganisms. It is also important to keep in mind that changes in one parameter can affect another one in the natural environment. Microorganisms must contend with the interactive variations among parameters that occur.

Natural variations in environmental parameters span a range of values, which for some parameters can be quite broad. Microorganisms also each have a range of acceptable values for environmental parameters, where each parameter has a minimum, a maximum and an optimum value. A microbe's range for a given parameter may be broad or limited, with the most rapid microbial growth typically occurring within a narrow optimum range. Bacteria, as a group, grow in a broad range of temperature (freezing to boiling); however, individual bacterial taxa require narrower ranges. Temperature affects microbial metabolism and therefore the growth range of organisms is determined by the

heat sensitivity of their enzyme systems (230). Another example is pH, where most bacteria have an optimum between 6.5 and 7.5, and eukaryotic microorganisms prefer an acidic environment, with optimum activities at a pH of 4 to 6 (173).

It is useful to distinguish between two different kinds of environmental parameters: ones that are resources and ones that are not. In ecology, resources are defined as substances required by living organisms for growth, maintenance and reproduction. A resource is transformed to produce a benefit to an organism, and in the process is made unavailable to other organisms or is consumed. Each organism has key resources that are required for optimal growth (173). A typical resource parameter is nutrient availability, for which microbes compete. Others include volume/surface area to colonize, access to light for photosynthesis, access to water, *etc*.

Non-resource parameters, in contrast, are environmental conditions, like temperature, salinity or pH, that influence and determine the surroundings of a microbe but are not resources used for growth. Microorganisms do not “compete” for temperature or pH because those parameters are imposed by the environment. Non-resource parameters are important because microorganisms are adapted to some conditions but not others, potentially having a strong influence on community makeup. Resources limit how many organisms can be supported, whereas non-resource parameters limit which organisms can live in that parameter range by imposing environmental stress. For example,

photosynthetic bacteria compete for light as a resource, while temperature or pH determines the metabolic activity range they can compete/live in.

Most studies in microbial ecology (as mentioned above) have examined variations in microbial diversity / richness / community makeup while considering resource and non-resource parameters simultaneously. This approach describes how communities vary in different environments. However, the separation of resource and non-resource parameters can help in understanding the reasons for variations in community makeup and diversity. Both types of parameters affect community makeup, but the underlying mechanisms by which they do so are fundamentally different.

When environmental parameters change, the change may influence just a subset of the microbial community. This is especially true for resource parameters that are only important to a subset of the community. For example, varying light levels can cause an immediate change in the primary production sub-community, whereas the heterotrophic community does not necessarily change. Changes across an entire community are more likely to be observed for a resource parameter (like water) that is required by all organisms. As discussed above, both abrupt and gradual temporal changes in environmental parameters can drive successional changes in community structure.

1.7.3 Selective pressures on communities

The environment and its parameters are dynamic, varying in their influence on microbial communities. These influences shape diversity and

community composition in complex ways. One parameter can have a stronger influence than another under a given set of conditions, but the relative importance of these parameters can change as conditions change. It is useful, then, to order environmental parameters according to their influence on microbial selection. The strongest parameter exerts the strongest selective pressure on the community (or a portion of it), followed by the second with less influence, and so on. All the parameters influence the selection simultaneously, but weaker parameters have less influence than stronger parameters.

Both resource and non-resource parameters can play essential roles in selecting a microbial community. Non-resource parameters set fundamental limits on the microbes that can be present, according to the minimum limit, optimal range, and maximum limit for each organism. Microbes that are closest to their optimal growth range will compete most successfully under a given set of conditions. For example, a soil pH of 7 will favor Cyanobacteria over eukaryotic algae (241). Microorganisms with a maximum or minimum pH near 7 may also be able to survive in this in environment, but will do so in much lower abundance than organisms for which the conditions are optimal. Because they set fundamental limits, critical non-resource parameters can place strong selective pressures on communities.

Resource parameters select microbial taxa according to their needs. For example, two soil samples with the same pH can have the same diversity, but very different community makeup depending on the availability of a critical resource like water (4, 6). And in general, the less abundant a critical resource is,

the stronger is the selective pressure that it places on the community. For example, in a desert with low water availability, water is a more important selecting parameter than in moderate climates where water is less restricting (199, 212).

The effect of resource parameter limitations has also been described in Liebig's "Law of the Minimum", which states that plant growth is controlled by the scarcest resource – the limiting factor – and not by the total amount of resources available (191). Although this law has been mostly applied to agriculture, it also governs other environmental settings. Community makeup is also controlled by V.E. Shelford's "Law of Tolerance" (242), which states that the abundance and distribution of species are limited by the range of tolerance (minimum and maximum) for an environmental parameter.

It is important to consider the interplay of these two laws. Specifically, different species within a community can have different resource requirements and tolerability, leading to different responses to changing environmental factors. Additionally, coexistence of organisms, such as primary producers and heterotrophs, influences the supply and demand of resources, perhaps resulting in a balance under optimal conditions.

These ecological concepts mean that selecting parameters tend to be weighted differently for aquatic and terrestrial environments. As noted previously, temperature can show more variability than other parameters in aquatic environments (*e.g.* marine surface waters and hot springs), having a greater influence than in terrestrial soils where other parameters vary more. Also,

because water is abundant in aquatic settings, it is not a limiting factor. In contrast, in a desert the scarcity of water exerts greater selective pressure than temperature. Stated generally, the probability that one resource parameter has a stronger selecting weight on the community than another depends on the scarcity of that resource parameter.

1.7.4 Ecological niches

An ecological niche provides conditions necessary for the persistence of a species, and determines its ecological role in the ecosystem (213). The term niche is defined in several different ways. A niche can be seen as a physical habitat, with conditions suitable for an organism, *i.e*., where that organism lives. Another type of niche is a functional niche that encompasses a particular role in an ecosystem. Several different species that serve the same ecological function can occupy a given functional niche. A third concept of a niche is one that enables coexistence of species within a habitat. In this concept, the ecological space is multi-dimensional, where each dimension is a resource or non-resource parameter important for species' persistence (129). In this concept, a niche is defined by a range of conditions and resources a species can use to survive and reproduce with no interference by other species. However, this niche range for each organism is typically narrower as a result of interactions with other species (*e.g*., competition) and environmental changes. I will use all three niche concepts in the chapters that follow, distinguishing among them as necessary.

1.7.5 Why study microbial ecology at Troll Springs?

Ecology is fundamental to understanding how all living organisms interact with their environment and with one another. A crucial aspect of ecology is how community structure (including diversity, richness, abundance and makeup) changes depending on environmental conditions. The relationships between organisms and their environment are driven by fundamental ecological principles, many of which are obeyed by both macro- and microorganisms. Many ecological models have been developed for macro-organisms, and now molecular techniques make it possible to extend this understanding to the microbial world. Ecological models are tested best in a natural setting, and microbial ecology offers many advantages for carrying out such tests. Environmental gradients are particularly valuable for studying the relationships between changes in the environment and the corresponding community.

Microbial ecology at Troll Springs addresses all of these scientific needs. In this dissertation I investigate the microbial communities at Troll along transitions from aquatic to terrestrial, warm to cold and mobile to immobile. These gradients are very steep, with major environmental changes over just a few tens of meters, eliminating other parameters that would complicate analysis over larger scales. Important temporal changes in environmental conditions at Troll are small and cumulative over time, driving gradual succession that is manifested across these gradients.

Troll also has two ecosystems, freshwater and endolith, together in close proximity, with intermediate environments between them. In the past, aquatic and

endolithic communities have typically been studied separately. The two have very different underlying environmental factors shaping their microbial communities, and at Troll it is possible to study both at once as well as the transitions between them.

The use of molecular methods and data analysis techniques in this dissertation provides detailed insight into community structure at Troll, and into how that structure changes across the ecosystems present. The result is a portrait of the relationships between communities and their environment that is nuanced enough to explore a range of ecological principles, yet simple enough to reveal the workings of these principles with some clarity.

CHAPTER 2: CALCITE PRECIPITATION AT TROLL SPRINGS LEADS TO ENDOLITH COLONIZATION AND DRIVES MICROBIAL COMMUNITY MAKEUP

2.1 INTRODUCTION

Environmental conditions influence communities of organisms, particularly along environmental gradients where environmental parameters change in a systematic way with geographic location. As described in Chapter 1, previous studies of microbial ecology have investigated changes in community structure along gradients in pH (80, 158, 231), temperature (183, 190, 278), water abundance (4, 6, 39, 212, 281, 298) and salinity (32, 51, 117, 164), among others.

Chapter 1 also summarized the advantages of conducting such a study at Troll. The environmental gradients there are very steep, with major changes over just a few tens of meters, meaning that changes in other parameters that might complicate analysis over larger scales are minimized or eliminated. At Troll it is possible to study aquatic and endolithic communities that are related to one another, as well as the transitions between them. Troll also provides a particularly useful setting for investigating the process of endolith colonization, with an unusual form of colonization in which microbial communities predate precipitation of the rock matrix. The process has some similarities to stromatolite formation, which can take place by precipitation of particles on microbial mats in carbonate environments.

In the sections that follow, I use a combination of optical and scanning electron microscopy and molecular methods to characterize the environmental gradients at Troll, determine the response of the microbial community to those gradients, and discover the processes by which endolith colonization takes place. I argue that the periphyton in the spring are a precursor to the endolithic communities, with major changes in community diversity and makeup taking place in response to environmental changes.

2.2 MATERIALS AND METHODS

2.2.1 Field site

The locations of each sample are shown in Figure 2.1, and schematic drawings and photographs of each sample type are shown in Figure 2.2.

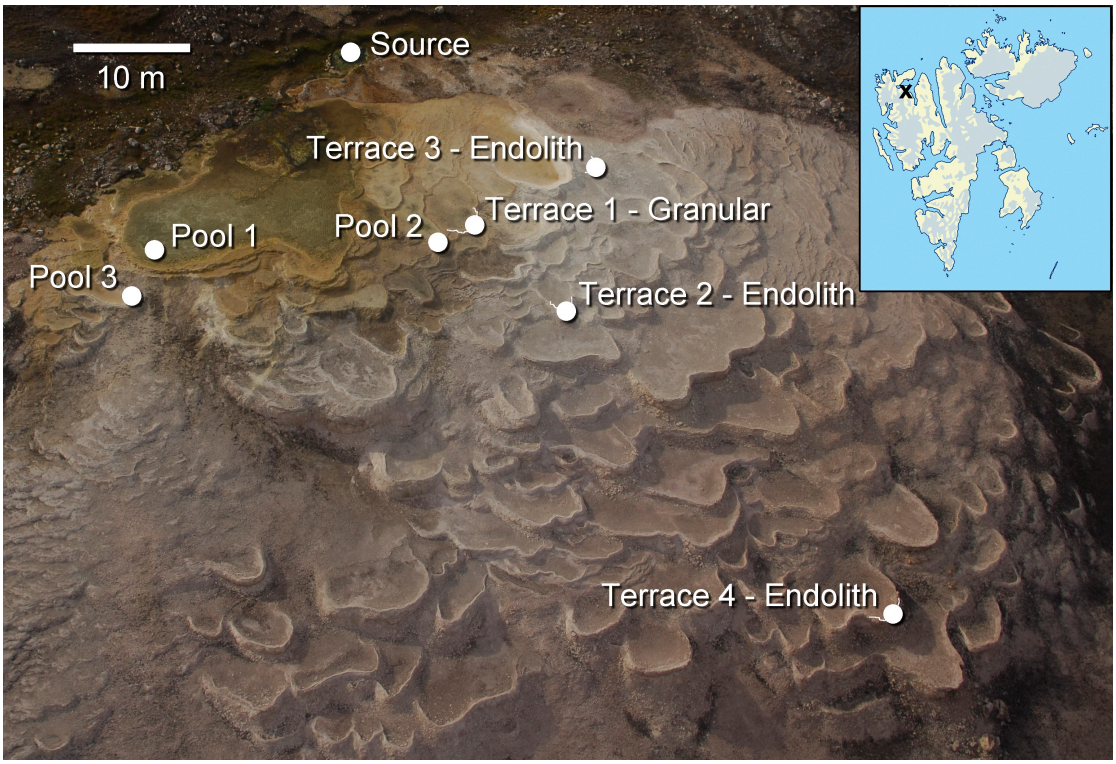


Figure 2.1: Aerial view of Troll Springs. Inset shows the location on Svalbard. Sample sites are indicated. Photo: K.O. Storvik/AMASE.

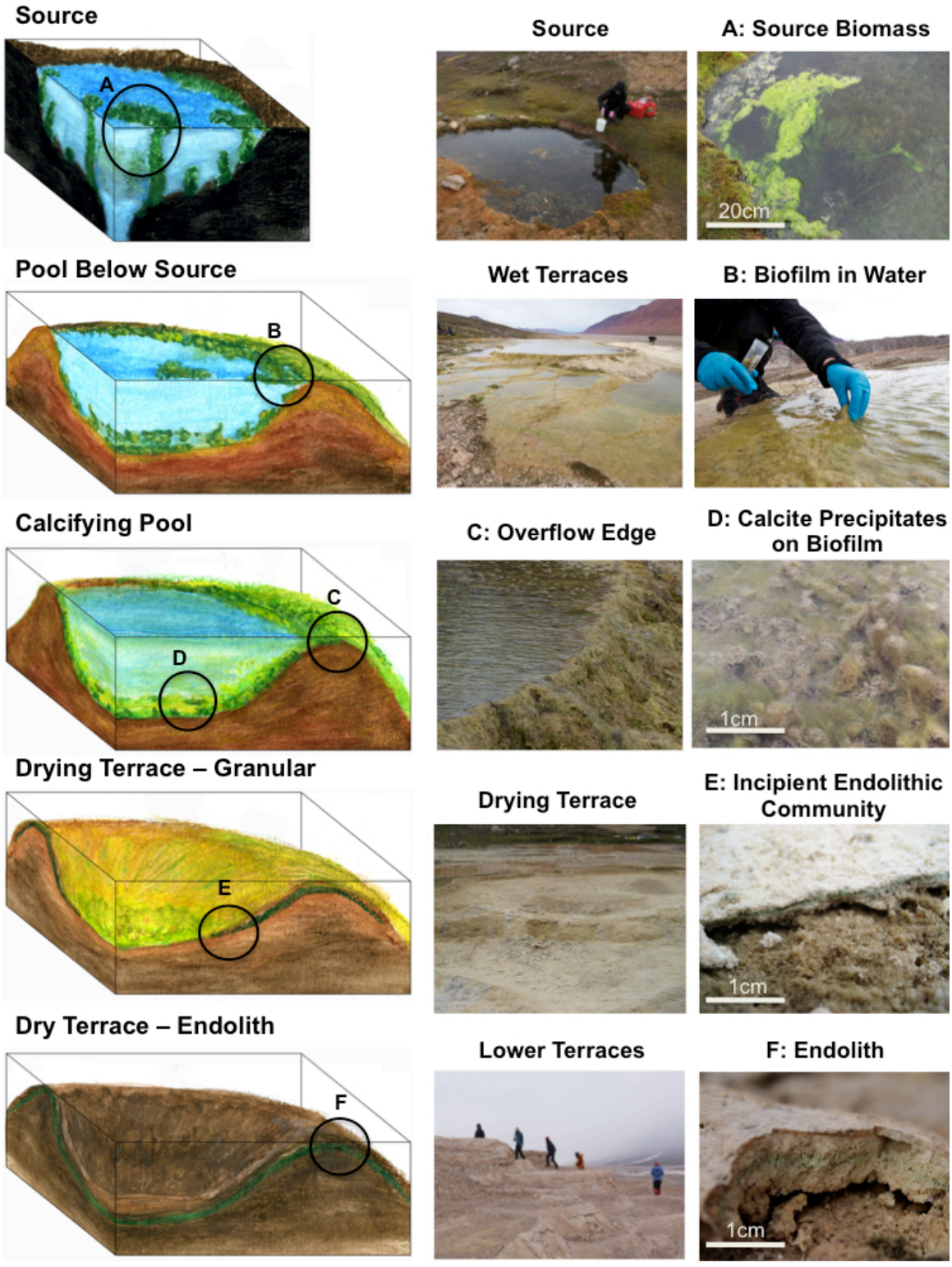


Figure 2.2: Schematic drawings and photographs of sample sites. Photos: V. Starke/K.O. Storvik/AMASE

2.2.2 Sample descriptions

Source periphyton consist of long strands and mats. I collected two dark green periphyton samples from the source (#1 was attached to the bottom, #2 was floating on the surface). Periphyton sampled from water-filled pools were dominated by filamentous organisms within a gelatinous matrix. Three yellow-green periphyton samples from water-filled pools were collected: pool 1, first pool below the source, pool 2, with the most visible precipitated carbonate, and pool 3, which also shows calcite precipitation. I also took a short core into mud from the bottom of the source using a sterile Falcon tube, and refer to it as “source mud”.

Granular samples were collected from terraces that were drying out, and had a weakly cohesive soil-like texture. I collected three granular samples from the center, edge, and rim of a drying terrace. These are referred to as “terrace 1 granular”. The center sample from this terrace was less lithified (*i.e.*, less cohesive) than the edge and rim samples. I found thin “green lines” below the yellow-white carbonate surfaces, most prominently in the rim sample.

Endolith samples were obtained from dry terraces with visible endolithic communities. Endolith samples are referred to as terraces 2, 3 and 4. I collected endolith samples from the center and rim of terrace 2. Terraces 3 and 4 are rim samples only. All terrace samples were intact but foliated travertine rock.

Endolithic samples were collected in August 2008 and aquatic and granular samples in August 2009. Periphyton samples were collected in sterile Falcon tubes, separated into sterile Eppendorf tubes, and frozen at -80ºC.

Granular and rock samples were collected in sterile Whirlpacks and stored at -20ºC.

Measurements of water temperature, pH, and other environmental variables were obtained concurrently with sample collection. These measurements are summarized in Table 2.1. Water content of granular and endolithic samples was estimated subsequently in the laboratory. To do this, I weighed approximately one gram of material per sample, dried the samples at 60°C for 72 hours, and then weighed them again and calculated mass loss per gram. Samples were measured in triplicate.

Table 2.1: Environmental parameters measured during sample collection. n.a. = not available.

Samples		Temperature [°C]	Water Content [wt%]	pH	eH [mV]	Dissolved Oxygen [%]	Conductivity [uS/cm]
Source mud	A	24.8	58.8	6.65	11.2	10	1616
Source periphyton 1	B	24.8	69.1	6.65	11.2	10	1616
Source periphyton 2	C	24.8	66.1	6.65	11.2	10	1616
Pool 1 periphyton	D	16.7	64.8	7.33	-29	126	1618
Pool 2 periphyton	E	9.4	61.2	8.06	-68.4	99	1545
Pool 3 periphyton	F	12.7	59.8	7.94	-62.2	105	1518
Terrace1 center granular	G	6	24.9	n.a.	n.a.	n.a.	n.a.
Terrace 1 edge granular	H	6	10.7	n.a.	n.a.	n.a.	n.a.
Terrace 1 rim granular	I	6	3.3	n.a.	n.a.	n.a.	n.a.
Terrace 2 center endolith	J	4	7.0	n.a.	n.a.	n.a.	n.a.
Terrace 2 rim endolith	K	4	1.9	n.a.	n.a.	n.a.	n.a.
Terrace 3 rim endolith	L	4	1.0	n.a.	n.a.	n.a.	n.a.
Terrace 4 rim endolith	M	4	0.3	n.a.	n.a.	n.a.	n.a.

2.2.3 Optical microscopy and SEM analysis

The samples were fixed in 2% glutaraldehyde, run through an ethanol series and then air-dried. Selected samples were etched for 20 s in 10% HCl, rinsed in three times distilled water, and air-dried prior to coating with platinum for electrical conductivity.

Prior to scanning electron microscope (SEM) observations, samples were imaged using an optical microscope Olympus SZH10 over a range of magnifications and focus positions. Images acquired at different focus positions were co-registered via an affine transformation using the StackReg routine from the Biomedical Imaging Group (BIG) at the École Polytechnique Fédérale de Lausanne. In-focus portions of the co-registered images were then merged via a complex wavelet transform using the BIG routine Extended Depth of Field (84). Subsequently, the same areas that had been imaged by optical microscopy were examined with a Zeiss Auriga FIB-SEM or JEOL JSM-6500 field emission SEM. For microscopy operating conditions I used 10 kV acceleration potential, 5 to 10 mm working distance and 0° tilt.

2.2.4 Nucleic acid extraction

I am particularly interested in determining the makeup of the bulk microbial community over a representative area of each sample. To achieve this, I collected samples from areas 5 x 5 cm in size, homogenizing each to minimize

within-sample variability over this area. Homogenized samples were separated into subsamples and DNA was extracted as duplicates, which then were pooled for bulk analysis. The same principle was applied for PCR amplification. DNA extractions were carried out on 1 g periphyton samples (2 g total), and on powdered granular and rock samples of approximately 3 g for each replicate (6 g total) using a Zymo Research Midi-Soil DNA isolation kit (Zymo Research Corp., Orange, CA, USA). The Midi kit was used in order to cover a large area/amount for each sample. Samples were homogenized using a Vortex BeadBeater Adapter (MoBio Laboratories, Carlsbad, CA, USA). An enzyme/detergent mixture of 20 mg/ml Lysozyme (Sigma Aldrich, St Louis, MO, USA), 20 mg/ml Proteinase K (Qiagen, Valencia, CA, USA), 10 mg/ml RNase A (Invitrogen, Carlsbad, CA, USA) and 20% Sarkosyl (Sigma Aldrich) was added after bead-beating and incubated for 45 min at 55°C. DNA was further purified according to manufacturer's instructions.

2.2.5 PCR amplification for 454 pyrosequencing

Universal primers 27F and 338R were used for PCR amplification of the V1–V2 hypervariable regions of 16S rRNA genes. The 338R primer included a unique sequence tag to barcode each sample. The primers were: 27F-5′-GCCTTGCCAGCCCGCTCAGTC**AGAGTTTGATCCTGGCTCAG**-3′ and 338R-5′-GCCTCCCTCGCGCCATCAGNNNNNNNNCAT**GCTGCCTCCCGTAGGAGT**-3′, where the underlined sequences are the 454 Life Sciences FLX sequencing

primers B and A in 27F and 338R, respectively, and bold letters denote the universal 16S rRNA primers 27F and 338R. The eight Ns denote the 8-bp barcode within primer 338R.

The 50 µl reaction mixture contained 10 mM (total) deoxynucleoside triphosphates (dNTPs), 0.5 µM (each) primer, 5 ng/µl of DNA template, 0.5 U of Phusion High-Fidelity DNA polymerase (New England BioLabs), and 5 x Phusion PCR buffer HF, containing 7.5 mM $MgCl_2$, and 3% DMSO. PCR conditions were 1 cycle of 30 seconds at 98°C, followed by 30 cycles of 5 seconds at 98°C, 15 seconds at 55°C, and 45 seconds at 72°C using a DNA Engine DYAD PCR machine (MJ Research). 10-min incubation at 72°C was the final step. Negative controls without a template were included for each barcoded primer pair.

Sequencing was done at the Genomics Resource Center at the Institute for Genome Sciences (IGS), University of Maryland School of Medicine, using protocols recommended by the sequencing system manufacturer as amended by the Center. The concentrations of amplicons were estimated using a GelDoc quantification system (Bio-Rad Laboratories), and approximately equal amounts (100 ng) of all amplicons were mixed in a single tube. Amplification primers and reaction buffer were removed using the AMPure Kit (Agencourt, Beckman Coulter Genomics). Emulsion PCRs were performed as described in Margulies *et al*. (175). Sequences were obtained using a Roche 454 GS-FLX sequencing system (Roche-454 Life Sciences). Assessment of the quality of the sequences and binning using the sample-specific barcode sequence was performed at IGS.

2.2.6 PCR amplification for Sanger sequencing

16S rRNA genes of 10 samples were amplified by PCR using the B27/1492R primer set. The amplified DNA fragments were gel-purified, cloned and sequenced in both directions (M13F/M13R primers) by Macrogen Inc. (Seoul, Korea) using an ABI3730 XL DNA Analyzer (Applied Biosystems, Renton, USA). Ninety-six clones from each clone library were randomly picked for sequence analysis.

2.2.7 PCR amplification for ARISA

To perform PCR for ARISA (see below), I used the PCR setup described above. PCR was performed in duplicates. The primers used were: universal bacterial primer 16S-1392F (5'-G[C/T]ACACACCGCCCGT-3') and 23S-125R labeled with a 5'TET (5'-GGGTT[C/G/T]CCCCAT- TC(A/G)G-3') (82). Amplification products were purified using a Qiagen PCR clean-up kit (Qiagen). Purified products were run at the same concentration for each sample for 45 min on an ABI310 genetic analyzer (Life Technologies). Electropherograms were analyzed using ABI Genescan software and custom ROX labeled 1500bp size standards with 100bp increments (Bioventures). Outputs were transferred to Microsoft Excel

2.2.8 Sequence, phylogenetic and multivariate analysis.

Sequences generated from pyrosequencing of bacterial 16S rRNA gene amplicons were processed using the mothur (240) pipeline. Sequences were checked for chimeras via Chimera Slayer, aligned to SILVA reference sequences, and clustered as operational taxonomic units (OTUs) using average neighbor clustering at overlap identity cutoffs of 90, 95, and 97% (Table 2.2). For all analyses presented here I used 90% similarity. Trial use of 95% and 97% did not lead to significant differences. I classified the taxonomy of OTU representative sequences using the RDP classifier as implemented in mothur 1.12, using the rdp6 taxonomic sequence database.

Table 2.2: Similarity-based OTU counts and diversity measure (Shannon Index *H*'). For Shannon diversity H'(1000) I used a randomly selected subset of 1000 sequences per sample in order to compensate for differences in sampling effort between samples. Chloroplast sequences were removed for further processing of bacterial diversity.

					Cluster Distance Bacteria				
					0.03	0.05	0.10		
Samples		**# Total Reads**	**# Chloroplast Reads**	**# Bacteria Reads**	**# of OTUs**	**# of OTUs**	**# of OTUs**	***H'***	**H' (1000)**
Source mud	A	3785	227	3558	1394	1254	1169	5.99	5.46
Source periphyton 1	B	8773	7348	1425	196	164	150	2.31	2.21
Source periphyton 2	C	6803	5709	1094	179	162	152	2.85	2.76
Pool 1 periphyton	D	4853	2055	2798	498	448	411	3.62	3.49
Pool 2 periphyton	E	4018	414	3604	999	876	798	4.91	4.56
Pool 3 periphyton	F	2542	202	2340	542	478	431	3.57	3.40
Terrace1 center granular	G	3393	17	3376	1393	1256	1157	6.12	5.62
Terrace 1 edge granular	H	3143	26	3117	1079	946	841	5.54	5.19
Terrace 1 rim granular	I	2709	40	2669	791	703	635	5.01	4.58
Terrace 2 center endolith	J	3874	28	3846	1369	1191	1063	5.69	5.18
Terrace 2 rim endolith	K	3572	250	3322	1077	935	821	5.23	4.83
Terrace 3 rim endolith	L	2354	61	2293	524	462	416	4.13	3.95
Terrace 4 rim endolith	M	3184	1	3183	688	576	492	4.04	3.79

I performed exploratory analysis of sequence data using nonmetric multidimensional scaling (nMDS) (40, 225) on OTU abundance data. nMDS calculations were performed using R (223). nMDS plots were generated from Bray-Curtis dissimilarities (20) calculated from both original and presence/absence transformed OTU abundances. I used isoMDS from the MASS package (268) supplemented by initMDS and postMDS from the vegan package (192). nMDS solutions were rotated to a common orientation using procrustes. Because very rare OTUs can obscure rather than illuminate trends in the data, I used only OTUs with counts of more than one for nMDS calculations. This thresholding typically reduced the number of OTUs by ~30%. It did not change the nature of the nMDS results, but it yielded lower stress scores, meaning that the solutions obtained were a better representation of the actual relationships among the samples.

The patterns revealed by nMDS were then explored in more detail via quantitative analysis of UniFrac (165) phylogenetic distances. A relaxed neighbor-joining tree was constructed from the filtered alignment using Clear-Cut (243). Weighted UniFrac distances, a beta-diversity metric that quantifies community similarity based on phylogenetic relatedness, were reoriented for visualization using principal coordinates analysis (PCoA) (100). I used weighted UniFrac because I am interested in how both the occurrence and abundances of microbes change with the measured environmental parameters. In order to investigate relationships between community structure and environmental parameters, I constrained the PCoA results using redundancy analysis (250), a

process known as distance-based redundancy analysis (db-RDA) (160). All PCoA coordinates were used for db-RDA. Results were computed both with and without chloroplasts. Statistical significance levels of the correlations between community structure and environmental parameters were determined via Monte Carlo permutations. All RDA analyses were performed using the Vegan Community Ecology Package for R.

Sanger sequence results from each amplified fragment were processed using Sequencher^{TM} ver. 4.0.5 (Gene Codes Corporation, Ann Arbor, USA).

2.2.9 ARISA data processing

I also investigated community makeup using automated rRNA intergenic spacer analysis (ARISA, (82)). Peaks with low (<50 units) fluorescence close to background were eliminated. Next, data were standardized and a peak height threshold was set to eliminate weak peaks. Data were then transformed to presence and absence. Thresholding was carried out in conjunction with outlier rejection (232). Reproducibility was checked by comparing replicate profiles for each sample over a range of thresholds. A threshold and replicate pair was accepted only if the Bray-Curtis similarity of the two replicates was great enough after thresholding. Reproducibility was good and no samples had to be rejected. I selected a threshold of 0.455%, so standardized peaks lower than this value were rejected. This value yielded a low threshold and low nMDS stress and, consequently, the best 2-dimensional representation. After thresholding, data

were binned using dpbin (232) and then transformed to presence and absence. I used the same nMDS parameters as for the sequencing data.

2.3 RESULTS

2.3.1 Aquatic environments

At Troll Springs calcite precipitation takes place downstream from the spring's source due to a decrease in water temperature and an increase in pH. X-ray diffraction analysis of Troll calcite detects only calcium carbonate, indicating high purity of the deposits (8). Figure 2.3 illustrates the growth and appearance of calcite grains and their interaction with the periphyton in the pools. In this figure I designate three stages of calcite precipitation, but note that these are points along a continuum. The order of the pools, from the least visible precipitation to the most, is Stage I (Pool 1: 17ºC, pH 7.3), Stage II (Pool 3: 13ºC, pH 7.8) and Stage III (Pool 2: 9ºC, pH 8.1). The rows in the figure show (a) the sample as observed in the field, (b) the sample while still wet under the optical microscope (c) the sample after drying, again under the optical microscope, and (d) SEM of the same area as shown in c.

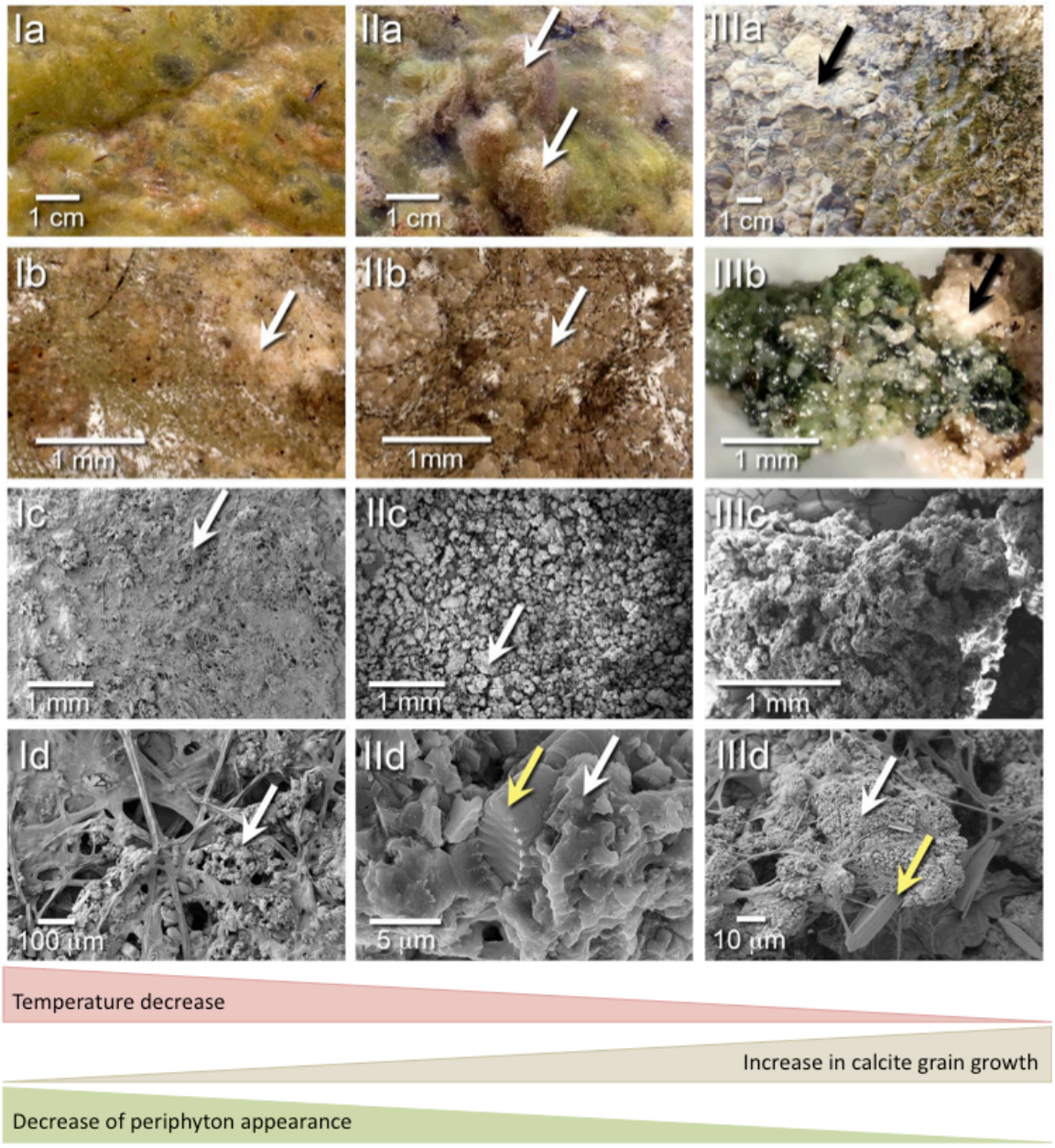


Figure 2.3: Calcite precipitation stages in three pools with increasing calcite presence (Stages I (periphyton pool 1), II (periphyton pool 3), III (periphyton pool 2)). a: field site; b + c: optical microscopy before and after drying; d: SEM after drying of the same area as in c. White arrows show calcite precipitates in and on top of the periphyton. Black arrows show contiguous calcite crust formed in Stage III.

Filamentous algae dominate the source periphyton samples, with no visible calcite precipitates (Fig. 2.4). Pool 1 (Stage I) is the first pool downstream from the source, and only shows small calcite flakes mixed in with the periphyton

(Fig. 2.3 Ib). The calcite flakes become more visible in Stage II, where flakes settle on top of the periphyton, partially covering them (Fig. 2.3 IIa). The periphyton in both stages are mostly loosely aggregated floating filaments within a gelatinous matrix. The periphyton at Stage I are mostly floating at the water-air interface due to trapped gas in the periphyton, whereas the periphyton at Stage II are submerged. By Stage III, the sample consists mostly of granular calcite with periphyton covering the grains and spaces in between (Fig. 2.3 IIIb). This periphyton-calcite mix can be found under a calcite crust that has formed on the bottom of the pool (arrow in Fig. 2.3 IIIa), perhaps limiting light access to it. The long green filaments are not observed at this stage. The abundance of calcite relative to periphyton increases through the three stages. Rows c and d in Figure 2.3 show the increasing abundance of calcite grains. In Stage III, the calcite flakes have merged to form bigger grains.

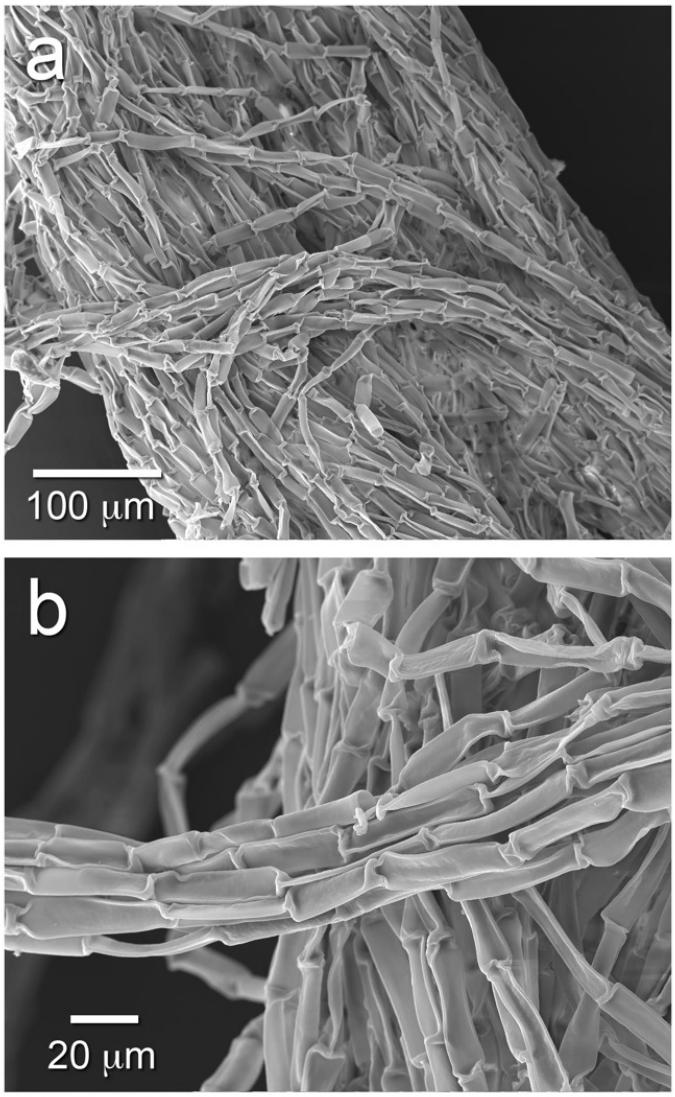


Figure 2.4: SEM images of filamentous algae in the source periphyton at low (a) and high (b) resolution.

High-resolution details of some of the physical relationships between the calcite crystals and the periphyton are shown in Figure 2.5. Extracellular polymeric substances (EPS) are evident as strands (relicts of dehydration), sheets, and sheaths associated with cells. EPS secretion/excretion anchors microorganisms to the surface and helps bind calcite grains together (Fig. 2.5a,b). Deposition of calcite is clearly visible on filaments. Diatoms and green

algae become partially enclosed by calcite, and when physically separated from the calcite can leave a mold behind (Fig. 2.5c-f). Figure 2.6 shows examples of calcite crystals becoming attached to eukaryotic cells and forming a calcite coat around the cell.

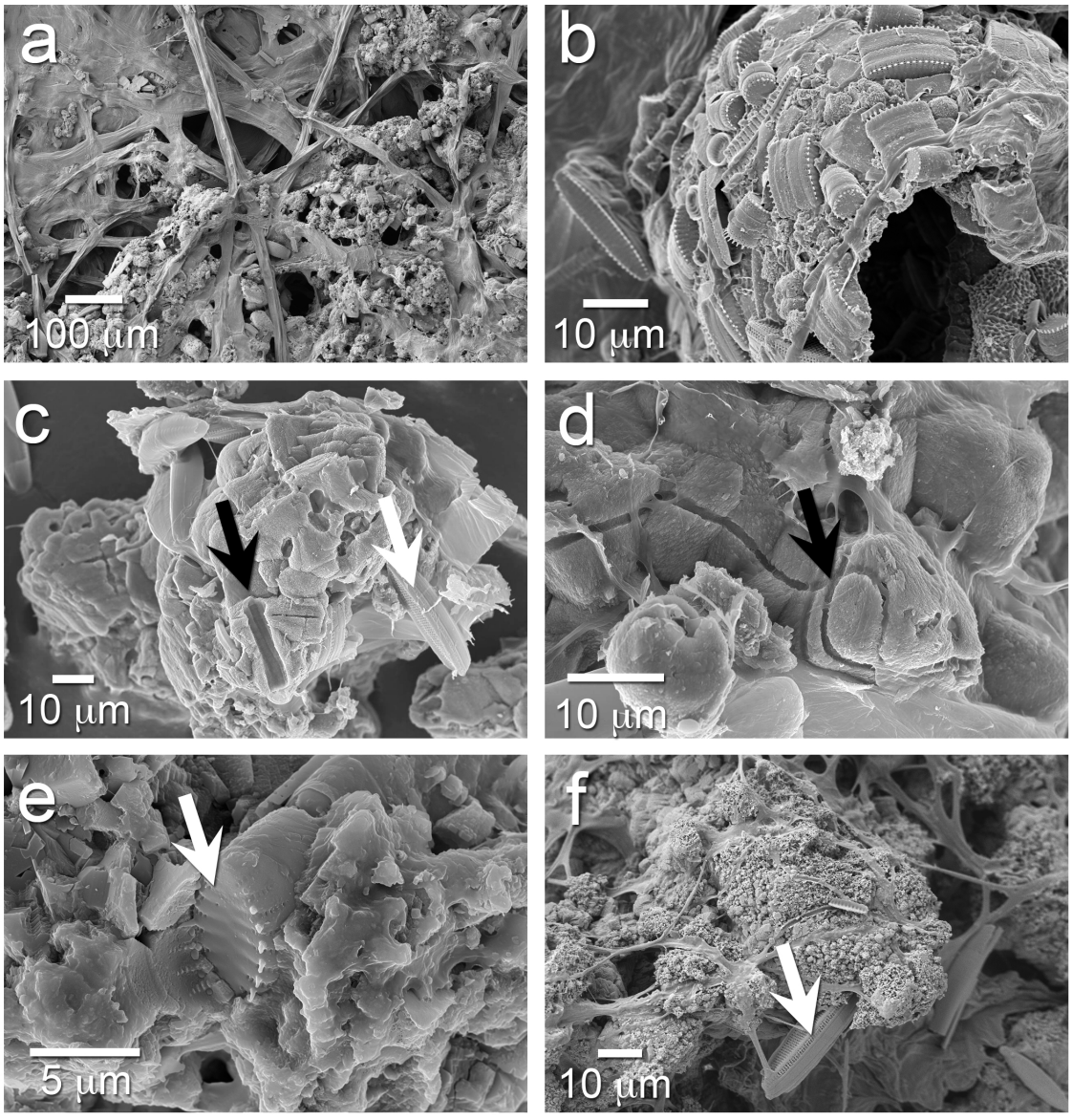


Figure 2.5: SEM images of interaction of periphyton with calcite precipitates. Diatoms, eukaryotic algae cells, and filamentous EPS related to eukaryotes are all common. As calcite precipitates, periphyton becomes partially entrapped in the calcite (white arrows). Separation of periphyton from calcite can leave molds behind (black arrows).

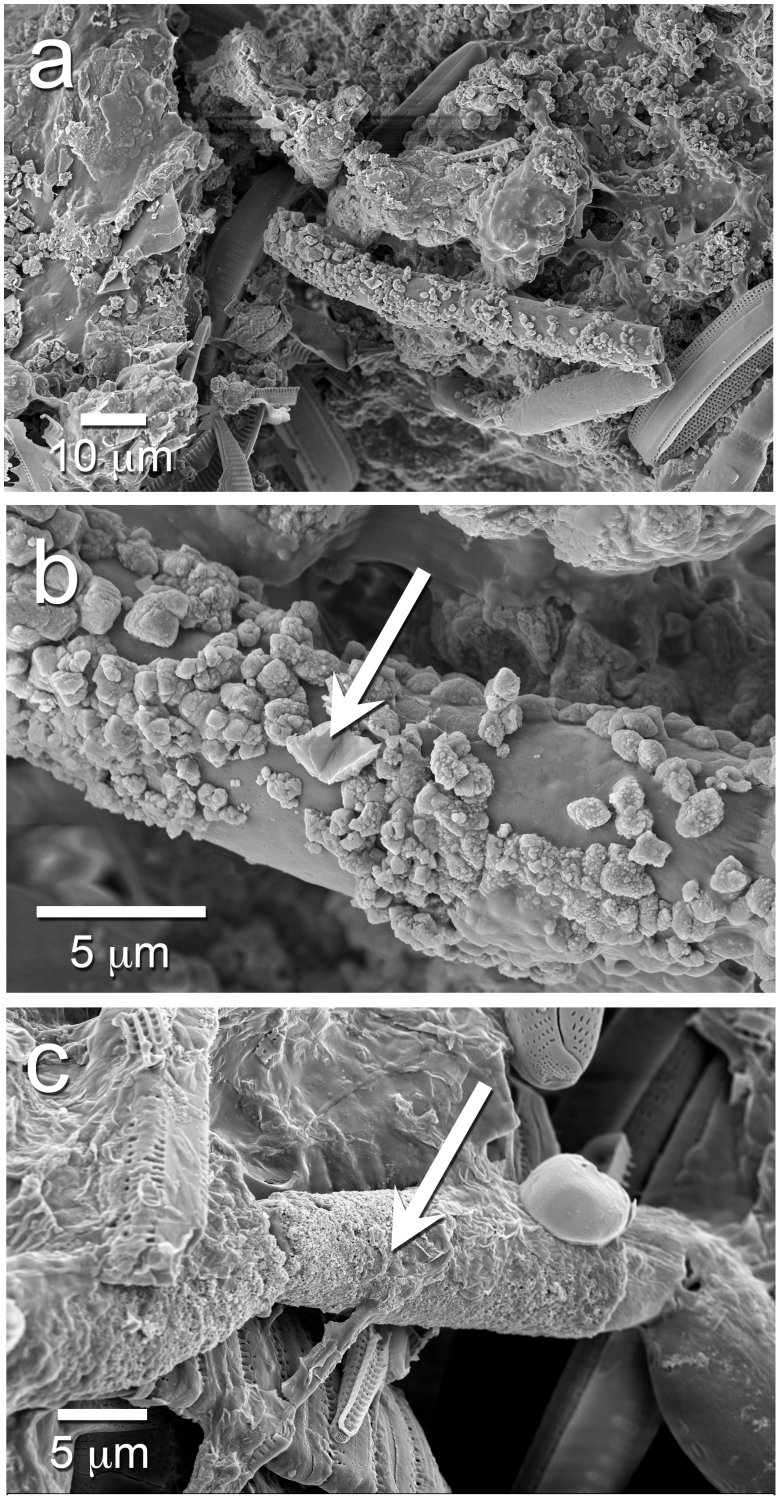


Figure 2.6: Entrapment of eukaryotic cells in calcite. Calcite crystals (a, b) ultimately merge to form a contiguous sheath (c) that accumulates around eukaryotic algae cells. White arrows point to calcite crystals and sheath.

The left side of Figure 2.7 summarizes the key observations among the aquatic samples at Troll Springs, including classifications derived from 454 sequencing. Further details from Sanger sequencing are provided in Figure 2.8. The results show clear and strong ecological patterns in microbial community makeup. Source and pool periphyton samples are mostly dominated by photosynthetic eukaryotes. There are also strong sample-to-sample variations in the dominant bacterial groups. For example, proteobacterial abundance and taxon composition in the source periphyton are different from those in other periphyton samples. Although the source samples share many of the same phyla and families as other samples, they differ markedly in their community makeup. Cytophagaceae, Cyanobacteria (GpIIb), unclassified Proteobacteria and Peptococcaceae are dominant in the source periphyton 1 and 2, pool 1 and pool 3 periphyton, respectively (Figs. 2.7, 2.8). Interestingly, the periphyton in pool 2, which exhibits significant carbonate precipitation, contains OTUs, such as Rhodobacteraceae and Caulobacteraceae, that become more dominant in the terrestrial (endolithic) environment.

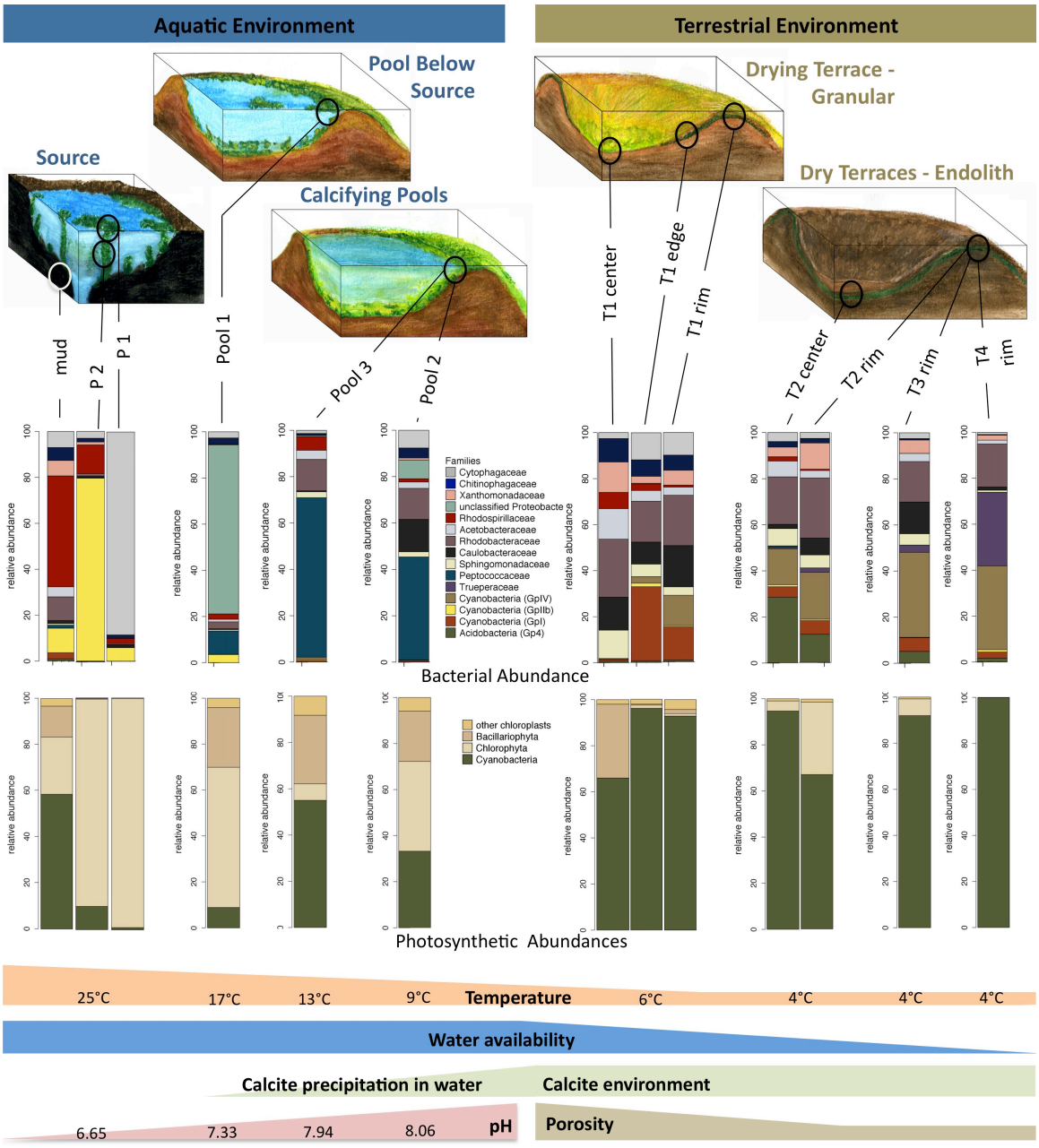


Figure 2.7: Summary of environmental parameters and community makeup at Troll Springs. Environment classifications at the top of the figure are from Konopka (150). Abundance plots include taxa with >500 counts summed across all 13 samples. Drawings of the pools and terraces have some vertical exaggeration. T = Terrace. Ovals show schematically where in a pool or terrace samples were collected. T1, T2, T3 and T4 are separate terraces. Source, Pool 1, Pool 2 and Pool 3 are separate pools.

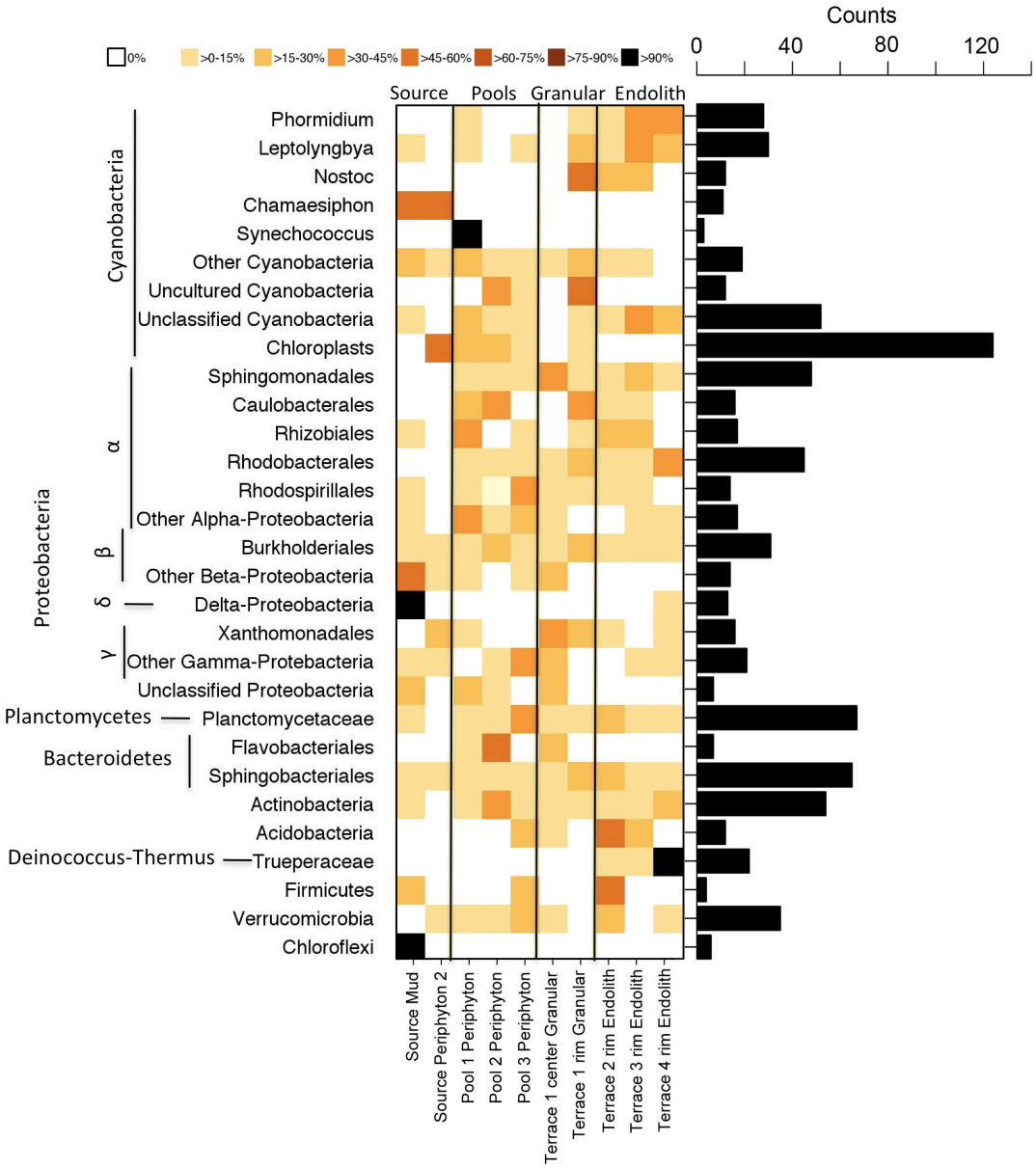


Figure 2.8: Taxa distribution and abundances of Sanger clone sequences for 10 Troll samples. The grid displays the proportional distribution of each taxon across all samples (not the proportion in each sample). The abundances are shown in the bar graph.

There are also strong trends in diversity (Table 2.2). Overall, the source periphyton and pool periphyton are significantly less diverse than other samples, with an uneven OTU distribution. The very low bacterial diversity of the source periphyton samples is particularly striking.

2.3.2 Terrestrial environments

As a pool dries out and the last water in it disappears, precipitation ends, leaving a surface covered in loose granular calcite. Calcite grain sizes are typically much larger than in the pool samples. A green line is visible 1-2 mm below the surface (Fig. 2.9a, Terrace 1 granular). This is the first step in endolith formation at a macroscale. The calcite grains have not lithified yet, instead showing a loose granular structure that easily falls apart when touched. The biological material is probably largely responsible for the cohesion of the calcite. Microorganisms occupy the spaces between the grains. Filamentous eukaryotic algae are a minor component in the optical and high-resolution images. However, diatoms remain abundant, sometimes in dense conglomerations (Fig. 2.9b). Filamentous bacteria are also visible (Fig. 2.9e,f).

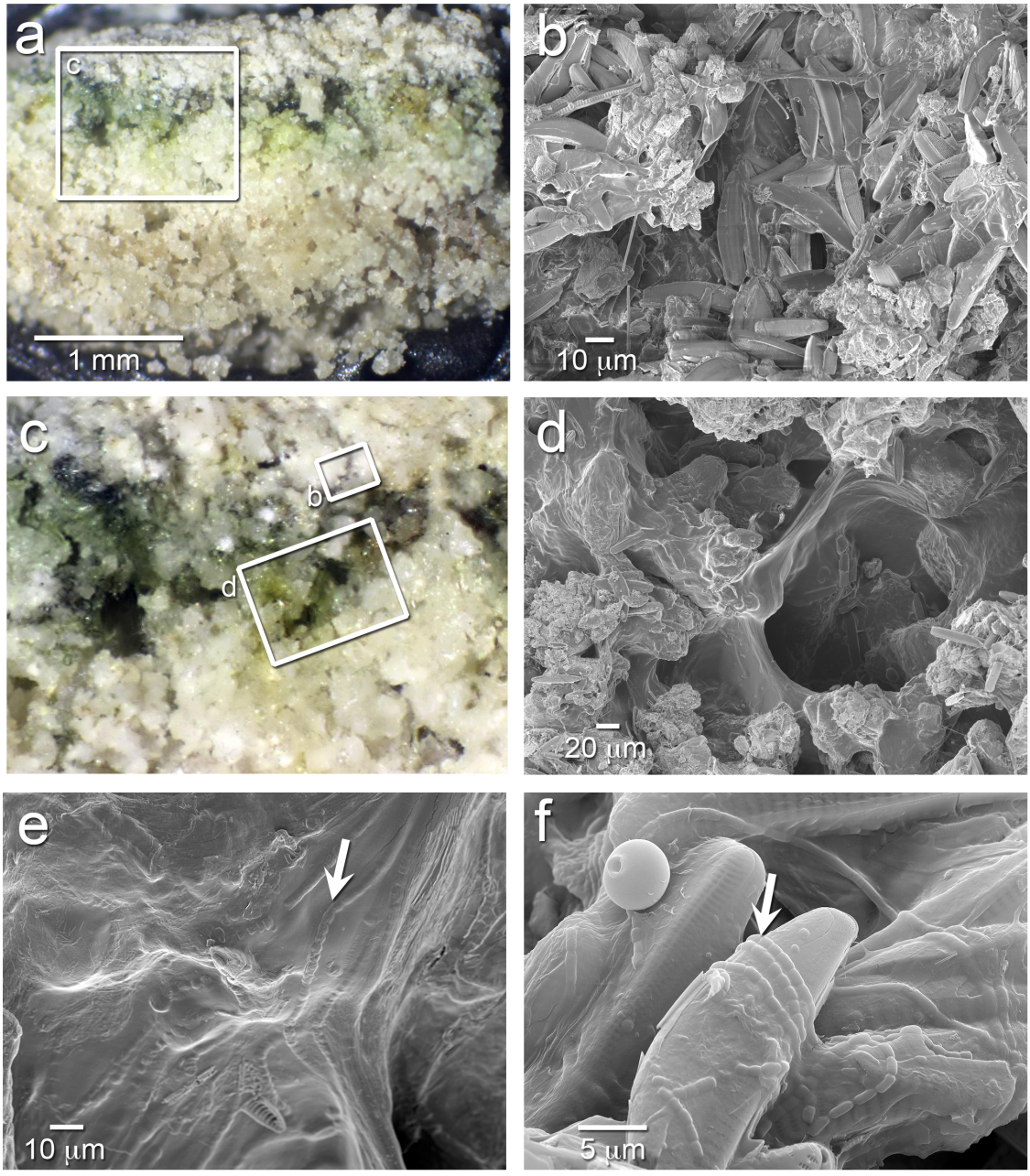


Figure 2.9: Optical and SEM images of a granular sample (Terrace 1 granular). A prominent green line is visible ~1 mm below the surface in a and c. This green zone represents the early stages of endolith formation. Diatom agglomeration similar to that in Figure 2.5 is still observed (b). Enlargement of green areas reveals dense sheets of EPS (d). Images e and f are etched granular samples showing that filamentous bacteria are prominent but eukaryotic algae are less common.

A prominent feature in Figure 2.9 is dense EPS sheets that cover areas up to a few hundred micrometers wide (Fig. 2.9d,e). Dense areas of EPS seen in

SEM images are prominently green in the corresponding portions of optical images (Fig. 2.9c,d), indicating chlorophyll production for photosynthesis. EPS can be highly hydrated, incorporating large amounts of water into its structure and inhibiting desiccation. Although the environment is dry, it is possible that when protected by EPS the endolith has enough water available to produce chlorophyll and conduct photosynthesis. In general, the EPS is more abundant in the drier environments than in the pool samples.

Calcite recrystallization and grain growth are the next stage of lithification, decreasing porosity and dramatically increasing cohesion. Lithified samples have higher calcite grain-to-grain contact areas, and the resultant cohesion makes it difficult to break them apart without force. Despite the low porosity relative to the granular samples, these samples still have green biofilm in the void spaces (Figs. 2.10: Terrace 2 rim endolith and 2.11: Terrace 3 rim endolith) at depths of 1–3 mm beneath the rock surface. Some near-surface carbonate can be removed by etching with HCl (Fig. 2.11c,d), exposing biomaterial and residual carbonate beneath. The etching process exposes diatoms that were encrusted/entrapped during initial carbonate precipitation in the water and subsequent lithification (Figs. 2.10d, 2.11b,c,e).

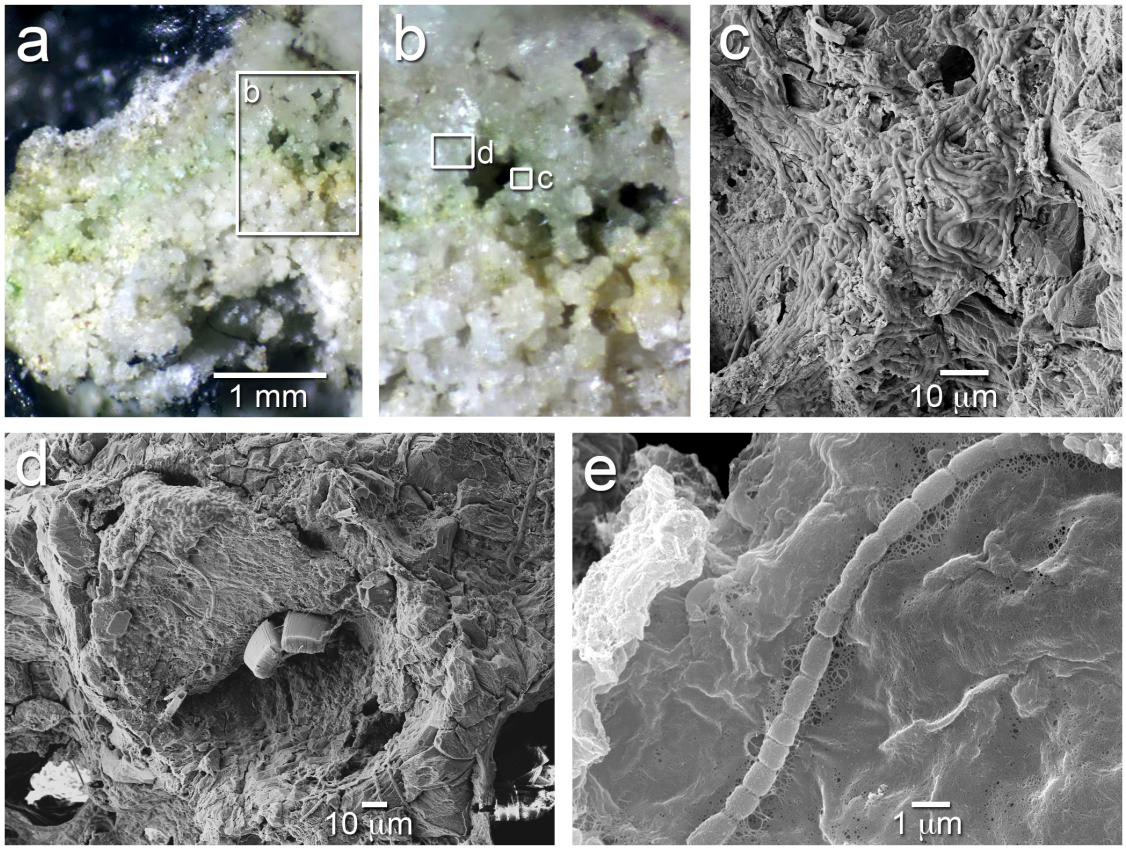


Figure 2.10: Optical and SEM images an endolithic sample (Terrace 2 rim endolith). Filamentous bacteria are visible (c,e), as are diatom frustules trapped in calcite (d). All SEM images are of an etched sample.

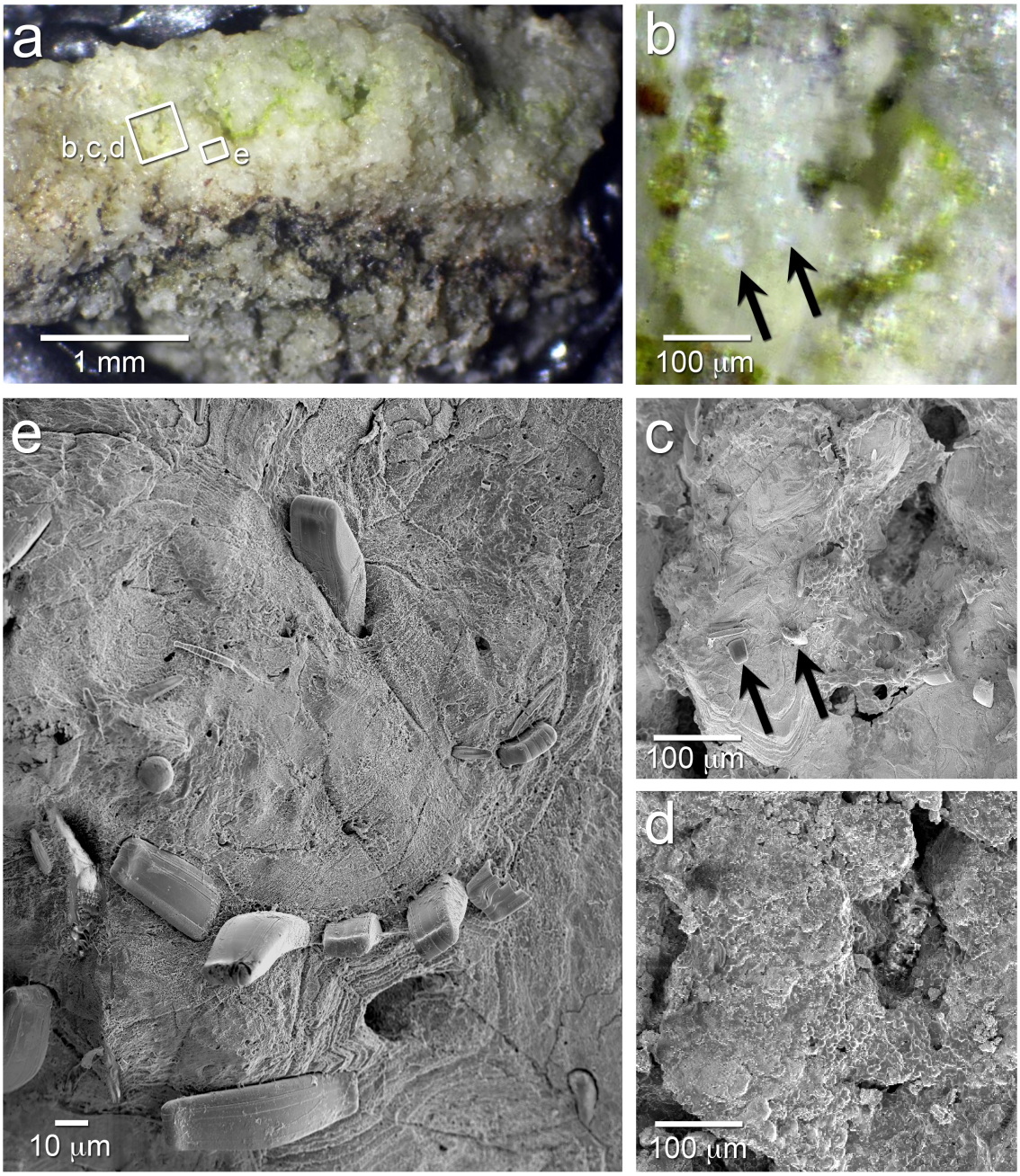


Figure 2.11: Optical and SEM images of a mature endolithic sample (Terrace 3 rim endolith). Images b, c, and d show the same area before (d) and after (b,c) etching. Images a and e are also etched. Green areas in b are seen to correspond to microorganisms in EPS. Diatom frustules revealed by etching are solidly embedded in a dense calcite matrix (e, black arrows in c and b).

The endolithic sample in Figure 2.10 shows abundant filamentous organisms, likely Cyanobacteria (Fig. 2.10c,e) that are present in green areas in

the rock. Cells within filaments are typically ~1μm long. The endolith in Figure 2.11 has less pore space and, I conclude, is therefore more mature than the endolith in Figure 2.10. Figures 2.11b, c and d all show the same area: an optical image and SEM images before and after etching. The rock material is covered with a dense film of biomaterial (Fig. 2.11d) that was mostly removed by etching to expose the surface beneath (Fig. 2.11c). Biomaterial that is not securely attached can be damaged and removed when the top calcite layers are disturbed during etching. Bacteria are prominent in these images; SEM observations of multiple samples show a general increase of bacteria relative to eukaryotic organisms as rock material becomes denser and drier.

Diatom frustules are prominent in microscopic images of wet samples at Troll. This observation is important because diatoms can function as non-biodegradable tracers of the original periphyton. As Figures 2.9-2.11 show, diatom frustules are indeed common in granular samples and some endolithic samples. Interestingly, however, they are largely absent in the lowest-porosity endoliths, which I interpret to be the most mature. A likely explanation for this is that diatom frustules, which are made of silica, can dissolve given sufficient time and exposure to modest amounts of water. Diatom frustule dissolution is well documented in a variety of settings (83, 163, 235). Dissolution rate is dependent on pH, salt concentration and temperature of surrounding media (83, 235). For example, dissolution rates double as pH increases from 6.3 to 8.1 (163), and decrease with decreasing temperature (266). It is clear from examination of SEM images that conditions in mature endoliths at Troll have been adequate to permit

dissolution. Figure 2.12 (Terrace 4 rim endolith) shows a mostly dissolved diatom frustule in a mature endolithic sample, resulting in an impression in the calcite (Fig. 2.12a) and a partially dissolved frustule leaving just a ribcage-like structure behind (Fig. 2.12b).

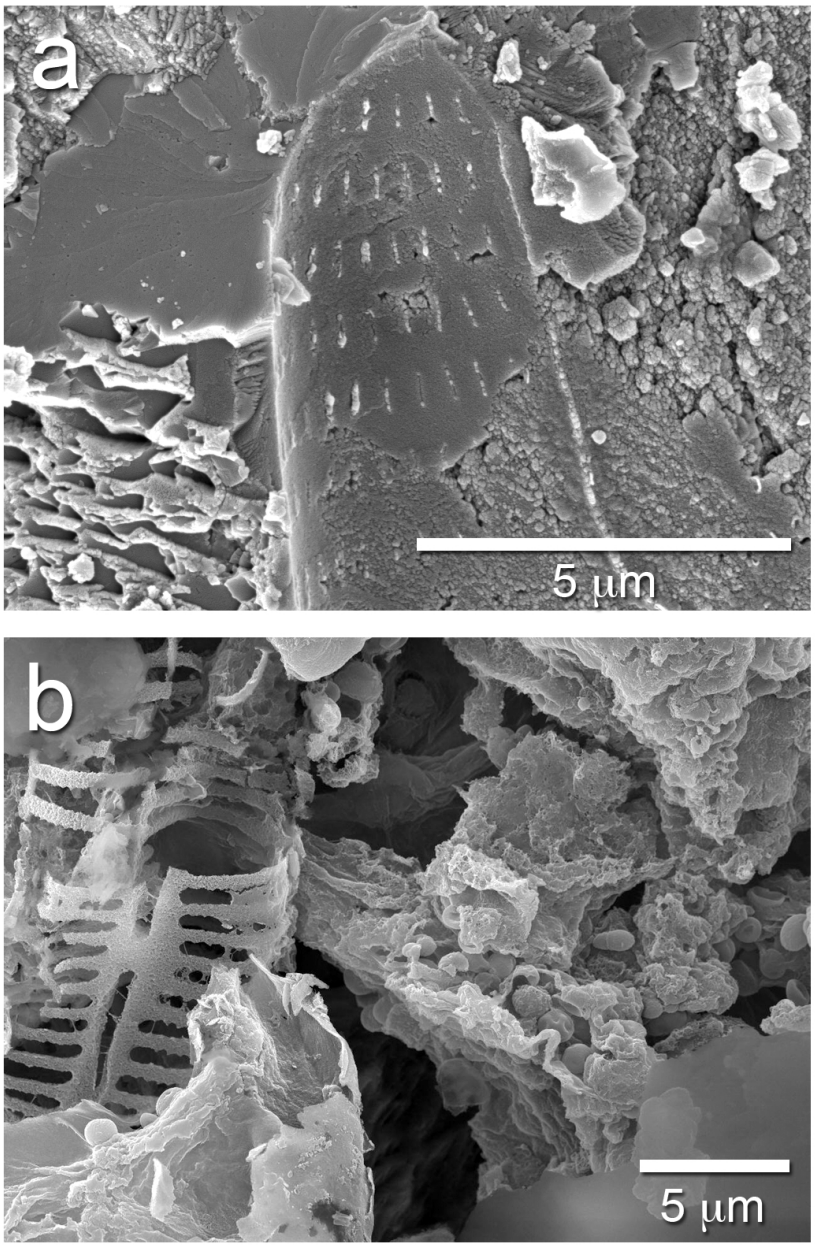


Figure 2.12: Dissolving diatoms in the terrace 4 rim endolith sample.

The right side of Figure 2.7 summarizes the key results for the terrestrial samples. The distribution of major bacterial sequences is more even than in the pool and source periphyton. Granular and endolithic samples share many major OTUs. Some taxa in the terrestrial samples are also found across most environments at Troll Springs. For example, sequences assigned to Cyanobacteria, Proteobacteria, Actinobacteria, Verrucomicrobia and Bacteroidetes are prevalent in all terrestrial samples and are also found in aquatic samples. Other taxa, such as Acidobacteria and Deinococcus-Thermus, are limited to granular and endolithic samples. Cyanobacteria are present in all samples, although sometimes at very low levels. Within phyla, clear abundance trends emerge: some taxa are mostly present in source and pool periphyton, while others, especially the genera *Leptolyngbya*, *Phormidium* and *Nostoc*, dominate in granular and endolithic samples.

Cyanobacterial groups show particularly clear abundance and diversity trends. Samples from the source as well as the driest endolith tend to be dominated by one or two Cyanobacteria groups, with greater diversity for samples with intermediate water content or in the pool environments. Granular samples are wetter than endoliths but dryer than pools, and along with the source mud sample have the highest diversity at Troll Springs (Table 2.2).

2.3.3 Chloroplast sequences as an indicator for eukaryotes

Chloroplast sequence counts, which are an indicator of eukaryotic

photosynthesis, provide an indirect indicator of photosynthetic eukaryotic presence. Comparison of chloroplast counts to bacterial counts therefore provides information regarding the balance of competition between photosynthetic bacteria and photosynthetic eukaryotes as environmental conditions change.

As noted above, SEM observations suggest that the concentration of phototrophic eukaryotes relative to bacteria is reduced in terrestrial environments. This observation is supported by chloroplast and cyanobacterial abundances. Figure 2.7 displays the distribution of all cyanobacterial counts and chloroplast counts from 454 sequencing. A similar distribution of chloroplasts is seen in the Sanger sequencing data (Fig. 2.8). Cyanobacteria increase in abundance in drier environments, while chloroplasts decrease. The drop in chloroplast abundance with calcite precipitation in the pools is particularly marked: ratios of bacteria sequences to chloroplast sequences are 0.19 for both source periphyton samples, 1.3 for pool 1, 8.7 for pool 2 and 11.58 for pool 3 (Table 2.2).

2.3.4 Aquatic and terrestrial samples are separated by OTU makeup

As a first exploratory step in analysis of sequence data analysis I used non-metric multidimensional scaling (nMDS) (40) on OTU abundance data. In nMDS, the ordination attempts to represent the differences in OTU makeup among communities on a two-dimensional plot. Figure 2.13 shows nMDS results

for bacterial sequence OTU abundance data (excluding sequences from chloroplasts), and for the same data after a presence/absence transformation. The plots show relative dissimilarities among samples projected onto a plane, such that samples with similar bacterial OTU compositions are closer to one another on the plot. Transforming the data from abundances to presence/absence influences this relationship, and, consequently, shifts the relative locations of points. These shifts give insight into the similarity of major and minor OTUs in the samples.

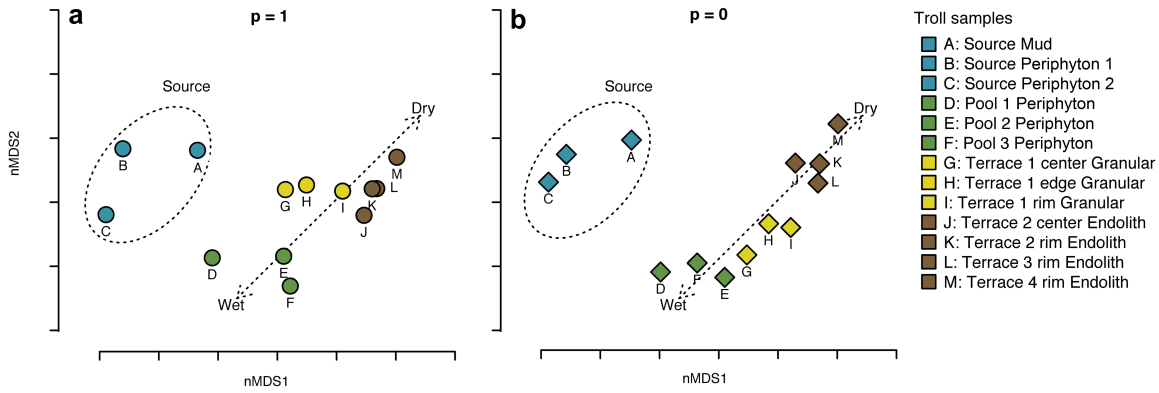


Figure 2.13: Non-metric multidimensional scaling of sequencing data. Figures 2.13a and 2.13b are for no transformation and a presence/absence transformation, respectively. Stress values are 4.98% for a and 5.38% for b. The arrow indicates the wet-dry axis, and the ellipse shows the position of the source samples off this axis.

The most striking feature of the nMDS plots is a clear and continuous transition from wet to dry samples. This “wet-dry axis” runs across each plot, extending from the Pool 1 periphyton (D) at lower left, through the pool 2 (E) and pool 3 periphyton (F) (which have some carbonate precipitates), then through the drier granular samples to the very dry endolithic communities at the upper right. While the source periphyton (B, C) and mud (A) samples lie toward the wet end

of the axis, they are distinctly to one side, suggesting that factors other than water availability affect their community makeup.

Even minor details of the plot are consistent with alignment along a wet-to-dry gradient. For example, the granular sample from the center of terrace 1 (G) is the most recently wet of the terrace 1 samples, because water stands longest in the low-lying center of a terrace as it dries out. The granular samples from the edge (H) and rim (I) of Terrace 1 are increasingly dry. The center, edge, and rim samples from terrace 1 appear in that order along the wet-to-dry gradient. Similarly, the terrace 2 rim endolith (K) lies farther along the dry direction of the axis than the sample from the center of terrace 2 (J).

OTU distributions for all samples are displayed in Figure 2.14. Panel a shows how some OTUs are specific to certain types of samples. Others are present through all samples, indicating that they persist through the transition from aquatic to terrestrial. This panel also displays a transition (blocks of shared OTUs) from periphyton to granular and from granular to endolith. This is the same pattern, a gradual transition of OTUs, seen in the nMDS plot. Note that many OTUs present in the granular samples are shared in the terrace 2 center endolith sample, but are not detected in the drier endolithic samples.

Panel b of Figure 2.14 emphasizes OTUs shared with the terrace 1 center granular sample, which has the highest diversity and water content of all the terrestrial samples. Because it was recently wet, this sample also represents a transition between pool and endolithic environments. Panel b exhibits a triangular pattern where samples plotted next to terrace 1 center granular share more

OTUs than the samples farther away, which are also more different in their environmental parameters (*e.g.*, source periphyton and driest endolithic samples). OTUs common to the periphyton are also found in the granular samples, the starting point of the endolithic community, and persist into the endolith with increasingly reduced presence.

Some OTUs are present in the periphyton and endolithic community, but absent in the center granular sample. This could be because the high diversity of that sample and reduced sequencing coverage caused some failures in detection. Nevertheless, the patterns in Figure 2.14 strongly support the overall conclusion that the periphyton acts as a precursor for the granular and endolithic communities.

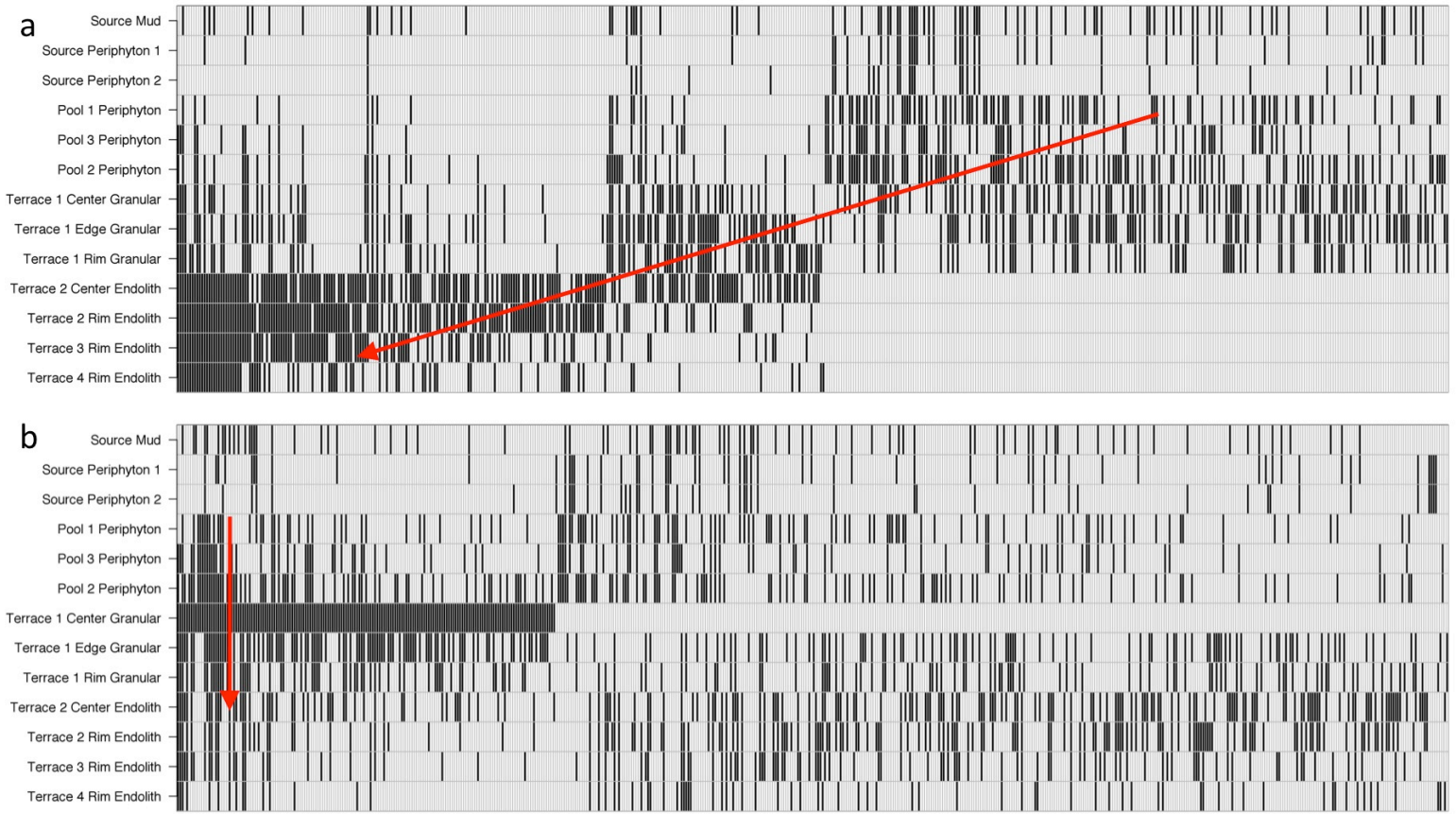


Figure 2.14: OTU distribution at 0.10 distance. OTUs are displayed as presence or absence. Only OTUs that are present in at least two samples are shown. a: OTUs have been ordered according to the number of shared OTUs in four endolithic samples (starting with four shared among the four samples, then three among the four, and so on). OTUs shared with other samples, such as periphyton and granular, are on the left side. All OTUs not shared with the endolithic samples, but shared between periphyton and granular samples, are on the right side. The terrestrial samples (rows) are ordered according to their water content. The arrow indicates the gradual transition of OTUs through all samples, from periphyton to endolith. b: OTUs have been ordered according to abundances in the terrace 1 granular center sample. All OTUs shared with that sample are located on the left. The arrow indicates the transition from the periphyton through the granular into the endolithic samples.

ARISA data show the same general trends (Fig. 2.15). The nMDS analysis of ARISA data shows higher stress and more scatter than the sequencing data, because ARISA is more susceptible to stress and scatter than sequencing due to the noise and base-pair shift variation in the peaks. However, the wet-dry axis and the location of the source samples off the axis are still clearly visible. ARISA data thus reveal the same overall picture of a transition in community structure over the wet to dry environmental gradient. The similar trends in the sequencing and ARISA analyses are important because the two methods target different regions on the bacterial ribosomal genes.

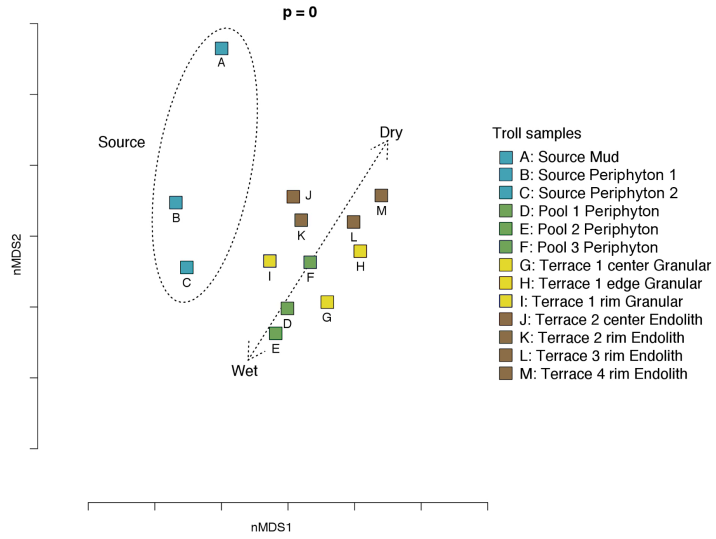


Figure 2.15: Non-metric multidimensional scaling of ARISA data. A peak height threshold of 0.455% is used, and the data are presence and absence transformed. The stress is 10.88%. The ellipse indicates the source samples and the arrow shows the wet-dry axis.

2.3.5 Aquatic and terrestrial samples are also separated by phylogeny

UniFrac distances quantify differences among microbial communities, measured by using the evolutionary differences in a phylogenetic tree (165).

Samples in close proximity to one another are more similar in their phylogenetic structure than samples farther away. I used weighted UniFrac distances, which take relative abundances into account, to gain insight into community changes related to both presence of taxa and their abundances.

PCoA can be used with UniFrac distances to represent samples as objects in ordination space. Eigenvalues measure how much variance is accounted for on each PCoA axis in descending order. The first PCoA component separates the data as much as possible, followed by the next, and so forth. Figure 2.16 displays phylogentic variations for the first three UniFrac principal coordinates for all samples. I analyzed the phylogenetic distances for bacterial sequences only, including chloroplasts (Fig. 2.16a), and also for the full data set excluding chloroplast sequences (Fig. 2.16b). In these analyses, the first three PCoA axes together represent 58.1% and 51.2% of the phylogenetic variations among the samples, respectively.

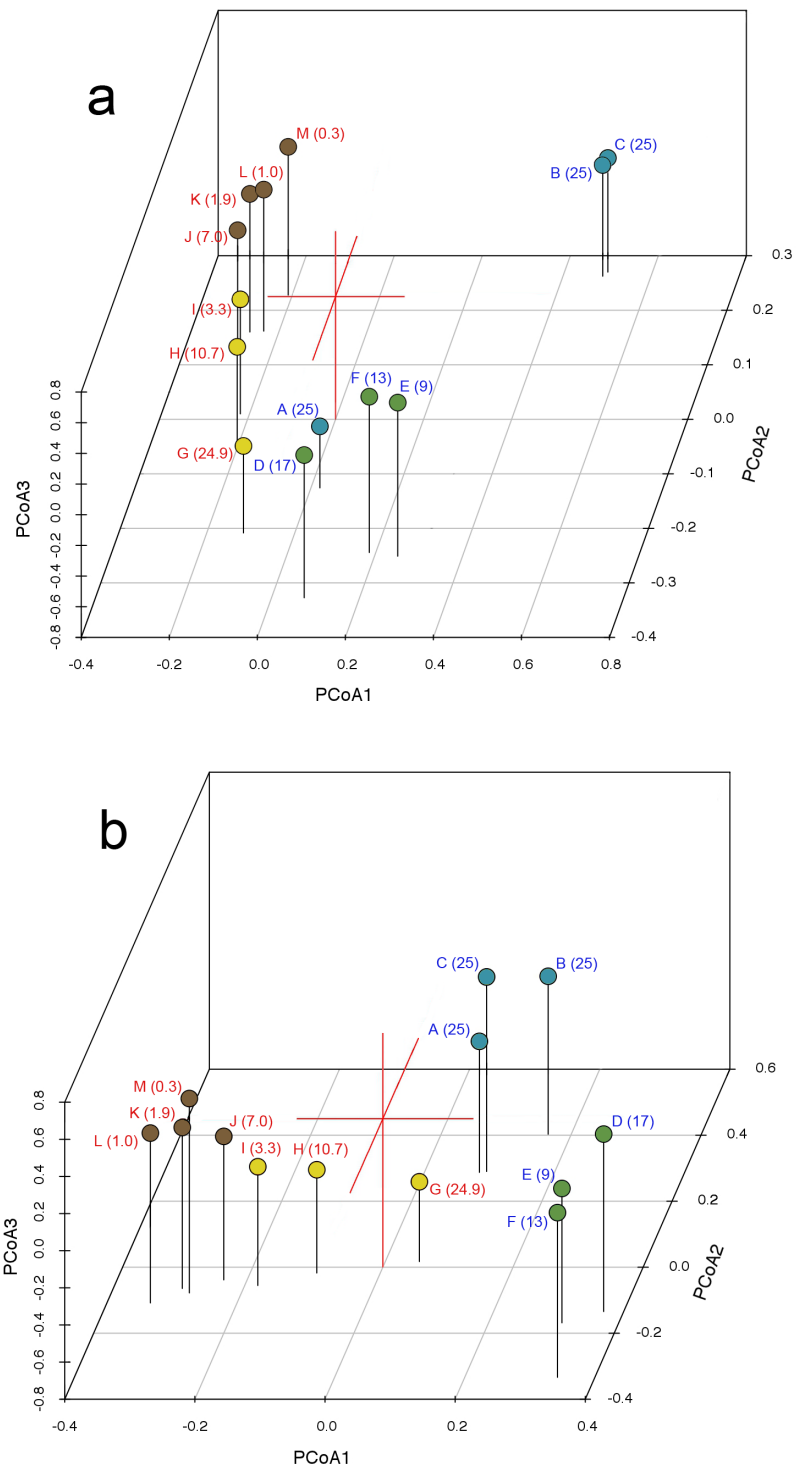


Figure 2.16: Three-dimensional plots of the first three weighted UniFrac principal coordinates for all samples, including (a) and excluding (b) chloroplast sequences. Red numbers give water content of terrestrial samples, and blue numbers give temperature at time of collection of aquatic samples.

Samples of the same type cluster together in the PCoA plots. In Figure 2.16a the aquatic and terrestrial samples are separated along PCoA1, indicating that water availability is an important factor for explaining community differences. The aquatic samples are further separated along the second PCoA axis, with pool and source mud samples plotting far from the source periphyton samples. This separation results from the strong influence of photosynthetic eukaryotes. The source mud sample is widely separated from the source periphyton samples due to the dominance of chloroplast sequences in the periphyton and their scarcity in the mud. The terrestrial samples (granular and endolithic) show a strong progression along the second and third PCoA axes that indicates a gradual change in their phylogenetic community structure. This progression appears to correlate with a decrease in water content, suggesting a relationship between water content and community formation in these samples.

Some phylogenetic relationships change when chloroplasts are excluded (Fig. 2.16b). The source mud sample groups with the other source samples, again indicating that the absence of chloroplasts in the mud is a major discriminator. The water-related transition among terrestrial samples is preserved, although it has moved to PCoA1 and PCoA3.

2.3.6 Quantitative relationship of environmental variables to community structure

To perform quantitative tests of the hypothesis that environmental variables are related to bacterial community makeup, I used distance-based

redundancy analysis (db-RDA; (160), constraining the UniFrac PCoA coordinates of groupings of samples with key environmental parameters. Statistical significance of the hypothesized relationships was evaluated via permutation tests.

The aqueous and terrestrial environments at Troll Springs are very different from one another: water is likely to have a much weaker influence on community makeup in aquatic settings, where its supply is effectively infinite, than in a terrestrial setting where its supply is limited. For this reason, the aquatic and terrestrial samples were analyzed separately. Additionally, I excluded the source mud sample from the water periphyton analysis due to the very different nature of that sample. All principal coordinates were used. Table 2.3 summarizes the db-RDA results.

Table 2.3: Relationship of microbial community structure to environmental parameters as determined by UniFrac db-RDA. All principal coordinates were used.

Samples	Environmental variable	Proportion of variance explained	p-value	Proportion of variance explained	p-value
One constraint variable, separate models		***Total Sequence Set***		***Bacterial Sequence Set***	
Terrestrial samples*	Water content	0.31	0.012	0.32	0.011
Aquatic samples**	Temperature	0.58	0.027	0.38	0.074
	pH	0.57	0.016	0.38	0.066
Several constraint variables applied sequentially, one model***					
Aquatic samples**	Temperature	0.58	0.017	0.38	0.166
	pH	0.20	0.046	0.20	0.613
	eH	0.20	0.082	0.19	0.693

* Terrestrial samples analyzed: granular (3) and endolithic (4) samples
** Aquatic samples analyzed: source periphyton (2) and pool periphyton (3) samples. The source mud sample was excluded.
*** Only first three selected parameters shown

For the terrestrial samples, water content was used to constrain the results. Water content was correlated with community makeup as represented by the UniFrac principal coordinates at a statistical significance level (probability of obtaining a test statistic as extreme as the one observed) of P=0.011. The RDA model showed that 32% of the variation in community makeup can be explained by variations in water content.

For the aquatic samples, both temperature and pH were used to constrain the results. These two parameters are very strongly correlated with one another (R^2=0.98), because of the temperature-dependent solubility of CO_2 in water and its controlling influence on carbonic acid concentration. The correlation of UniFrac-derived community makeup with pH (or, equivalently, temperature) had a statistical significance of P=0.016 (P=0.027 for temperature). The significance of the correlation weakened to P=0.066 (P=0.074 for temperature), however, when chloroplast sequences were excluded in the distance matrix, indicating that the presence of bacteria and photosynthetic eukaryotes is strongly influenced together by temperature and/or pH. The total variation in community makeup explained by pH (temperature) when chloroplasts are included is 57% (58% for temperature). Other environmental parameters measured have weaker correlations with community makeup when applied sequentially.

2.4 DISCUSSION

Variations in environmental parameters, particularly the availability of water, clearly have a major influence on community makeup at Troll Springs.

Konopka (150) has described four types of microbial ecosystems: surface-associated with saturated water, surface-associated with unsaturated water, planktonic, and macroorganism-associated. The first two are relevant here. In a surface-associated ecosystem with saturated water, hydrodynamic processes determine nutrient fluxes. Subsurface sediments, microbial mats, and periphyton are examples. Unsaturated water ecosystems, in contrast, are characterized by patchy nutrient distribution where water availability limits microbes' activity and dispersal.

My data, which were summarized in Figure 2.7, show clear variations in microbial community makeup with ecosystem type. In particular, community makeup is dramatically different between the saturated (aquatic) and unsaturated (terrestrial) ecosystems. The water-saturated periphyton samples tend to be dominated by one or two taxa that differ from one sample to the next. In contrast, the water-unsaturated granular and endolith samples have more major taxa in common.

2.4.1 Aquatic environments

Microbial community structure in aquatic environments, such as oceans, lakes and hot springs, tends to be controlled by different environmental parameters than in terrestrial environments. Whether a resource is unlimited or scarce is important; water is less likely to have a strong influence on community makeup in an aquatic setting, where its supply is effectively infinite, than in a

terrestrial setting, where its supply is limited. With water being abundant in the source and pools at Troll, other non-resource environmental parameters like temperature and pH are more likely to influence community makeup.

As discussed in detail in Chapter 1, dependence of microbial community structure on temperature and pH is common in freshwater systems. Fierer *et al.* (81) found that freshwater streams with distinct pH levels have distinct bacterial communities. Similarly, community makeup patterns are strongly correlated with pH in lakes in northern Europe, followed in importance by temperature (161). Lakes in Wisconsin also show a strong dependence of community makeup on pH (294).

As shown by the db-RDA results, pH and temperature show the strongest relationship to the makeup of aquatic communities at Troll Springs. The left side of Figure 2.7 shows a large variability in microbial taxa, with a few dominant organisms, in the aquatic samples, indicating that the periphyton communities can be highly heterogeneous from one part of the spring to the next.

Temperature and pH alone do not fully account for these variations, however. Environmental stability may also play a role. The source is not just warmer than the other sample locations, but also provides a more disturbance-free thermal environment, with a nearly constant temperature year round (135). The water pools, in contrast, are more prone to temperature fluctuations, sometimes freezing in the winter (135).

Pool temperatures and pH also fluctuate with weather conditions (Fig. 2.17). In particular, high winds and cold air temperatures can cause temperature

drops and corresponding pH increases. The sensitivity to weather conditions varies from pool to pool, and is greatest for pool 1 (which is broad and shallow) and least for the source (which is narrow and deep). Warmer pools, such as pool 1, would also tend to cool more sharply in response to cold air and winds because of their warmer initial temperatures. Pool 2, in contrast, has a cooler initial temperature, resulting in lower temperature and pH variations. The organisms in the source therefore experience small temperature changes and relatively constant pH, whereas organisms in the pools experience temperatures that can vary by several degrees from day to day, and by 10 degrees or more with season. These unsampled environmental variations may be responsible for some of the observed community variations.

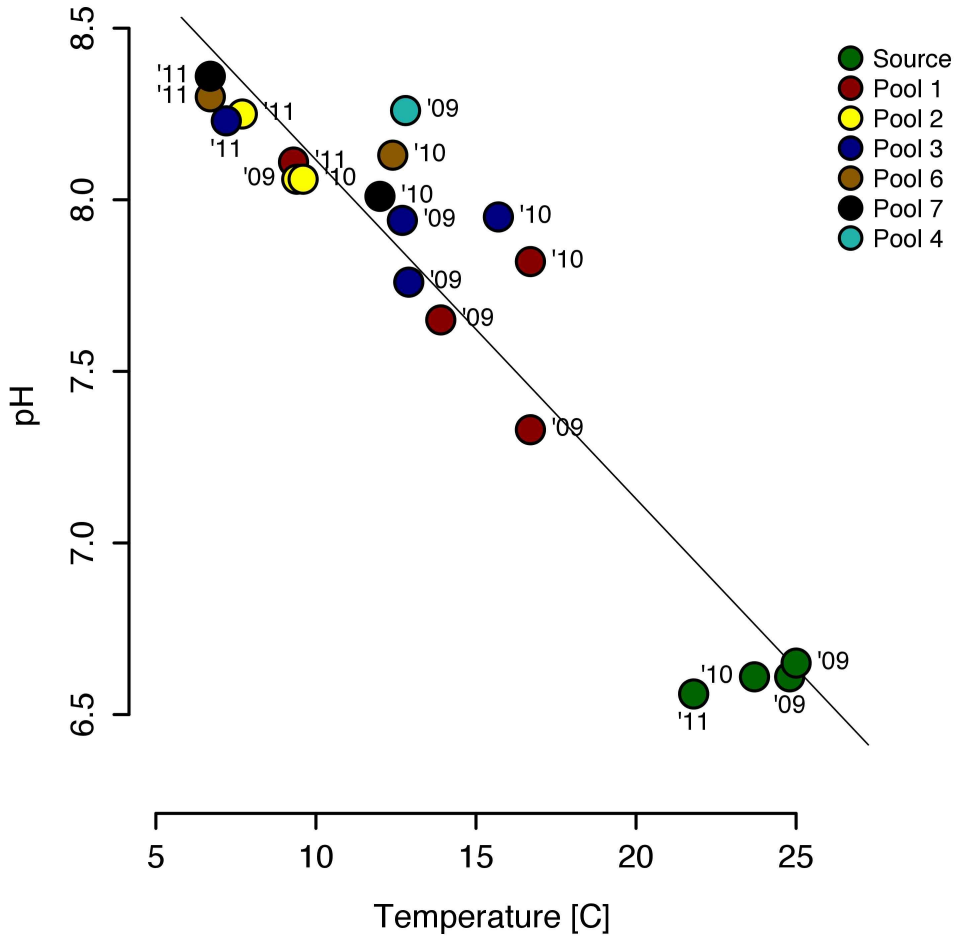


Figure 2.17: Temperatures measured for the source and several pools at Troll in August of three different years. Data are shown for several pools not otherwise included in this study (pools 4, 6 and 7). Three locations (the source, pool 1, and pool 4) were sampled on two different dates in 2009, three days apart. The colder temperature of each 2009 pair was measured under high wind conditions. A regression line is shown fit through all points; the correlation coefficient is -0.94.

2.4.2 Terrestrial environments

As Chapter 1 described in detail, in terrestrial settings where water is in short supply, its availability can become a strong selecting factor for microbial communities. For example, Zeglin *et al.* (298) studied parafluvial sediments across moisture gradients in stream–soil transition zones in a cold (Antarctica) and hot (New Mexico) desert, finding in each case that bacterial community

makeup differed between wet and dry sediments. Bachar *et al*. (6) and Angel *et al*. (4) studied a variety of soils with similar pH, finding that of all measured parameters, water content had the strongest influence on community makeup. Similarly, the db-RDA results show a strong relationship between community makeup and water content in the granular and endolithic samples at Troll. As periphyton become more firmly entrapped via calcite grain growth, the enclosed microbial community becomes integrated into the rock material. Water content decreases as lithification progresses, and this changing availability of water exerts a strong influence on community makeup.

Despite being from different stages of lithification and age, major taxa are shared among all endolithic communities at Troll (right side of Fig. 2.7). The community differences are mostly the result of changes in the relative proportions of major taxa, rather than major changes in the taxa themselves. Endolithic samples are protected from the surface environment, and this environmental stability may contribute to development of similar and stable communities across multiple samples. Walker and Pace (273) have argued that endolithic communities experience relatively static environmental conditions, leading to resistance to disturbances and low turnover rates.

2.4.3 Endolith colonization, community makeup and distribution

As noted earlier, most reported studies of endolith formation involve organisms occupying pre-existing rock. At Troll, however, the reverse is true: the

rock develops in the presence of microorganisms. Calcite precipitates interact with microbial material, particularly periphyton, entrapping it in travertine. I suggest that the entrapped periphyton become a precursor for endolithic communities, occupying space that is later colonized by other organisms that are minor OTUs in the periphyton but better adapted to the cold, dry environment of the endolith. The nMDS and UniFrac PCoA plots are consistent with this interpretation, showing a transition of microbial community makeup from the periphyton in the pools to the endolithic stage.

One of the most fundamental characteristics of an endolithic community is the source of primary production. Photosynthetic bacteria and eukaryotes compete for the same space and resources, and fulfill the same function in the ecosystem. Under ideal conditions they can coexist, but it is more typical for endolithic communities to either be lichen (algae) or Cyanobacteria dominated. Primary production in the endolithic community at Troll Springs is dominated by Cyanobacteria.

In moderate climates like the Rocky Mountains (274) and others, cyanobacterial diversity is high (120, 155, 245, 274) compared to drier environmental conditions (211, 290). Significantly, a number of studies have shown that endolithic settings with similar environmental parameters, such as nutrient availability, water availability, and UV intensity, have similar community makeup (91, 274), even if they are from different geographic locations. In terms of both Cyanobacteria diversity and the specific taxa present, the endoliths at

Troll are more similar to alpine and other Arctic endoliths than they are to hot desert or Antarctic endoliths.

The similarity of the endolithic communities at Troll Springs to other alpine and Arctic endoliths is noteworthy, because of the uncommon way in which the endoliths at Troll Springs formed. This observation indicates that it is the nature of the endolithic environment itself that primarily drives community makeup, not the mechanism by which that environment was produced.

Acidobacteria, Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes are found in the endoliths at Troll, and in other endoliths elsewhere. These same groups are also among the most important and widespread in soil communities (158). The right side of Figure 2.7 shows that these taxa are common across most of the terrestrial samples at Troll, exhibiting some changes in abundance but relatively little change in presence. They are apparently able to adapt to a range of conditions in these samples; it is the change in their relative proportions that is largely responsible for the variation of community structure displayed in the nMDS and PCoA plots.

Many prominent taxa in the endoliths are also present at low levels in aquatic samples, gaining a competitive advantage as conditions change. A counter example is Deinococcus-Thermus, which is present in the endolithic community, with the highest abundance in the driest endolith, but is not detected in any of the wet samples. Organisms like these that appear only in the endolithic community can be interpreted as ones that entered the endolith during formation and maturation, occupying physical and nutritional niches created by the death

and decay of organisms that cannot compete successfully under the drier conditions.

2.4.4 The competition between eukaryotic algae and Cyanobacteria

Eukaryotic algae and Cyanobacteria are both phototrophic organisms, providing the same ecological function and competing for the same resources. The balance of this competition changes across the spring system, with a shift from eukaryotic algae in the wet, high-illumination pool environments to Cyanobacteria in the drier, lower-illumination endolithic environment.

One of the most important resources for both eukaryotic algae and Cyanobacteria is access to light. Barranguet *et al*. (10) showed that mature biofilms on filtration dunes have significantly reduced microalgae abundance under low light conditions. Cyanobacteria are a common organism in many endolithic ecosystems (89, 194, 274), in part because of their ability to photosynthesize under low light conditions. The endolithic environment at Troll Springs has reduced irradiance, allowing Cyanobacteria to outcompete eukaryotic algae there.

Another factor in the competition between eukaryotic algae and Cyanobacteria may be fluctuating water levels. When terraces are filled with water to create pools, nutrient and light availability are sufficient for the organisms there. As water availability decreases, however, both dissolved nutrients and access to light decrease owing to increased calcite precipitation,

potentially affecting community makeup. Benthic microbial mats in the Florida Everglades that undergo frequent desiccation are dominated by Cyanobacteria, whereas diatoms and green algae are common at sites with standing water (21). Their greater resistance to the effects of desiccation will also tend to favor Cyanobacteria in the drier environments at Troll Springs.

The source mud sample is a special case. It was collected 0.5 m below the water surface, and has a much lower abundance of algae relative to Cyanobacteria than the other source samples. While there is no calcite precipitation at the source, it is likely that illumination levels in the mud are reduced both by the depth of the water and opacity of the mud itself. Given the dominance of eukaryotic algae in the source periphyton and optimal temperatures and pH for algae growth in the source, it is likely that lower illumination levels in the mud are responsible for the poorer competitive performance of algae there.

2.4.5 Diatoms

Diatoms (a type of photosynthetic algae) are abundant in freshwater and marine ecosystems and are observed in nearly all environments at Troll, ranging from wet pools to dry endoliths. In general, diatoms are able to survive a wide range of irradiance (210), water availability and water quality conditions (219), consistent with my observations. Diatoms show a particularly high presence in the calcifying periphyton and in the wettest granular samples. Light-limited

biofilms in other settings are largely composed of diatoms due to their better photosynthetic efficiency relative to green algae (10).

The diatom cell walls (frustules) are preserved in granular and endolithic samples. Diatoms cannot penetrate into an established rock matrix, so the presence of diatom frustules within endolithic samples clearly shows that some material from the periphyton in the pools becomes trapped by calcite precipitation. Diatom chloroplast sequences were not found in the rock, indicating that the entrapped frustules were non-living remnants. As noted above, the absence of diatom frustules in the most mature endolithic communities can be readily attributed to silica dissolution over time.

2.4.6 Extracellular polymeric substances

Extracellular polymeric substances (EPS) are observed across a range of environments at Troll, and their abundance and appearance vary with environment. An enormous number of microbial organisms are capable of producing EPS, which can vary greatly in composition and chemical and physical properties. EPS are mainly composed of polysaccharides but may also contain a variety of proteins and lipids depending on the organisms and environment involved (61). Generally speaking, EPS become more prevalent under drier conditions and are known to render Cyanobacteria more desiccation tolerant (257, 281). Consistent with this function, networks and sheets of polysaccharides are extensive in the Cyanobacteria-dominated terrestrial samples at Troll, with

relatively few microbial cells within it, whereas EPS only appear as a thin layer surrounding the microorganisms in the aquatic environments. Green algae also produce EPS in form of gels, mucilage, and slimes that encapsulate cells or are secreted for motility (63). The EPS of Cyanobacteria can range from loosely bound polymers, such as capsules and slimes, to tightly bound sheaths (14). Diatoms as well produce EPS in the form of sheaths, tubes, stalks, pads, and mucilage for attachment and motility, or during nutrient-starved conditions (61).

Microscopic images suggest that EPS also plays a role in the early stages of endolith development (Fig. 2.9), binding together and providing weak cohesion for granular materials. Similar observations have been made elsewhere. Studies in sediments have shown that EPS formation can result in significant sediment stabilization (13, 267). In endoliths, Hoppert *et al*. (118) have argued that forming a tight endolithic community with an EPS matrix can stabilize the structure, preventing rapid decomposition of the substratum.

2.4.7 Similarities and contrasts to stromatolites

Endolith colonization at Troll has some interesting similarities to formation of stromatolites. The sediments in many stromatolites are detrital, but there are also instances where stromatolite sediments are locally precipitated carbonates (11, 67, 85).

The entrapment of periphyton by calcite precipitation at Troll is broadly similar to stromatolite formation. However, there are also important differences.

In typical stromatolites, microorganisms repeatedly re-colonize the growing sedimentary platform, forming layer upon layer in a repetitive process that can build large domical or laminated structures. This process requires environmental stability, and abundant water, nutrients, and sediment.

At Troll, water, nutrients, and sediment are all in short supply. Calcite precipitation onto periphyton is similar to the initial stages of stromatolite formation, yielding microbial communities that are mixed and layered with calcite. However, dwindling resources rapidly limit both further calcite precipitation and further microbial growth. The water-filled terraces at Troll are small bodies of water subject to significant water fluctuations, including complete drying. Such conditions are not conducive to either the continued growth of microbial mats or the continued sedimentation required for stromatolite development. Instead, the typical result is an endolithic community consisting of a single thin zone of microbe-rich material under at most a few mm of calcite. Even when terraces are reactivated after prolonged dry periods, the result is simply layered calcite, with gaps between the layers corresponding in perhaps some instances to former zones of biological colonization (143). The cold, dry, and nutrient-poor conditions at Troll therefore lead to an unusual form of microbe-sediment interaction, producing mature and hardy endolithic communities, rather than stromatolites.

2.5 SUMMARY AND CONCLUSIONS

By combining microscopy with molecular methods, I have been able to show both how the endolithic community at Troll Springs develops, and how microbial community structure changes with environmental parameters across the spring system. The periphyton in the water and the endolith in the rock have many taxa in common, but abundances vary dramatically among the communities. As water becomes more scarce and as calcite precipitation progresses, periphyton become entrapped in calcite, becoming a precursor for an endolithic community. Much of the pore space originally occupied by periphyton becomes occupied either by organisms that were already present in minor quantities in the periphyton, or by new organisms that colonized an environment for which they became well suited. Environmental parameters exert differing influences on the aquatic and terrestrial environments at Troll Springs. Water is abundant in the aquatic settings, and so is not a limiting factor. Instead the combined influences of temperature, pH, illumination as limited by calcite precipitation, and annual thermal stability appear to be most important. In contrast, in the granular and endolithic samples, the scarcity of water exerts the strongest selective pressure.

CHAPTER 3: MICROBIAL COMMUNITY STRUCTURE AND SUCCESSION AT TROLL SPRINGS

3.1 INTRODUCTION

In Chapter 2, I showed how community makeup – that is, the particular grouping of taxa in each sample – varies with environmental parameters at Troll Springs. In this chapter I look at the larger issue of variations in microbial community structure at Troll, and tie those variations back to the ecological principles I enumerated in Chapter 1.

3.1.1 Evenness and richness

As described in Chapter 1, the richness and evenness of taxa can be used as fundamental descriptors of microbial community structure. Richness and evenness are often combined into the single parameter called diversity, which is widely used to describe the structure and dynamics of communities in ecosystems.

The relationship between richness and evenness has been discussed extensively in the ecological literature. It has been argued that richness regulates evenness (115), implying that richness, evenness and diversity should be strongly and positively correlated. Others argue that richness and immigration rate (part of the neutral diversity theory) predict evenness distribution (122) and that an absence of interaction (*e.g.*, competition) among taxa should lead to

positively correlated evenness and richness (34).

Observations do not always conform to models, however, and in practice a variety of relationships between richness and evenness have been observed. The relationship of evenness to richness is governed by inter-species interaction, migration and dispersal as well as changes in environmental parameters. Migration can influence richness where new species are added to the community, whereas competition can affect evenness due to changes in the relative abundances of dominant species. For example, Stirling and Wilsey (251) found a positive correlation of richness and evenness in vertebrate communities, but negative correlations in plant communities. For plant communities they also found that evenness accounts for more variation in diversity than richness. Ma (171) studied a grassland site in which there was no consistent relationship between these two variables, and Wilsey *et al*. (287) only found weak negative correlations between richness and evenness. Ma (171) also investigated the impact of environmental conditions, showing that richness and evenness can each be negatively correlated to different edaphic parameters. In a study of several taxa, Bock *et al*. (17) found that species richness and evenness were uncorrelated or weakly negatively correlated for each taxonomic group. They suggested that large and/or dominant species influence causal relationships among abundance, richness, and evenness in the species assemblages of which they are a part.

In fact, there are a number of scenarios for how evenness and richness can be related:

- Migration and dispersal: Addition of rare species leads to an increase in richness and a reduction in evenness.
- Local extinction of rare species: This leads to a decrease in richness and an increase in evenness of remaining species.
- Inter-taxa interaction (*e.g.*, competition): Large changes in the relative abundances of dominant taxa lead to lower evenness and no changes in richness (if there is no local extinction); a weak relationship between evenness and richness results.
- Competition with extinction: Highly abundant taxa outcompete rare taxa, which become extinct, leading to a decrease in richness and evenness.
- Niche differentiation: Addition of niches can promote the coexistence of taxa, avoiding competition and leading to an increase in richness and evenness.

All these scenarios depend on ecosystem circumstances and the organisms present. So despite the predictions of models, both observations and consideration of the full range of possibilities show that the relationship between richness and evenness is likely to be environment- and organism-specific.

3.1.2 Ecological succession

Chapter 1 also introduced the principle of ecological succession, in which changes in community makeup occur over time in response to disturbances in the environment. I will argue below that the community variations observed at Troll are a consequence of succession, driven by changing environmental conditions, and by shifts in the competitive balance among organisms that use differing strategies to deal with the stresses that threaten their survival.

Grime (103) and Tilman (260), have studied the effects of competition on succession. Grime suggests that stress is a property of a habitat, that competition is highest in productive (*i.e.*, low-stress) areas, and that stress tolerance becomes more important in less productive (*i.e.*, high-stress) areas. Grime's model predicts how species abundances change via succession in conditions of differing resource availability and productivity.

Tilman (262) argues that low resource availability may be stressful for some species but optimal for others. Tilman's "resource competition" model defines competition as the ability to deplete resources to a level that is not sufficient for other species to persist. This model predicts rapid succession with minimal species change in productive areas, and slow succession with greater species change in less productive areas. In contrast to Grime's model, competition in Tilman's model is important at all levels of productivity, with the outcome of the competition depending on which resource is most limiting (*e.g.*, light in productive environments and nutrient availability in unproductive environments).

Another topic that is pertinent to ecological succession is r/K selection theory (172). In this theory, species called r-strategists are those, typified by high reproduction and growth rates, that do well in unstable environments. K-strategist species, in contrast, have lower reproduction and growth rates, and thrive in more stable environments. Macro-organisms that are r-selected typically have short life spans, are generally small, mature quickly and use energy inefficiently. K-strategists are larger in size, have longer life expectancies and are energy efficient. K-strategists produce a few progeny each with a high probability of survival, while for r-strategists the reverse is true. In ecological succession following a disturbance, r-strategists dominate among the pioneering species, and then tend to be replaced over time by K-strategists. While r/K selection theory was originally developed for macro-organisms, r-selected and K-selected traits involving reproduction rate, growth rate, resource use and response to environmental stability can also be applied to microorganisms (71, 153, 167, 236).

Disturbances to an environment are important in ecology, but the term “disturbance” is used inconsistently in the ecological literature. Therefore, it is important to define “disturbance” and its consequences as I will discuss them in this chapter. Disturbances can have a range of outcomes, depending their frequency and intensity. For example, in plant ecology, disturbance is often described as a major event that leads to removal of biomass and mortality (248), and consequently to secondary succession. However a small disturbance can take place without major associated damage or mortality, and small, frequent

disturbances can also drive succession if they are cumulative – that is, if their effects accumulate with time.

Disturbances of different intensities affect community structure and succession in different ways. Major disturbances lead to widespread mortality, instantaneously reducing the diversity of a community, altering the successional trajectory and initiating secondary succession. Small, cumulative disturbances change resource availability, and alter diversity more slowly by affecting growth, reproduction and competition, leading to successional transitions. A similar phenomenon has been described in the intermediate disturbance hypothesis (45). Small, cumulative disturbances also create selective pressures that promote survival of adapted species and elimination of sensitive ones, again leading to progressive successional changes.

Environmental gradients and succession are related, and the histories of ecology along gradients and succession theory are intertwined. For example, Cowles (49), Clements (42) and Gleason (98) presumed an association of temporal changes in plant communities based on observed spatial patterns of community structure along gradients. Pickett (208) proposed that sorting of species along changing environmental gradients is the driving mechanisms for succession. Communities at different locations along allogenic gradients will not converge to the same composition unless environmental conditions become uniform over time and the physical gradient disappears (128).

Figure 3.1 shows a simplified concept of community changes along a succession, an environmental gradient and a combination of both. A succession

(Fig. 3.1a) leads to a sequence of community variations through time (*e.g.*, development of a forest) at a given location. An environmental gradient (Fig. 3.1b) leads to community variations through space (*e.g.*, along a salinity gradient) at a given time. Either can happen independently, but both can also happen together (Fig. 3.1c) as is the case at Troll Springs. At Troll, a pool-filled terrace is built by calcite precipitation, entrapping the periphyton present in the pool, which progresses into endolithic communities as the water disappears. This process is a succession at that terrace. At any given time, Troll has many terraces at different stages in this succession, leading to environmental gradients such as changes in pH and temperature, evaporation of water, reduction of light during precipitation and so forth. The changes over time at a given terrace are all represented along the observed environmental gradients (Fig. 3.1c), so the full ecological succession from pool to endolith is revealed at the spring system today.

My data permit limited investigation of temporal changes. While most samples were collected in 2009, the endolithic samples were collected in different years (terraces 1 and 2 in 2009, terraces 3 and 4 in 2008), potentially revealing some year-to-year variations. For all samples collected in all years I performed bulk analyses of microbial community makeup over large sample areas, homogenizing materials to minimize within-sample variability (see section 2.2.4).

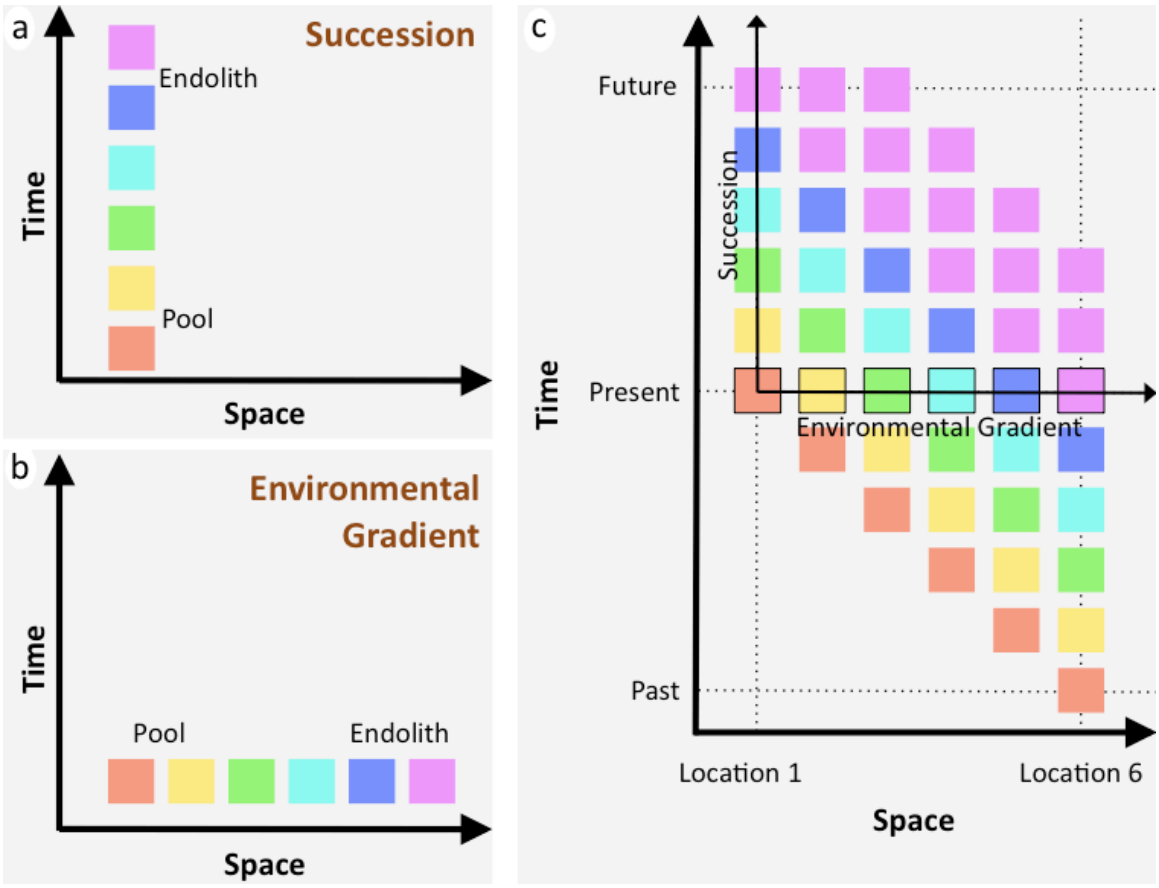


Figure 3.1: Simplified representation of ecological succession through time (a) and environmental gradients through space (b), and the combination of both at Troll Springs (c). Each color represents a different community. An environmental gradient can be observed at any given time (b), whereas succession can only be observed over time (a). Recognition of succession along environmental gradients at Troll makes it possible to hypothesize about past and future communities (c).

All samples collected along the gradient represent the state of the spring today. However, because the transitions observed today represent a progression in terrace development, they also represent a succession through time. Figure 3.1c shows a schematic depiction of the spring system's possible development over time. In this particular depiction, the spring source has moved gradually uphill, from the place labeled "Location 6" to "Location 1", with pools and terraces developing and evolving below it. Endoliths form as terraces dry up, and

ultimately are removed by erosion. This depiction is not unique, of course, and a range of histories is possible. The point is that observations in community transitions along today's environmental gradient also reveal the character of past and future environmental successions.

Figure 3.2 shows a simplified depiction of the samples collected at Troll. In this figure, the vertical axis represents the current age of the materials in which the samples were collected. The biological samples collected at Troll are associated with geological materials of different ages, and therefore represent a sequence of events. The molecular data reveal transitions in community makeup along those environmental gradients today. Relative ages are determined by microscopic observations, such as grain growth through time in the pools and lithification of the granular samples to mature endolith. The figure shows that different communities reside in materials of different ages. The associated communities (colors) along the diagonal change along an environmental gradient, and also mimic an ecological succession over time.

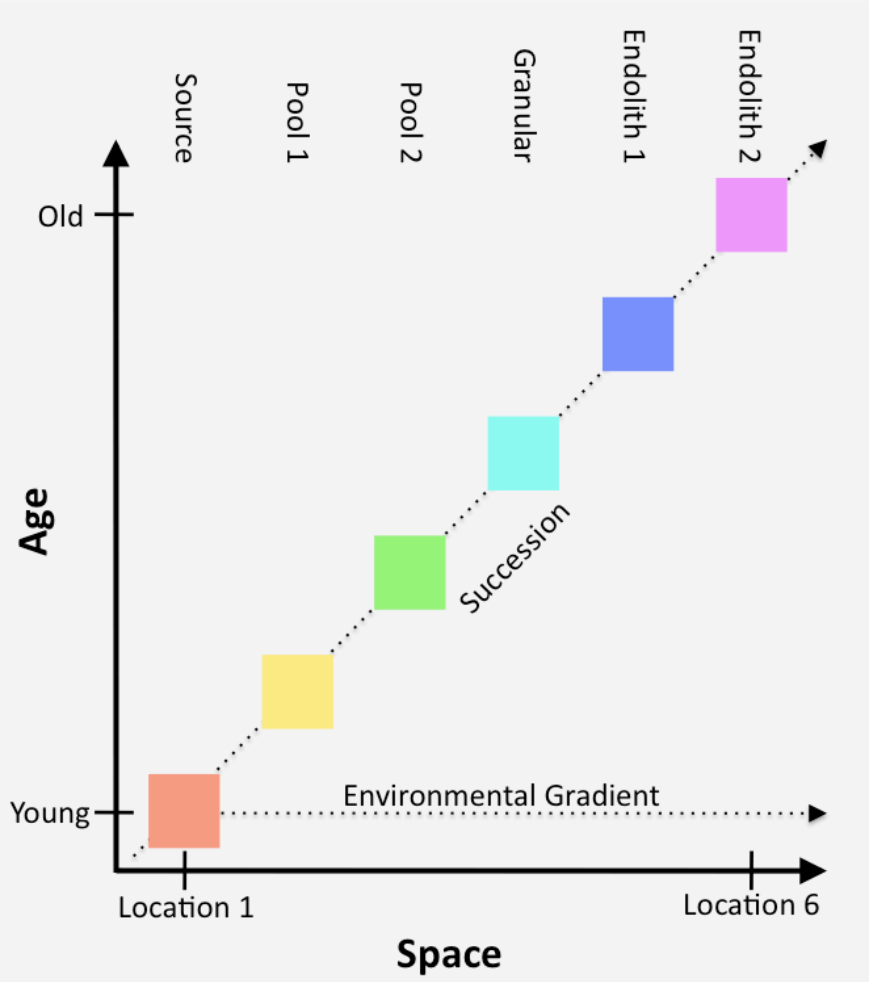


Figure 3.2: Schematic depiction of succession along an environmental gradient at Troll. In contrast to Figure 3.1, the vertical axis here represents the age of the geologic material in which the community is found. Each color represents a different community. Different communities reside in materials of different ages, so species composition changes in both time and space.

Environmental gradients and succession can be related in complex ways. As discussed in Chapter 1, it is possible to have gradients in both resource and non-resource parameters. In the case of a gradient in a resource parameter, a critical resource can be depleted along some portions of the gradient. Such a depletion is a form of small, continuous disturbance that can drive succession there. The gradients at Troll are not static, but undergo such small, continuous and often cumulative disturbances. The communities along these environmental

gradients undergo change as a consequence of these disturbances and experience succession as a result.

3.2 MATHEMATICAL DEFINITIONS

The sections that follow will make extensive use of the terms richness, diversity, and evenness. Richness *R* for a given sample is defined here to be the number of OTUs present in the sample, calculated at the 90% similarity level.

Diversity is parameterized using the Shannon Index *H'* (also known as the Shannon-Wiener Index or Shannon-Weaver Index):

$$H' = -\sum_{i=1}^{R} p_i \ln p_i$$

where p_i is the proportion of individuals belonging to the *i*th OTU. Diversity (*i.e.*, Shannon index) is an often-used index to measure the structure and dynamics of communities in ecosystems (122, 172, 174), and it encompasses both evenness and richness.

Evenness is parameterized using the Pielou Index *J'* (209):

$$J = H'/\ln(R)$$

where *R* again is species richness.

The Pielou Index ranges from 0 (highly dominant taxa) to 1 (even distribution resulting in equally abundant taxa) (246).

3.3 RESULTS

3.3.1 Variations in community structure: Richness, evenness, and diversity

As described in Chapter 2, Troll includes both aquatic and terrestrial environments, with very different underlying parameters that shape the microbial communities in each. Water content has a major impact on the community makeup in the terrestrial environments, whereas temperature/pH has the strongest influence in the aquatic environments.

Figure 3.3 shows community richness, evenness, and diversity as a function of temperature for the aquatic samples, and as a function of water content for the terrestrial samples. These analyses are for the bacterial data set only. I also included the source mud samples in the analysis of the terrestrial samples due to its sedimentary nature.

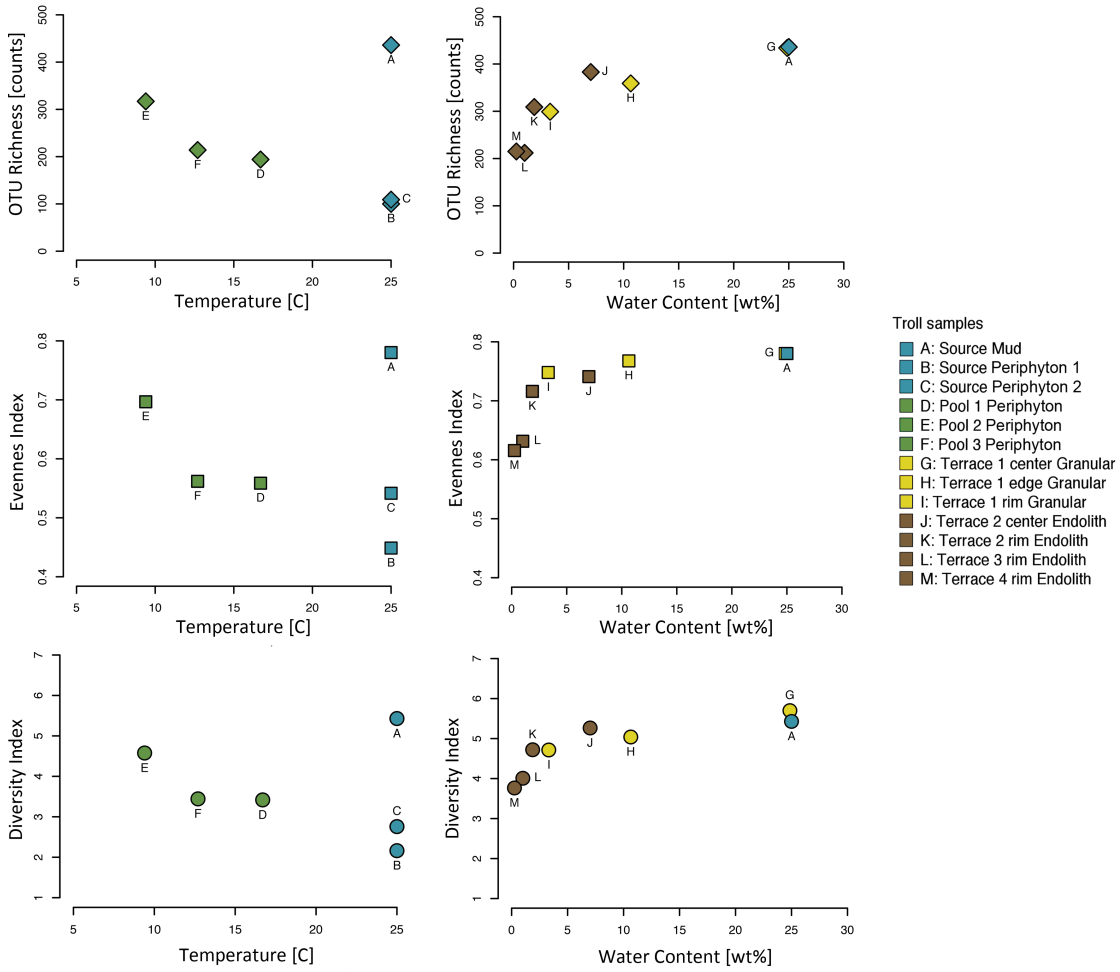


Figure 3.3: Plots of richness, evenness, and diversity of bacterial sequences *vs*. temperature for aquatic samples, and *vs*. water content for the terrestrial samples. All calculations were performed on a randomly selected subsample of 1000 sequences from each sample.

The left side of Figure 3.3 displays the relationship of richness (top) and evenness (middle) to temperature for the aquatic samples. For all aquatic samples but the mud, both richness and evenness decrease sharply (r = -0.97 and -0.83, respectively) with increasing temperature. Due to the strong anticorrelation of temperature and pH, richness and evenness show a similar relationship to pH, although with opposite sign (r = 0.91 and 0.75, respectively).

Interestingly, the source periphyton 2 (C) sample and the pools 1 (D) and 3 periphyton (F) samples are similar in evenness, but are somewhat less rich and

sharply more uneven than pool 2 (E). These very uneven periphyton samples are mostly dominated by one or two taxa, whereas the pool 2 periphyton sample has more equally distributed taxa (Figure 2.7). Pool 2 is also distinguished by its particularly high calcite precipitation rates relative to the other pools (Figure 2.3).

The source mud sample is a sediment sample, and exhibits very different richness and evenness than the periphyton samples.

For the terrestrial samples, richness and evenness increase with increasing water content ($r = 0.74$ and 0.59, respectively). Interestingly, the source mud sample has a richness and evenness similar to the wettest terrestrial sample, terrace 1 granular center (G). In terms of richness and evenness, then, the mud sample, which was submerged in water at the bottom of the source pool, exhibits properties more like those of a terrestrial sample than an aquatic one.

At the other end of the water content scale, mature endoliths (L, M) show the lowest evenness and richness of all terrestrial samples. Apparently, low water eliminates taxa in a fashion that leads to a more uneven distribution of abundances.

Table 3.1 summarizes richness, evenness and diversity for both the complete data set and the bacterial data set discussed above. The addition of chloroplast sequences decreases the evenness for the source periphyton samples significantly, consistent with the high abundance of eukaryotes there. However, although inclusion of chloroplast sequences reduces evenness, it does not have a major influence on the correlations of richness and evenness for the

aquatic samples with temperature (r = -0.98 and -0.99, respectively) and the terrestrial samples with water content (r = 0.72 and 0.60, respectively).

Community diversity is plotted vs. temperature and water content in the bottom row of Figure 3.3, and shows patterns similar to those seen for richness and evenness.

Table 3.1: Richness, evenness and diversity measures for the complete sequence set and a subset of 1000 randomly selected sequences (total abundance) from the complete data set and the bacterial data set in each sample. Diversity = Shannon Index (H'), Evenness = Pielou Index (E), Richness = OTU count at the 10% distance level (R).

		All Sequence Data						**Bacterial Sequence Data**					
		Complete data set*			Subset 1000 seq.**			Complete data set***			Subset 1000 seq.****		
Samples		**R**	**E**	**H'**	**R**	**E**	**H'**	**R**	**E**	**H'**	**R**	**E**	**H'**
Source mud	A	1211	0.84	6.00	427	0.77	5.47	1169	0.85	5.99	443	0.78	5.53
Source periphyton 1	B	211	0.26	1.39	52	0.25	1.33	150	0.46	2.30	109	0.46	2.28
Source periphyton 2	C	231	0.30	1.63	60	0.28	1.52	152	0.57	2.85	111	0.57	2.88
Pool 1 periphyton	D	478	0.56	3.46	156	0.50	3.09	411	0.60	3.62	180	0.54	3.23
Pool 2 periphyton	E	862	0.74	4.97	285	0.68	4.59	798	0.73	4.90	292	0.68	4.54
Pool 3 periphyton	F	475	0.61	3.76	221	0.57	3.55	431	0.59	3.57	207	0.55	3.31
Terrace1 center granular	G	1165	0.87	6.15	438	0.79	5.57	1157	0.87	6.12	467	0.80	5.63
Terrace 1 edge granular	H	862	0.82	5.57	373	0.76	5.13	841	0.82	5.54	356	0.76	5.10
Terrace 1 rim granular	I	655	0.78	5.05	282	0.73	4.72	635	0.78	5.01	276	0.72	4.62
Terrace 2 center endolith	J	1082	0.82	5.73	389	0.75	5.25	1063	0.82	5.69	391	0.75	5.21
Terrace 2 rim endolith	K	868	0.78	5.30	334	0.73	4.94	821	0.78	5.23	331	0.73	4.91
Terrace 3 rim endolith	L	432	0.69	4.30	221	0.66	402	416	0.69	4.13	230	0.65	3.94
Terrace 4 rim endolith	M	498	0.65	4.05	201	0.61	3.76	492	0.65	4.04	212	0.60	3.72

*Correlation coefficient: $r_{richness}$= -0.93, $r_{evennes}$= -0.99, $r_{diversity}$= -0.99 to temperature for the aquatic samples (excluding mud), $r_{richness}$= 0.72, $r_{evenness}$= 0.60, $r_{diversity}$= 0.64 to water content for terrestrial samples
**Correlation coefficient: $r_{richness}$= -0.98, $r_{evennes}$= -0.99, $r_{diversity}$= -0.99 to temperature for the aquatic samples (excluding mud), $r_{richness}$= 0.72, $r_{evenness}$= 0.60, $r_{diversity}$= 0.65 to water content for terrestrial samples
***Correlation coefficient: $r_{richness}$= -0.94, $r_{evennes}$= -0.84, $r_{diversity}$= -0.92 to temperature for the aquatic samples (excluding mud), $r_{richness}$= 0.71, $r_{evenness}$= 0.62, $r_{diversity}$= 0.65 to water content for terrestrial samples
****Correlation coefficient: $r_{richness}$= -0.97, $r_{evennes}$= -0.83, $r_{diversity}$= -0.93 to temperature for the aquatic samples (excluding mud), $r_{richness}$= 0.74, $r_{evenness}$= 0.59, $r_{diversity}$= 0.64 to water content for terrestrial samples

One of the most striking characteristics of Figure 3.3 is the similarity of the relationships of richness and evenness to environmental parameters. The implication of this is that richness and evenness are strongly correlated at Troll. Figure 3.4 displays the relationship between richness and evenness for all samples. The correlation is positive and strong ($r = 0.92$), albeit with some scatter.

Some of this scatter may be biologically significant. For example, samples B and C, which are periphyton samples collected from the surface and the bottom of the source, respectively, have similar richness but the surface sample is substantially less even.

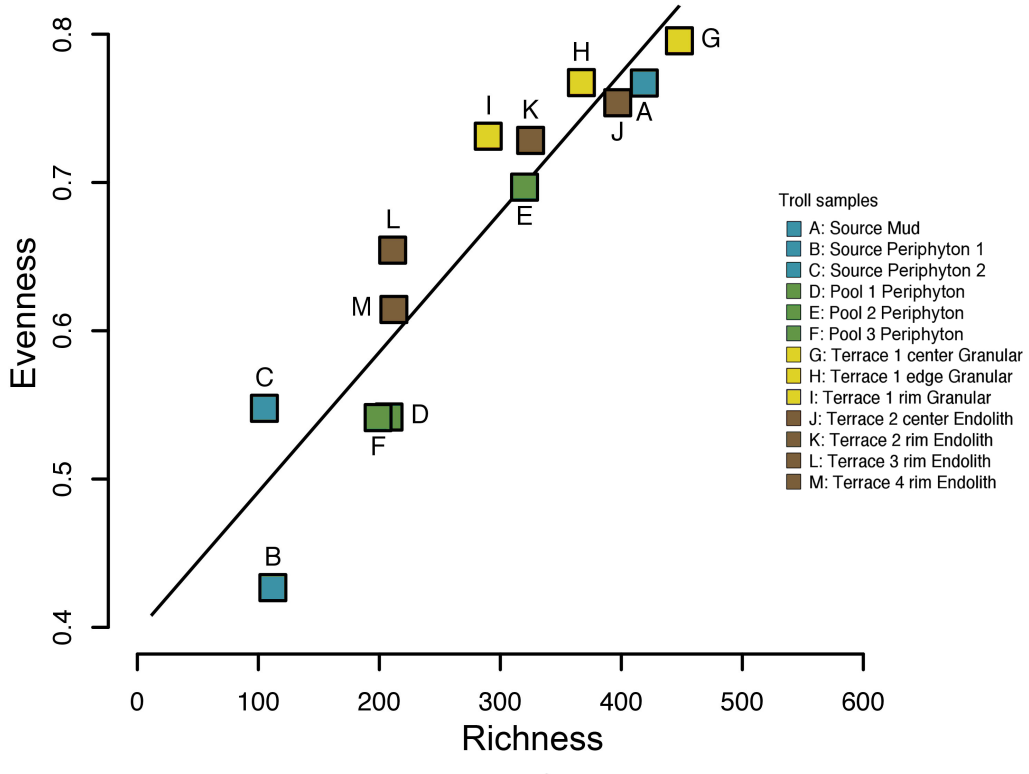


Figure 3.4: Correlation of richness and evenness for all samples. Bacterial sequences only. All calculations have been performed on a randomly selected subsample of 1000 sequences in each sample. The correlation coefficient is 0.92.

The correlation of evenness with richness at Troll is important. Such a correlation is not a general characteristic of ecosystems; as noted above, in some settings the two parameters are unrelated (17) or anticorrelated (251). If evenness increases with increasing richness, as it does at Troll, this typically means that as new taxa are recruited, their numbers increase more sharply than those of taxa already present.

3.3.2 Variations in community structure: OTU makeup

Richness and (especially) evenness are fundamentally related to the amounts and relative proportions of major and minor OTUs in a sample. To explore this relationship in more detail, I use nonmetric multidimensional scaling (nMDS) combined with a data transformation that allows for a variable emphasis of the influence of major *vs*. minor OTUs.

As described in Chapter 2, nonmetric multidimensional scaling reduces many-dimensional relationships to a few (typically two) dimensions, making it useful for visualizing complex relationships among objects. Prior to nMDS analysis, I applied a transformation to the data, assigning weighting factors to the abundances of OTUs. Typical measures of sample dissimilarity can be dominated by the most abundant OTUs, and may not reflect the relationships among samples well (41, 76). Transformations down-weight the influence of abundant OTUs.

In order to fully explore the effects of transformation on the data, I used a power-law transformation of the form

$$x' = x^p$$

where x' represents the transformed value of OTU abundance x, and p is an exponent that I varied from 1 to 0. Use of $p = 1$ means no transformation, so abundant OTUs are weighted heavily. Decreasing values of p increasingly down-weight abundant OTUs; for example, $p = 0.5$ is the commonly-used square root transformation (193). Use of $p=0$ means that only the presence or absence of an OTU, not its abundance, is used. (I used $0^p = 0$ for the special case of $p = 0$.)

After transformation, I used Bray-Curtis dissimilarity to quantify the compositional dissimilarity between samples. As *p* varied from 1 to 0, each nMDS calculation was "seeded" with the result of the previous one, reducing the tendency of nMDS to fall into local minima (41).

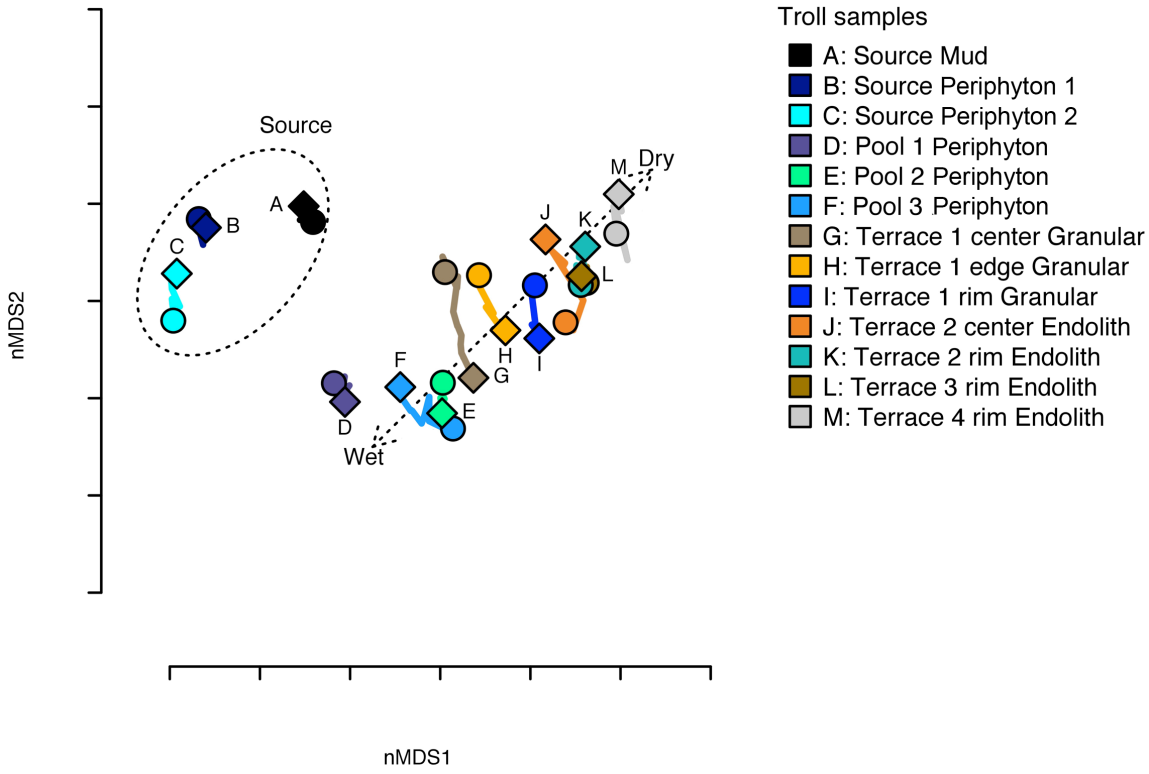


Figure 3.5: Trajectory plot showing the change in nMDS configuration as the exponent *p* in the power law transformation is varied from 1 (circles) to 0 (diamonds) in steps of 0.1.

Figure 3.5 shows how relationships among samples change as a result of this transformation. Small, abrupt changes in the trajectories are artifacts of the calculation process, but the trends in the trajectories are biologically significant. For example, the granular samples from terrace 1 move closer to the periphyton samples with carbonate precipitates from pools 2 and 3 as *p* goes from 1 to 0. This shows that those samples are more similar to one another when all OTUs

are weighted equally – *i.e*., that they share many common minor OTUs even though there are significant differences in their major OTUs. The same is true for the pool 1 periphyton, which also moves closer to the pool 2 and 3 periphyton. In contrast, the terrace 2 rim and terrace 3 rim endolith samples move apart as *p* goes from 1 to 0, suggesting that they share major OTUs but have differences in their minor OTUs.

These changes can also be shown by plotting the untransformed *vs*. presence-absence transformed Bray-Curtis dissimilarities for selected sample pairs (Fig. 3.6, left). Displayed in this form, points above the 1:1 equality line show an increase in dissimilarity upon transformation, whereas points below the line show a decrease (*i.e*., become more similar). The points that plot below the 1:1 line are mostly for aquatic pairs, and the points above the line are mostly for terrestrial pairs.

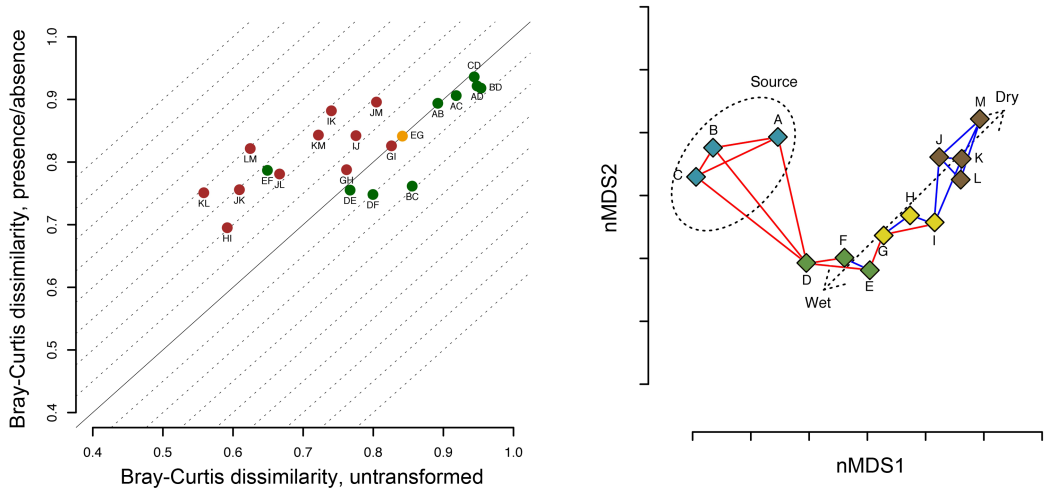


Figure 3.6: Changes in dissimilarity for transformed and untransformed Bray-Curtis dissimilarities. The different colored dots in the left panel represent different samples pairs: brown = terrestrial pairs, green = aquatic pairs and orange = transitional pair from aquatic to granular. The solid line represents the 1:1 ratio between the transformed and untransformed dissimilarities. In the right panel, dissimilarity changes are displayed on the presence/absence transformed nMDS plot. Blue lines connect sample pairs that become more similar when abundances are taken into account, and red lines connect pairs that become less similar. The legend for sample labeling in the right panel is the same as in Figures 3.3 and 3.4.

To display how these dissimilarity measures vary for different sample pairs, I used the presence/absence transformed nMDS plot from Figure 2.13, connecting sample pairs according to the changes in their Bray-Curtis dissimilarity (Fig. 3.6, right). Blue lines connect pairs that become less similar with transformation, and red lines connect pairs that become more similar.

The red lines on the right side of Figure 3.6 are concentrated among the source periphyton, pool periphyton and a granular sample pair, while the blue lines are located among the drier granular and endolith samples. This pattern reveals a fundamental trend in the data: Aquatic samples at Troll tend to become more similar to one another when presence/absence transformed, while terrestrial samples tend to become more dissimilar. This pattern confirms trends seen in the trajectory plot of Figure 3.5.

The explanation for this behavior is that aquatic sample pairs tend to have few common major OTUs and many common minor OTUs, whereas for terrestrial sample pairs the reverse is true. When common OTUs are mostly minor, and few common major OTUs are present, then samples decrease in dissimilarity as *p* approaches 0 because the weight on abundances is reduced by transformation. The reverse is true when samples share major OTUs but not many minor OTUs.

This trend can be visualized by examining the distribution of OTUs for each sample in a pair, enabling the OTUs that are common to both samples, as well as the distribution of counts among the OTUs, to be displayed. Figure 3.7 shows an example for two sample pairs that exhibit decreasing and increasing dissimilarity when using Bray-Curtis dissimilarities – a periphyton pair and an endolith pair, respectively. To produce these plots, data were first square-root transformed for better graphic visualization of high counts, and then sorted in decreasing order of counts. I generated two plots for each pair, with each plot displaying all OTUs for one sample (the “base sample”) in the pair, and overlaying on those just OTUs for the other sample that are common to both samples. Specifically, only common major OTUs are overlaid on major OTUs, and only common minor OTUs are overlaid on minor OTUs. For purposes of this comparison I define a major OTU to be one with more than 10 counts (untransformed), and a minor OTU one with 10 counts or fewer. The dashed line in each figure represents that limit for the base sample; *i.e.*, major OTUs lie to the left of this line, and minor OTUs lie to the right.

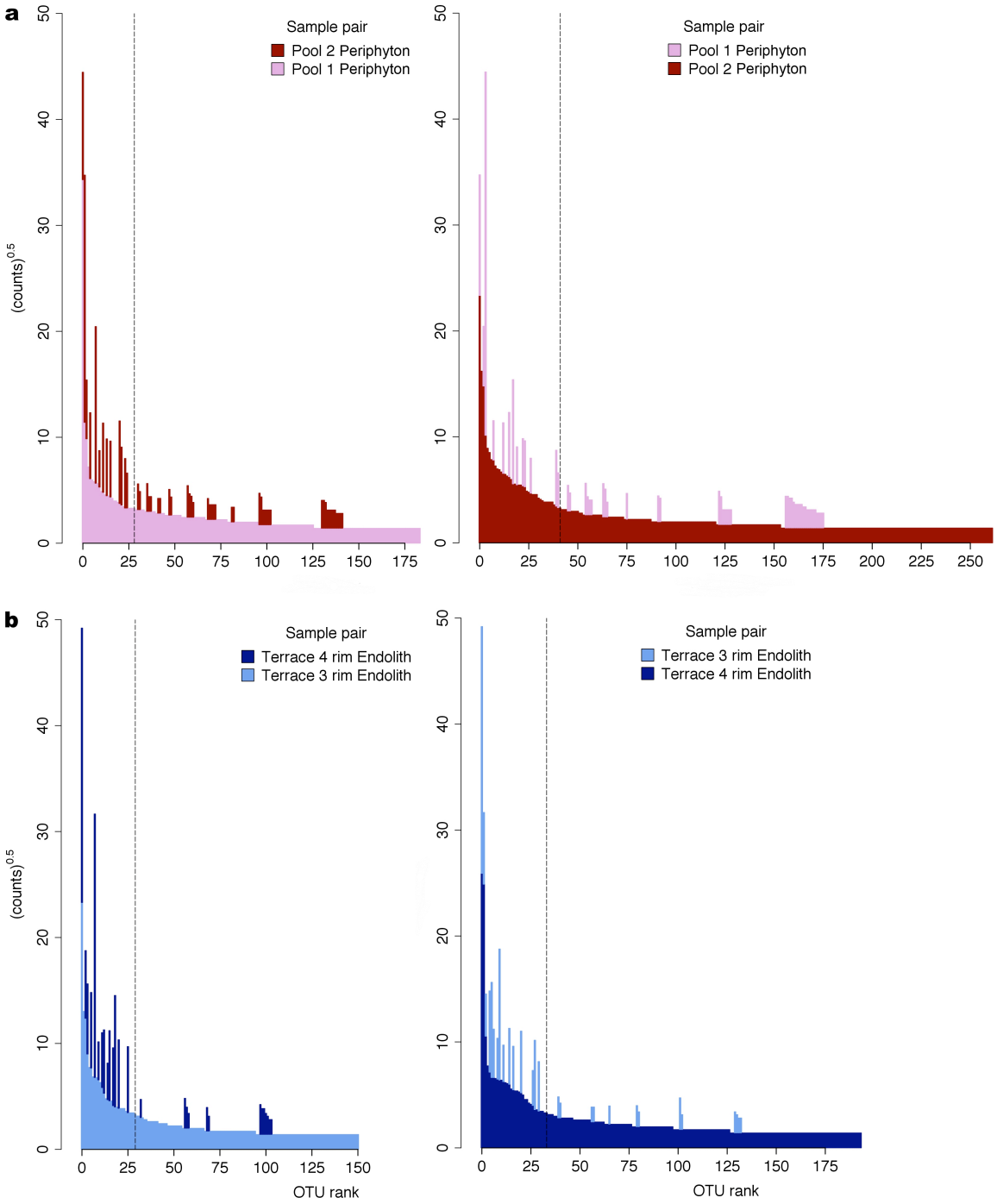


Figure 3.7: OTU distribution plots for an example periphyton pair (*a*), and an example endolith pair (*b*). Data are square-root transformed for better graphic visualization of high counts, and then sorted in decreasing order of counts. The y-axis therefore gives the square root of the number of counts for each OTU. The figure shows that aquatic samples tend to have many common minor OTUs and fewer common major OTUs, while the reverse is true for terrestrial samples. The dashed line represents the division between major and minor OTUs. For the non-base sample, only common major OTUs are displayed left of the line, and only common minor OTUs right of the line.

The plots in Figure 3.7 display a major difference between terrestrial and aquatic samples: Aquatic sample pairs tend to have few common major OTUs and many common minor OTUs, whereas for terrestrial sample pairs the reverse is true. For the pool 1 and pool 2 periphyton samples in Figure 3.7a, only 17% of the common OTUs are major OTUs in both, and a large fraction, 52%, are minor in both. In contrast, for the terrace samples in Figure 3.7b, 34% of the common OTUs are major OTUs in both samples, and 30% of the common OTUs are minor in both. So although the periphyton samples have many OTUs in common, most of them are low in count. Additionally, the periphyton samples have a small number of OTUs that contain a high percentage of the counts. The terrace samples, in contrast, have much of their similarity in their shared major OTUs.

These differences explain the changes in Bray-Curtis dissimilarity with p seen in Figure 3.6. When common OTUs are mostly minor, and few common major OTUs are present, then samples show a decrease in dissimilarity when all OTUs are weighted more nearly equally (*i.e.*, as p approaches 0). In contrast, when samples share major OTUs but not many minor OTUs, then their dissimilarity increases as p approaches 0. So the red and blue lines in Figure 3.6 reveal a fundamental trend in the makeup of microbial communities at Troll: terrestrial samples tend to have common major OTUs, whereas aquatic samples are less likely to.

3.3.3: Variations in community structure: Phylogeny

While Bray-Curtis dissimilarity based on OTU counts is one way of assessing community differences, it has some limitations. OTUs are defined by the degree of sequence similarity (e.g. 97%, 95%, 90%), where sequences that are similar to each other at some percentage level are assigned to the same OTU. A 90% similarity of sequences is equivalent to the bacterial family level (28, 38, 123, 258), such as Nostocaceae, Oscillatoriaceae and Rhodobacteraceae, where the first two belong the phylum Cyanobacteria and the last to Proteobacteria. The resulting OTUs are then used to cross-compare samples and to calculate dissimilarities. A disadvantage of OTU-count-based dissimilarity measures is that all OTUs are considered to be equally different from each other. For example, Nostocaceae, Oscillatoriaceae and Rhodobacteraceae would all be considered equally different, although the two Cyanobacteria families are phylogenetically closer to each other than to the Proteobacteria families.

UniFrac, in contrast, is a measure that takes phylogenetic distances into account and exploits the degree of divergence between different sequences (165). UniFrac measures the phylogenetic distance between a set of taxa as the fraction of branch length in a phylogenetic tree that leads to descendants from either one environment or the other, but not both, capturing the amount of evolution that is unique to each environment. When comparing environments, it is likely that closely related organisms have similar biological properties. Very similar environments share much of the branch length in the tree (descendants from both communities) because only few adaptations would be needed to

transfer from one community to the other. In contrast, if lineages were not shared, such as in very distinct environments, then the branch length in the tree would lead to descendants from only one of the two communities, resulting in adaptation to different environments and a large phylogenetic distance.

The transformation of OTU data used above weights the importance of abundances, which are then analyzed by nMDS. UniFrac offers a similar capability, with unweighted UniFrac distances not accounting for abundances and weighted UniFrac distances accounting for them (166).

The left side of Figure 3.8 plots unweighted and weighted UniFrac distances in the same way that transformed and untransformed Bray-Curtis dissimilarities are shown in Figure 3.6. Because of the way that UniFrac weighting is performed, the weighted distance is less than the unweighted distance for all samples. The pattern is strikingly similar to the Bray-Curtis plot, however, with the difference between the unweighted and weighted distances having a consistently more positive value for terrestrial sample pairs than aquatic sample pairs.

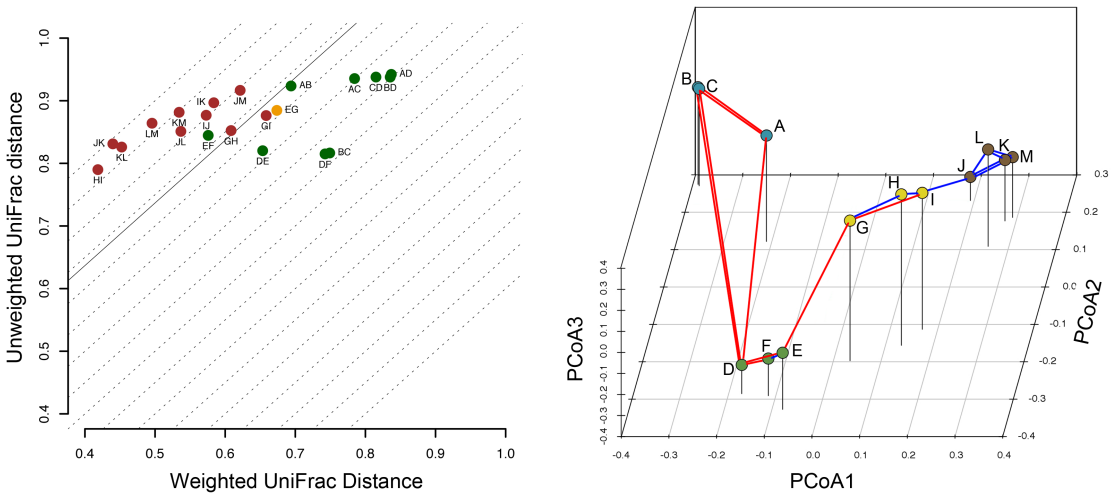


Figure 3.8: Changes in dissimilarity for weighted *vs*. unweighted UniFrac distances. The different colored dots in the left panel represent different samples pairs: brown = terrestrial pairs, green = aquatic pairs and orange = transitional pair from aquatic to granular. The solid line in the plots represents the median. In the right panel, dissimilarity changes are displayed on an unweighted UniFrac principal coordinates plot for the pairwise UniFrac distance values (PCoA1=14%, PCoA2=12.7%, PCoA3=9.8%). Blue lines connect pairs for which the decrease in distance is greater than the median when abundances are taken into account, and red lines connect pairs for which it is less than the median. The legend for sample labeling in the right panel is the same as in Figures 3.3 and 3.4.

The right side of Figure 3.8 is analogous to that of Figure 3.6, with blue lines on a UniFrac principal coordinates plot connecting sample pairs with UniFrac distances below the median, and red lines connecting pairs with distances above the median. Despite the very different ways in which Bray-Curtis dissimilarity and UniFrac distance are calculated, the patterns are very similar.

The similarity between samples as determined by weighted UniFrac depends on the relative abundances of phylogenetic lineages as well as the types of lineages present. Abundances in shared dominant OTUs that are phylogenetically similar to each other put weight on shared tree branch lengths, leading to similarity of samples and low UniFrac distances. Terrestrial samples share many OTUs and are more evenly distributed, which leads to smaller

distances when abundances are accounted for. If those weights are removed, distances increase. Aquatic samples, in contrast, do not share many major OTUs, but the OTUs they do share phylogenetically weight the shared tree branches, resulting in weighted distances that are closer to the unweighted distances compared to terrestrial sample pairs. The resulting pattern therefore resembles the pattern from the nMDS analysis.

3.3.4 Summary

The data presented above and in Chapter 2 show a number of strong variations in microbial community makeup at Troll Springs:

- Aquatic periphyton samples have many common minor taxa, but tend to be dominated by a few major taxa that differ from one sample to the next.
- Terrestrial granular and endolith samples have more major taxa in common, and fewer minor ones.
- The same variations in community structure revealed by comparing major and minor taxa are also seen when differences are computed phylogenetically.
- Community richness and evenness are positively correlated among all samples.

- For terrestrial samples, richness, evenness, and diversity all increase with increasing water content.
- For the aquatic periphyton samples, richness, evenness, and diversity all increase with decreasing temperature (or, equivalently, with increasing pH).
- The source mud sample, which is sedimentary in character, falls on the same richness, evenness, and diversity trends as the terrestrial samples.

3.4 DISCUSSION

In the discussion that follows, I will attempt to explain the observations that were summarized in the previous section in terms of ecological principles that were presented in Chapter 1. In particular, I will argue that community evenness at Troll is affected mostly by the balance of competition, and richness by the availability of physical niches. I will also apply ecological succession concepts to my data, comparing their predictions to some of the trends observed.

3.4.1 Microbial succession at Troll Springs

A successional change is influenced by environmental parameters, the interaction of taxa present and site conditions at the time of the initial

disturbance. Troll Springs shows a progression of environments, starting at the source with fresh emerging water that is immediately colonized by periphyton (Stage I), a disturbance of the pool environment by calcite precipitation (Stage II), followed by water evaporation (Stage III) and eventual lithification and the appearance of endolithic communities (Stage IV). Through all these stages I see a succession of microbial communities. The succession at Troll is fundamentally allogenic, occurring because the habitat is altered by environmental factors, although I will suggest that autogenic succession also plays a role. The different succession stages and associated observations are summarized in Figure 3.9. (Note that these successional stages are distinct from the stages of calcite precipitation discussed in section 2.3.1.)

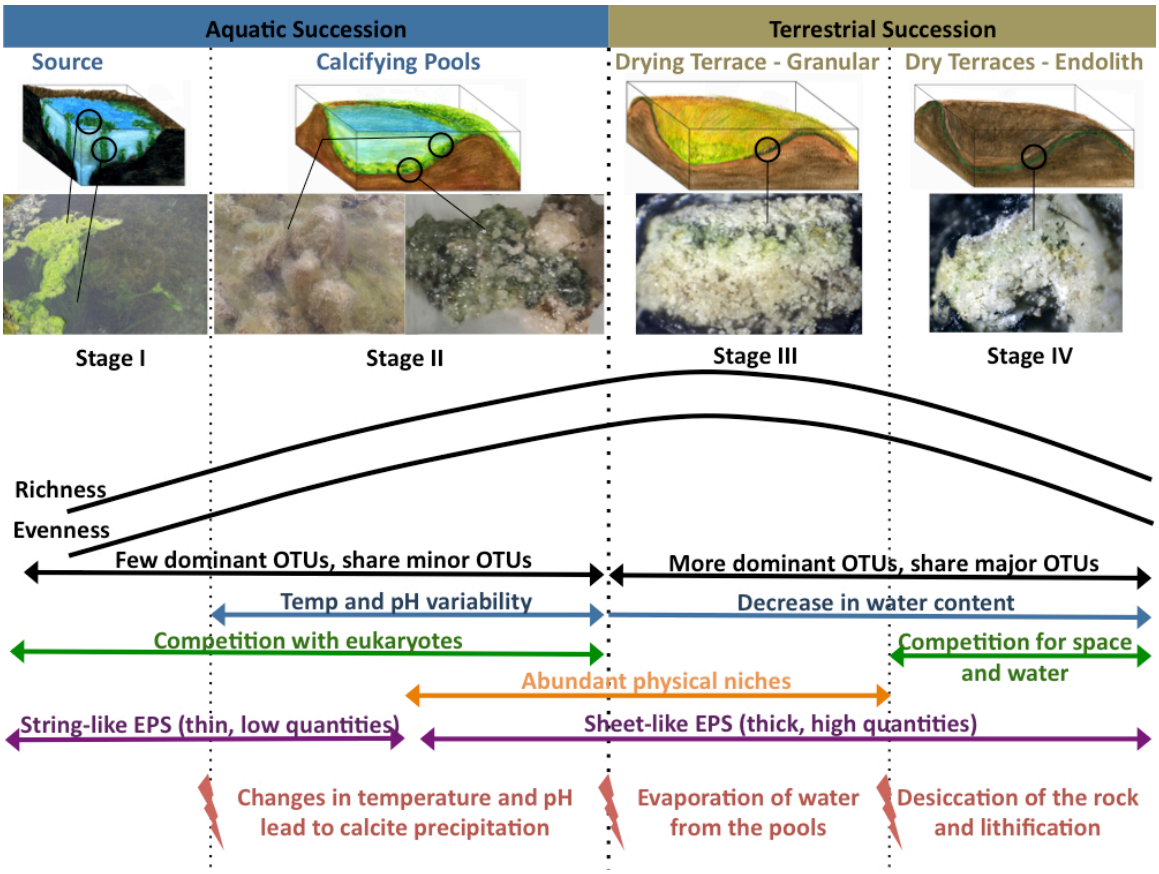


Figure 3.9: Microbial succession at Troll. The succession of the communities from the source to the endolith encompasses 4 major stages in a sere.

3.4.2 Aquatic succession

When the water first emerges from the source, only organisms that were picked up from the subsurface can have colonized it. However, colonization of this water by photosynthetic eukaryotes probably takes place rapidly. This initial colonization is a form of primary succession where organisms make use of inorganic nutrients and sunlight, and organic matter rapidly accumulates (Stage I). The accumulation of organic matter can subsequently lead to colonization by heterotrophic organisms.

Calcite precipitation downhill from the source leads to construction of terraces and ultimately to formation of rock as the water evaporates, driving allogenic succession. The drop in temperature, increase in pH and calcite precipitation can be seen as small, continuous, and cumulative disturbances in the pools (Stage II). The communities in the pools in Stage II are richer and more even than the source periphyton of Stage I, which have only one or two major OTUs. A few dominant bacterial taxa replace the original source communities, although photosynthetic eukaryotes still outcompete photosynthetic bacteria before calcite precipitation becomes advanced. With continued calcite growth as the temperature drops and pH increases, organisms colonize the gaps between the growing calcite grains, displacing thick filamentous organisms originally submerged in the water. These organisms grow close together and bind calcite grains together by producing thick EPS sheets. This aquatic succession is continuous process in which calcite precipitation increases and the community makeup changes in response.

During the early stages of an ecological succession, a community is typically dominated by r-strategists, characterized by high reproduction rates and rapid growth rates, allowing them to outcompete other taxa when resources are abundant. Tilman (262) suggests that fast reproducing and growing organisms have a competitive advantage over slower-growing organisms. As shown in Chapter 2, the source environment in particular shows a high abundance of filamentous eukaryotic algae that outcompete bacteria, which are possibly better and faster colonizers under these conditions. A similar situation is found in some

freshwater stream environments, where algae drive the development of biofilm that is later colonized by bacteria during succession (15).

In general, communities dominated by r-strategists tend to have a small number of species with high reproduction and growth rates, so the earliest stages of succession are often characterized by low diversity, richness and evenness (57, 73, 202). In plant successions, evenness tends to increase with succession age (238, 288). Similar trends are seen in the aquatic environments at Troll, where the source periphyton show low bacterial richness and evenness and these parameters increase in the other pools as succession proceeds, possibly indicating a similar process.

A useful concept in understanding some of the trends observed through the aquatic succession may be the competitive exclusion scenario (107), in which superior competitors outcompete inferior ones by using limiting resources more efficiently. Inferior competitors are excluded or replaced as a consequence. The observed trend in evenness may result in part from the changing competitive balance between bacterial and eukaryotic photosynthetic organisms. As described in Chapter 2, Cyanobacteria gain a competitive advantage over eukaryotes as illumination decreases. It is noteworthy, then, that the source periphyton sample collected at the water surface, which has the highest abundance of eukaryotic algae of all samples, as seen in SEM observations (Fig. 2.4), has substantially lower bacterial evenness than the periphyton sample that was attached to the bottom of the source. The comparison between these two samples is particularly useful because their environments are effectively identical

other than their depth. The difference in evenness may result from eukaryotes outcompeting photosynthetic bacteria in the well illuminated floating sample, driving their abundances down and thereby driving bacterial evenness down. Similarly, the increase in evenness as temperature decreases among the pool samples may reflect the diminishing influence of eukaryotes with increasing carbonate precipitation and decreasing access to illumination, allowing bacterial taxa, particularly Cyanobacteria, to flourish.

The trend in richness through the aquatic succession may be explained primarily by variations in niche availability. As shown in Chapter 2, precipitation of calcite with decreasing temperature (and increasing pH) produces physical niches, in the form of grain surfaces and intergranular spaces, which can be occupied by microbes. I suggest, then, that the observed relationship between richness and temperature in the aquatic samples is a direct consequence of the increased availability of physical niches produced by calcite precipitation in the lower temperature samples. As discussed in Chapter 1, an environment with many niches (soil is a good example) can support many coexisting organisms with similar ecological functions. This enhanced coexistence comes about by niche differentiation, where spatial displacement of two species that use the same resource reduces competitive pressure. The consequence is high richness and diversity. Species diversity increases during the early stages of succession due to addition of new species.

As usually defined in plant ecology, a secondary succession follows a severe disturbance to a pre-existing community, which is not the case in the

pools. Instead, the aquatic succession in the microbial community at Troll is a more gradual and continuous one, driven by calcite precipitation that can be seen as a progressive disturbance to the aquatic system. As discussed in section 3.1.2, a series of small, cumulative disturbances can also drive succession, resulting in an increase in diversity over time.

3.4.3 Terrestrial succession

Once the water has evaporated from a pool, terrestrial succession begins. During this succession, the community structure shows a general trend towards lower diversity and evenness as water content decreases (right side of Fig. 3.3). As described in Chapter 2, this process is not a completely new colonization of the drying material; rather, it is a gradual shift in community makeup from periphyton to endolith, with the periphyton serving as a precursor to the endolith. As the granular samples desiccate, aquatic-originated organisms are mostly displaced, with OTUs that were minor in the periphyton gaining importance. Endolithic communities begin to form, becoming visible as a green line immediately below the surface.

During lithification of granular material to form rock, access to both water and light diminish, leading to a shift in the competitive balance among the organisms present. Organisms that are tolerant to low levels of nutrients and other resources outcompete fast growing organisms. Smith and Huston (247) argue that r and K strategies entail compromises that dictate competitive abilities

under varying resource levels. The model postulates that an organism can be competitive under high resource levels or tolerant of low levels, but not both. Light and water are critical resources for photosynthetic taxa at Troll, especially during endolith formation when their availability becomes sharply reduced. According to the model, taxa that grow best under high light and water conditions will be outcompeted by taxa adapted to drier and more poorly illuminated environments. Interestingly, in the model of Smith and Huston, the tolerant species do not outcompete light-competitive species due to their own best growth under these stress conditions, but rather because they persist when light-competitive species could not.

As the terrestrial succession proceeds at Troll, organisms seem to be better able to live under nutrient-limited conditions, possibly similar to the K-strategist life style as described in the classic succession model. As the water evaporates and the endolithic communities form, the communities share more major taxa and become more stable and widespread over a broader range of samples. K-strategists are more competitive in their specific environment, and form a more permanent and stable community.

The granular samples, particularly the center terrace sample, show the highest richness and evenness of all samples studied other than the source mud. These types of sample, in particular, provide many resources, such as organic matter from phototrophs, light for photosynthesis, water, protection and niches. In all the terrestrial samples, evenness and richness both decrease with decreasing water content. The competitive exclusion scenario possibly explains this

dependence of evenness on water content. In this case, the critical resource is water. As water becomes scarce, the community becomes increasingly dominated by a relatively small number of taxa that are able to use water particularly efficiently. This same effect is seen in a wide variety of arid settings, as described in Chapter 1. In this context, the details of the relationship between evenness and water content (left middle panel of Figure 3.3) are noteworthy. There is little variation in community evenness across most of the range of water content, but a sharp drop at the driest values. This observation suggests that water only becomes a limiting resource (thereby reducing evenness) below values of a few weight percent.

The relationship between richness and water content in the terrestrial samples is again probably related to the availability of physical niches. Richness is highest in the granular samples, which the SEM images in Chapter 2 show to have abundant calcite grain surface area and pore space. As materials lithify, however, calcite recrystallization and grain growth lead to a reduction in pore space. The reduced pore space limits the amount of water that the rock can hold, and at the same time also reduces the availability of physical niches for colonization. A decrease in richness can be expected as a direct result. In other settings, species diversity can decline in later stages of succession as competition or harsh environmental conditions eliminate taxa.

3.4.4 Application of ecological succession to Troll Springs

During succession of a plant community, richness and biomass increase, stabilizing when the climax stage is reached. However, at Troll the visible biomass, from the thick filamentous periphyton in the source and pools to the thin, sparse endolithic community, decreases during succession. Of course, there are limitations when comparing plant succession to microbial succession (79). In fact, some studies of microbial succession have found that the accumulation of biomass is high in the earlier stages of succession and then tapers off as resources become more limited (79, 140, 244). But as described above, the clear decrease in biomass with succession at Troll can be related to the nature of the disturbances there, which gradually reduce the availability of key resources like water and access to light over time.

Although some aspects of plant succession cannot be applied to microbial succession at Troll, some processes are equivalent. For example, although direct growth rates have not been measured, Troll may display some aspects of the r- and K-strategists concept, reflected by the OTU distributions. During the early succession phase, the pools exhibit major differences in community makeup with a small number of dominant OTUs and only a few major OTUs in common, as shown by UniFrac and nMDS analysis (Figs. 3.6 and 3.8), perhaps due to different nutrient availabilities and niches in the different pools. Generally, organisms that are r-strategists tend to be subject to extreme population fluctuations when conditions change (such as nutrients or physical conditions), leading to unstable populations of low metabolic efficiency. As shown in Chapter 2, the pools are prone to seasonal- and weather-driven temperature and pH

fluctuations that can affect community makeup and the distribution of taxa. However, some OTUs in the pool periphyton persist and become integrated into the developing endolithic community during terrestrial succession, aided by niche differentiation and reduction in competition. OTUs in the endolithic community are more stable and are probably diverse in their metabolic phenotypes, leading to functional ecosystem stability and therefore could be considered possible K-strategists. Heterotrophs are less abundant early in the succession, but become more prevalent in the later stages, with an increase in richness and diversity.

An important characteristic of the early stages of plant succession is that competition among organisms is less pronounced than later in succession. Organisms in the early stages colonize harsh and unstable environments, and do not face severe competition there. Once a more stable community develops, competition for resources becomes more intense. This observation suggests that pioneer species may be less competitive than species in more established communities. However, this theoretical concept of competiveness may not apply in all situations, because early competition may be intense if conditions are actually favorable at the beginning of the succession.

At Troll Springs succession is influenced by environmental conditions that change from moderate to harsh as the succession proceeds. In the early stages of the aquatic environment, bacteria must compete with photosynthetic eukaryotes, leading to a few dominant bacterial OTUs. Richness is low and primary producers are the first to colonize. As environmental conditions like temperature and pH change and calcite precipitation begins, photosynthetic

eukaryotes get outcompeted. While they are strong competitors under a particular set of environmental conditions, they become inferior as disturbances accumulate, so succession proceeds. Their susceptibility to an increase in pH and decreases in temperature and illumination may be the key factors in reducing their competitive ability and preventing their persistence. Near the end of the succession, in the most mature endolith, pore space and water are in short supply, and competition becomes intense, limiting richness. The important point is that it is necessary to evaluate relative competitive abilities together with environmental parameters in order to understand successional changes and their directionality.

The classical model of ecological succession includes an end-stage called “climax” that is far more stable than its predecessors. At Troll, the steady accumulation of ecosystem disturbances prevents the arrival at a climax state across most of the spring system. Community distribution is determined by the environmental tolerances and growth constraints of taxa, resulting in continuous changes of microbial community makeup with changing environmental parameters. Although the endolithic ecosystem appears to be relatively stable, even the community there is prone to changes as the environment dries out and erodes, and all the organisms die. So perhaps even the endoliths are not a climax community in the conventional sense of the term. However, the terrestrial ecosystem shows more stability in their microbial community makeup then the aquatic environment.

A prominent feature at Troll Springs is an increase in richness and evenness during most of the succession, but a decrease of the same parameters during the final maturation of the endolithic community. This hump-shaped feature of richness and evenness differs from most established models for plant succession. There are similar exceptions in the microbial literature, however. Sigler *et al*. (244) observed a similar decrease in richness and evenness with successional age where the bacterial community structure in soils changed with distance from a receding glacier and, consequently, with increasing soil age. Schipper *et al*. (238) found that heterotrophic evenness declined in more advanced successional stages soils, citing consistency with the intermediate disturbance hypothesis (45, 102). The explanation they offered was that an increase of cell density driven by a decrease in easily available substrates leads to increase competition and survival of a limited number of species. I suggest that a similar process of competition and also adaptation occurs in the mature endoliths at Troll, with sharp reductions in reduced niche availability along with reduced water availability and hence richness.

3.4.5 Ecosystem stability

Succession influences species composition, and also changes the structure and functioning of an ecosystem. For example, low intensity disturbances, such as calcite precipitation, influence patterns in resource

availability, and also create additional habitats, or niches. All these changes are linked during succession.

Every environment imposes conditions on microorganisms, influencing microbial community makeup, including richness and evenness. Species evenness is not necessarily required for ecosystem stability. Even where there is a single dominant species in a particular role, ecosystem stability could be high. Other species (minor OTUs) from the same functional group may have been outcompeted by the dominant species (major OTUs), but can become prominent and take over the function if the dominant species disappears for some reason.

Higher diversity leads to functional redundancy, and consequently to increased ecosystem stability (162, 263). In diverse ecosystems, ecological functions like photosynthesis are distributed across a large number of taxa that have a range of traits and environmental tolerances, leading to redundancy and higher resilience to environmental stress and changes (186). Although new redundant taxa may not add novel capabilities, higher diversity within functional groups means that species can replace or compensate for one another and secure ecosystem functioning during disturbances. A climax community is typically tolerant to changing environmental conditions, with taxa replaced by others with the same functional traits, maintaining equilibrium. Although a real climax community is unlikely at Troll, a functional climax community is perhaps possible. Willebolle *et al*. (289) showed that highly uneven communities, which are dominated by one or few species, are less resistant to environmental stress.

They conclude that greater evenness results in a more robust ecosystem stability.

3.5 SUMMARY AND CONCLUSIONS

The richness, evenness, and diversity of microbial taxa are all strongly correlated at Troll Springs. These parameters all increase with decreasing temperature across the aquatic samples, and all decrease with decreasing water content across the terrestrial samples. I attribute the trends in evenness to the balance of competition, with evenness limited in the most calcite-free environments by competition with photosynthetic eukaryotes, and in the driest endolith by competition for water. I attribute the trends in richness to the availability of physical niches, with niche availability first increasing as calcite grain surfaces become available for colonization, and later decreasing as pore volume becomes scarce.

Periphyton samples in the aquatic environments have many common minor taxa, but are dominated by a few major taxa that differ from one sample to the next. In contrast, terrestrial samples have more major taxa in common, and fewer minor ones. The same pattern is revealed whether comparing communities by their OTU makeup, or by their phylogenetic composition.

Succession is a major theme in ecology, and while it has been particularly studied for plants, it is also becoming important in microbial ecology. At Troll Springs, it is possible to observe an ongoing microbial succession process along

environmental gradients. The succession at Troll is characterized by gradual changes in environmental parameters that produce a sequence of small, incremental environmental disturbances. These disturbances drive a succession in microbial community makeup. The succession is governed by inter-taxa relationships, such as competition for resources, with well-adapted taxa thriving under the environmental conditions that most favor them.

The succession at Troll begins at the spring source with a few dominant phylotypes, progressing as conditions change into a more stable and even community. Community richness and evenness are strongly correlated through this progression. They also both increase with increased successional age, except in the most mature endolith where they diminish because of the sharply reduced availability of resources and niches.

By analyzing microbial samples from travertine terraces of different ages, it has been possible to deduce the process of ecological succession at Troll Springs. The community patterns revealed in the molecular data show how changing environmental conditions and the resultant competition for resources and niches have caused succession, culminating in mature endolithic communities.

CHAPTER 4: CYANOBACTERIAL CULTURING FROM ENDOLITHIC COMMUNITIES

4.1 INTRODUCTION

Cyanobacteria are simple organisms that can inhabit virtually any aquatic environment, as well as many types of soil and rock. They are important primary producers at Troll Springs, as they are in many endolithic communities (88). At Troll, Cyanobacteria form an isolated community subjected to a range of unusual environmental conditions, including constant light in the summer and constant darkness in the winter. It is possible that some Cyanobacteria at Troll are endemic.

Because Cyanobacteria are a major component of the endolithic community at Troll, it is desirable to culture these organisms. Culturing is an important technique in microbiology that provides two benefits: amplification of microbial material and purification of single organisms. I therefore undertook an effort to culture Cyanobacteria from the endolith and the spring under a range of conditions and characterize them. My hope was that these cultured Cyanobacteria might be better suited to metagenomic analysis than environmental samples, due to their reduced species diversity.

To determine how well representative Cyanobacteria can be cultivated from environmental samples, endolithic rock samples and periphyton collected from pool 1 were used. Because the periphyton are from an aquatic environment that has low richness (see Chapter 3), I wanted to determine whether their

Cyanobacteria might be easier to cultivate than the endolithic cyanobacteria, and whether their cultured isolates might represent the environmental samples better than for the endoliths.

While the goal of this study was to culture representative organisms from endolithic samples, the vast majority of organisms are not culturable. For example, Amann *et al*. (2) showed that culture-based techniques typically cultivate only about 1% of organisms actually present in the environment. So when organisms are cultured from the environment, it is often unclear to what extent the cultured isolates will resemble either the community from which it was drawn or a targeted taxon from it. As will be seen below, the organisms that thrived in the cultures that I grew from Troll endoliths were not representative of the endolithic communities from which they arose.

4.2 RESULTS AND DISCUSSION

The analysis focused on three sample pairs: endolithic samples from terraces 3 and 4 and their corresponding cultured isolates, plus a periphyton sample from pool 1 and its cultured isolate. I also included three more cultured endolith isolates from Troll, two from a previously collected endolithic sample cultured at different salinities (0 and 3%), and one that was a sub-culture originally cultured by Feng Chen. Data from corresponding rock samples for those three cultures are not available. Finally, data from two other endolithic samples (terrace 2 center and terrace 2 rim) were included for comparison.

4.2.1 Culturing

The terrace 3 rim and terrace 4 rim endolith samples were cultured under several different conditions. Culturing is typically performed under conditions similar to those in the microbes' natural habitat. Cyanobacteria experience a range of temperatures at Troll, and are exposed to extended periods of continuous illumination in summer and darkness in winter. Therefore, the culturing study included four different combinations of illumination and temperature: continuous illumination at room temperature (RT, ~25ºC) and 4ºC, and continuous darkness at RT and 4ºC.

Diverse Cyanobacteria grew from the illuminated endolith samples, with significant variability in visible morphology and color among the isolates from both. Cultures failed to grow in total darkness at both RT and 4ºC for both endolith samples.

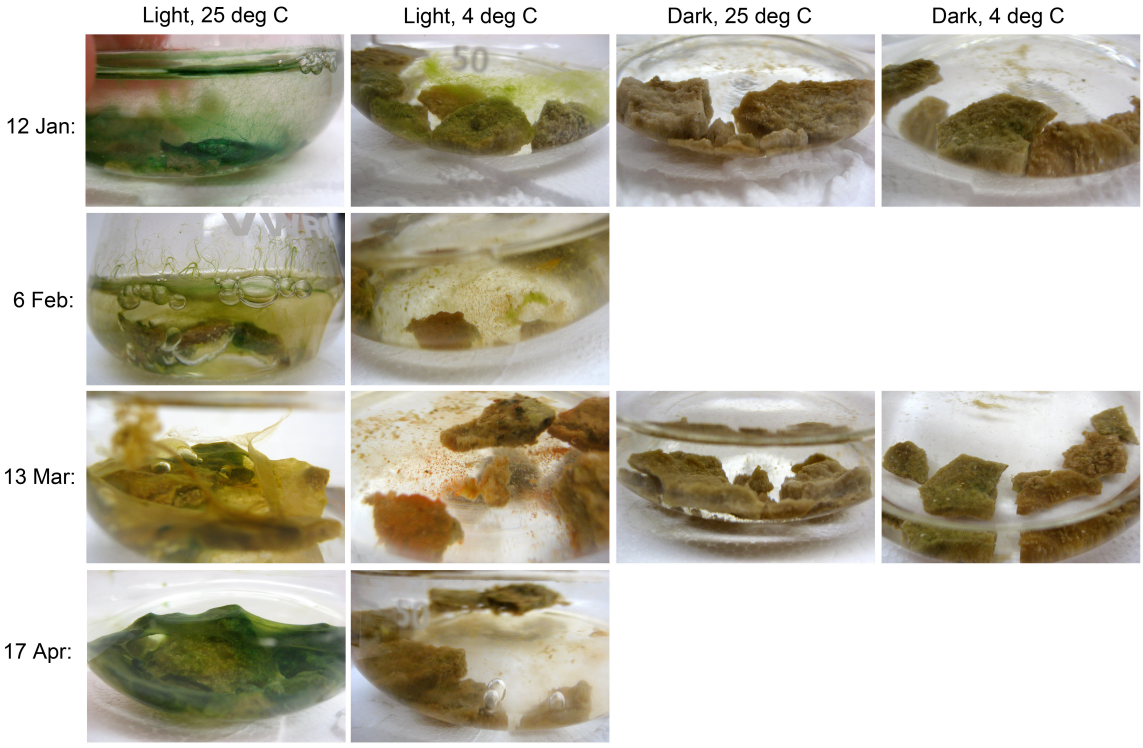


Figure 4.1: Culturing of terrace 3 rim endolith under different environmental conditions. Culturing began on 8DEC2008; all dates shown are in 2009. Cyanobacteria were cultured with continuous illumination at room temperature (~25ºC) and 4ºC, and in continuous darkness at the same temperatures. The constant light source was turned off on 9APR2009.

Growth of Cyanobacteria from terrace 3 at RT began as a sheet-like structure adhering to the glass surface (Fig. 4.1, 12JAN and 6FEB). On 6FEB "hyphae" were growing above the water surface, and air bubbles were trapped within the EPS sheet structure. All rock pieces in the flask were engulfed in the same sheet structure. During later development, the sheet released from the sides of the flask. The cultures were exposed to constant light during the initial growth, but I turned the light of on 9APR2009 due to bleaching of the cultures (Fig. 4.1,13MAR), which resulted in recovery of the culture.

The culture at 4ºC produced a light green "fluff" (Fig. 4.1, 12JAN), but not an EPS sheet structure. These isolates turned orange later in the progression,

indicating a change in pigments. The cultured isolates slowly disappeared as time progressed.

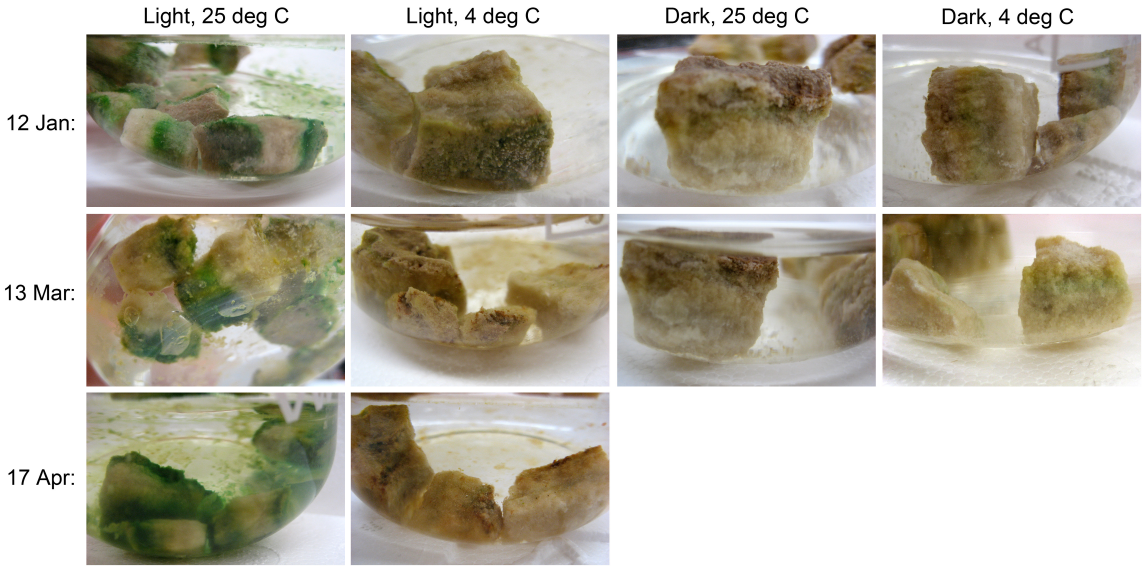


Figure 4.2: Culturing of terrace 4 rim endolith under different environmental conditions. Culturing began on 8DEC2008; all dates shown are in 2009. Cyanobacteria were cultured with continuous illumination at room temperature (~25ºC) and 4ºC, and in continuous darkness at the same temperatures. The constant light source was turned off on 9APR2009.

The growth of Cyanobacteria from the terrace 4 sample differed from terrace 3. There was no visible EPS sheet structure, but rather green “fluff” that developed where the endolithic community is located below the rock’s surface. Most of the Cyanobacteria accumulated on the underside of the rock fragments (Fig 4.2, 13MAR), suggesting possible light sensitivity. There was minor growth of orange-pigmented isolates at 4ºC, which did not progress.

Culturing of Cyanobacteria from two different endoliths resulted in growth at RT and 4ºC under illumination, with growth occurring more rapidly at RT. It is possible that only a small fraction of the species present were culturable via the

technique used. Additionally, factors such as incubation time and growth medium may have significantly influenced growth.

Cyanobacteria, especially from extreme environments, can grow slowly under laboratory conditions. The samples discussed above were part of a batch culture. In batch cultures, the medium is typically continuously altered by the metabolism of the growing organisms, which can result in conditions that are no longer suitable for growth. Conditions may remain stable during the early stages of growth, but as the composition of the media changes, the number of cells will decrease or the appearance of the culture will change.

Castenholz (33) has shown that some Cyanobacteria cannot be isolated easily by standard cultivation methods. This failure is attributed to their sensitivity to high nutrient concentration (72), to high illumination, or to contamination by inhibiting ingredients in the media (33). The Cyanobacteria of the terrace 4 endolith grew best on the underside of the rock, suggesting sensitivity to the high light levels in the laboratory.

Four additional cultures were included in this study, three of which are shown in Figure 4.3. I received a culture from Feng Chen, which he grew from a Troll endolith years before. I grew a subculture, which grew as a sheet structure (Fig. 4.3a). Two other cultures were produced from a single endolithic rock, but differed in the salinity of the growth media, where one represented seawater (Fig. 4.3b) and the other freshwater (Fig. 4.3c). Both of these cultures resulted in “fluff”-like growth.

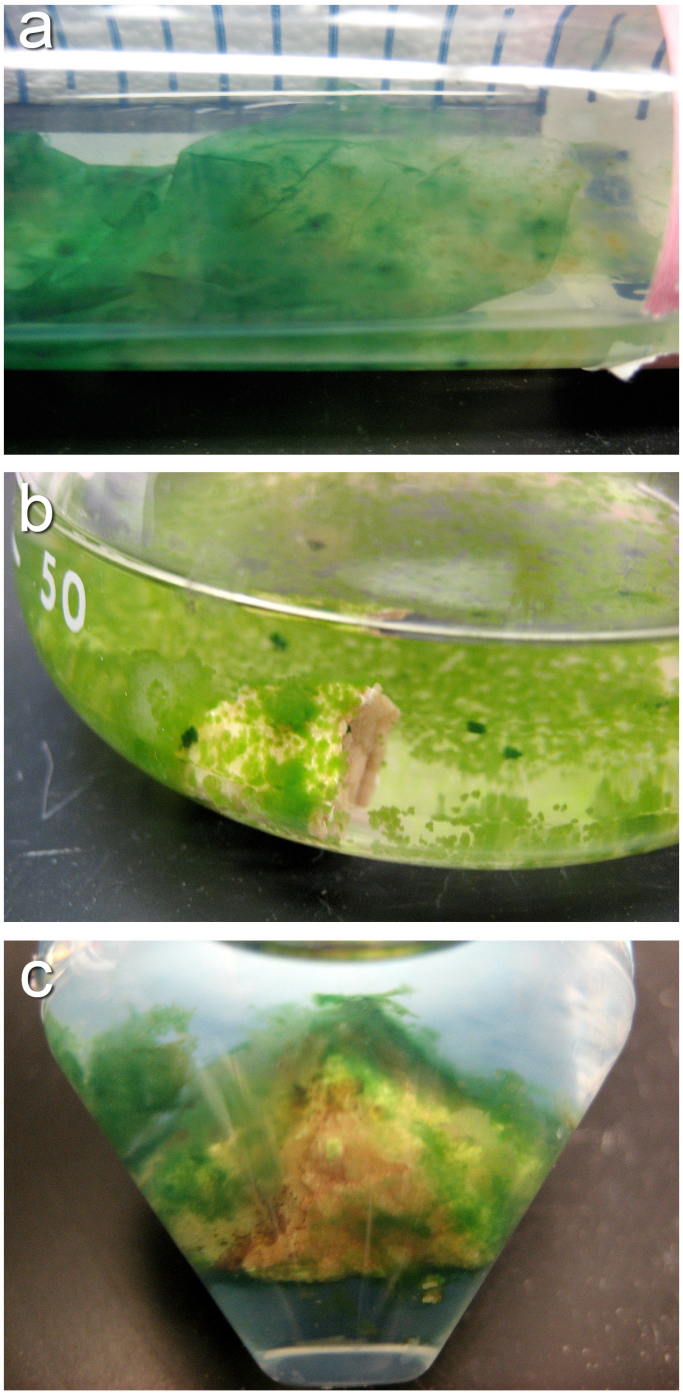


Figure 4.3: Growth of three endolithic cultures. a: subculture from Feng Cheng, b: culture grown at 3% salinity and c: at 0% salinity.

4.2.2 Ordination

Cultures grown under illumination at room temperature were used for DNA extraction, 454 sequencing and compared to their rock counterparts where possible. To explore variations in community makeup I used non-metric multidimensional scaling (nMDS) of OTU abundance data, and principal coordinate analysis (PCoA) of UniFrac distances (see Chapter 2 for details).

Figure 4.4 displays the dissimilarities and phylogenetic variations for the first two nMDS axes and first three UniFrac principal coordinates for all of the samples discussed above. The left column shows nMDS for untransformed and presence/absence transformed data, and the right columns display PCoA for weighted and unweighted UniFrac distances. In general, samples of the same type cluster together in the nMDS and PCoA plots, especially in the phylogenetic analysis.

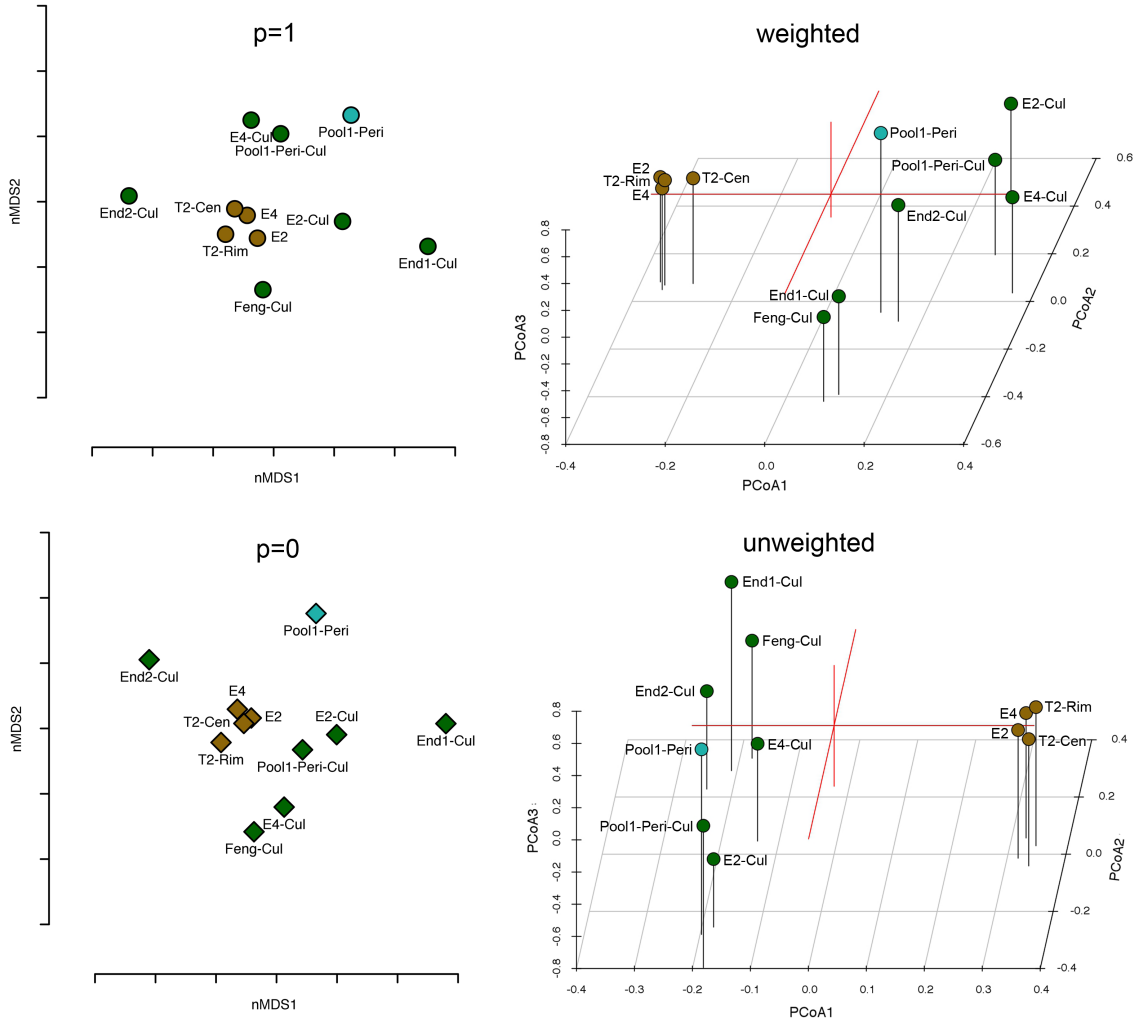


Figure 4.4: nMDS with Bray-Curtis dissimilarities (left) and UniFrac distances in PCoA space (right) for the culture isolates and endolithic communities. The nMDS results are shown for both untransformed (*p*=1) and presence/absence-transformed (*p*=0) data. The PCoA results are shown for both weighted and unweighted UniFrac distances. Only Cyanobacteria sequences were used in this analysis.

The Cyanobacteria from the endolith cultures are not similar to the uncultured endolithic communities. Cyanobacteria cultured from endoliths cluster with each other rather than with their environmental counterparts. The first principal coordinate for the UniFrac distances clearly separates the cultured and uncultured cyanobacteria, explaining 27.2% and 15.4% of the variability in the weighted and unweighted analyses, respectively (Fig. 4.4). The cyanobacterial

cultures are separated along the second PCoA axis, whereas all endolithic samples cluster tightly together. This result suggests that the cyanobacterial cultures do not represent the endolithic communities, and that the cultures have large variations among one another. This effect could be a result of the media used or an indicator that some Cyanobacteria are able to outcompete others under culture conditions.

A particularly interesting observation is that the environmental periphyton sample from pool 1 falls among all the cultured samples on the PCoA plot. I speculate that this may indicate that some of the organisms that were cultured from endolithic samples persist in those samples from periphyton earlier in the succession. While they may by minor OTUs in the natural environment of the endolith, perhaps they were “re-energized” by the culturing process, in a water and nutrient-rich medium that more closely resembles Troll’s pools than its endoliths.

The overall results of the culturing are similar to those of Lozupone and Knight (165), who showed that cultured isolates from seawater and sediment resemble each other rather than uncultured samples from the same environment.

4.2.3 Comparison of OTUs of cultures, endolith and periphyton

The UniFrac analysis in Figure 4.4 shows that cultured samples tend to cluster with periphyton samples, indicating similar phylogenetic composition. Figure 4.5 explores this relationship further, showing the distribution of OTUs in

cultures compared to endolithic and periphyton samples. Panel a shows all of the samples used in the culturing study, and panels b and c show portions of panel a in greater detail.

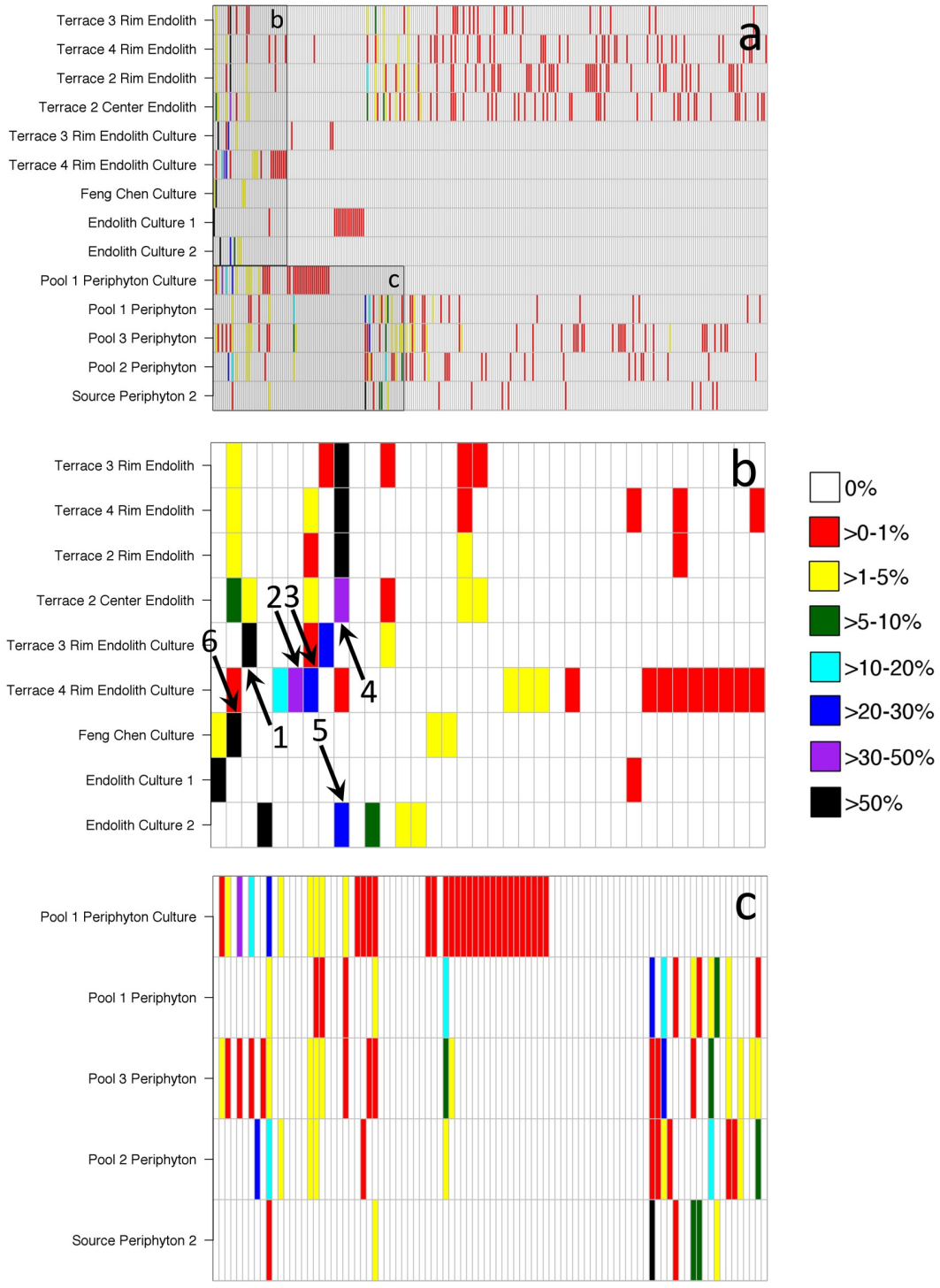


Figure 4.5: OTU distribution of cyanobacteria at 0.10 distance for all cultured samples compared to endolithic communities and periphyton. Panel a shows an overview. The OTUs are ordered according to the cultured samples, where all OTUs shared with these samples are on the left and unshared OTUs are on the right of the plot. Panel b and c are subsets. Arrows point to OTUs discussed in the text. Colors represent percentage contribution of each OTU to the whole cyanobacterial community in each sample.

A striking feature of Figure 4.5 is the low richness of cyanobacterial OTUs in the cultured endolithic samples compared to the environmental samples. Evenness is also low in the cultured endolithic samples, with high counts in just one or two OTUs. This reduction in richness may result from the change from dry conditions in the endolith to wet conditions of the culture.

Inspection of Figure 4.5 panel b suggests that most endolithic Cyanobacteria do not grow well in the culture medium, and are outcompeted there by Cyanobacteria taxa that are minor in the dry endolith. For example, the highest count OTU in the terrace 3 rim endolith culture (arrow 1) is not detected in the terrace 3 rim endolith. The highest count OTU in the terrace 4 rim endolith culture (arrow 2) is not present in any other samples, and the second highest count OTU (arrow 3) is found only in minor quantities in other endolithic samples. Interestingly, the major OTU (arrow 4) that is common to most endolithic samples is only present in low counts in a corresponding endolithic culture (terrace 4 rim endolith).

There are also significant differences between periphyton culture and the corresponding periphyton samples (panel c). For example, the pool 1 periphyton culture and the corresponding periphyton are not similar in their high count OTUs, but share some minor OTUs. Interestingly, some of the major OTUs of the culture that are absent in the pool 1 periphyton are found in other periphyton samples, such as pool 2 and 3 periphyton. Laboratory culturing conditions are different from the environmental conditions, possibly shifting the balance of competition and affecting the growth of organisms. Perhaps the pH of the media,

which probably is closer to the pH conditions in Pool 2 and 3, selected Cyanobacteria that were outcompeted and not detected in pool 1.

The Feng Chen culture and endolith culture 2 were grown in media with 3% salinity. Endolith culture 2 shows high counts in OTUs that are not present in other samples, perhaps because of selective pressures imposed by the salinity. It also shares one high count OTU (arrow 5) with all endolithic samples, some of which have very high counts as well. The Feng Chen culture also shares its highest count OTU (arrow 6) with the endolithic samples, although most of them are low count.

When considering the relationships among communities displayed in Figure 4.4, it is important to keep in mind how the OTUs were constructed. As pointed out in Chapter 2, OTU construction is strictly by percentage dissimilarity, whereas UniFrac makes use of phylogenetic relationships. The clustering of the endolithic samples in the UniFrac PCoA plots indicates that their Cyanobacteria are phylogenetically similar to one another, and different from Cyanobacteria in the cultures and periphyton. However, in the nMDS analysis the endolithic communities cluster in the middle among the cultured cyanobacteria. The nMDS analysis used the distribution of OTUs shown in Figure 4.5. The endolithic communities share some OTUs, mostly minor, with the cultures.

Figure 4.6 shows a comparison of cultures with several periphyton samples, to test the hypothesis that the media conditions simulated the aquatic environment, selecting for organisms that are well adapted to these conditions. The environmental periphyton samples are richer than all the cultured samples.

The cultured periphyton sample is richer than the cultured endolithic samples, perhaps resulting from cultivating organisms that were well adapted to an aquatic environment. The endolithic cultures are mostly dominated by one high count OTU (black bars), similar to the source periphyton 2. Interestingly, many cyanobacterial culture OTUs are shared with minor OTUs from pool 3 periphyton.

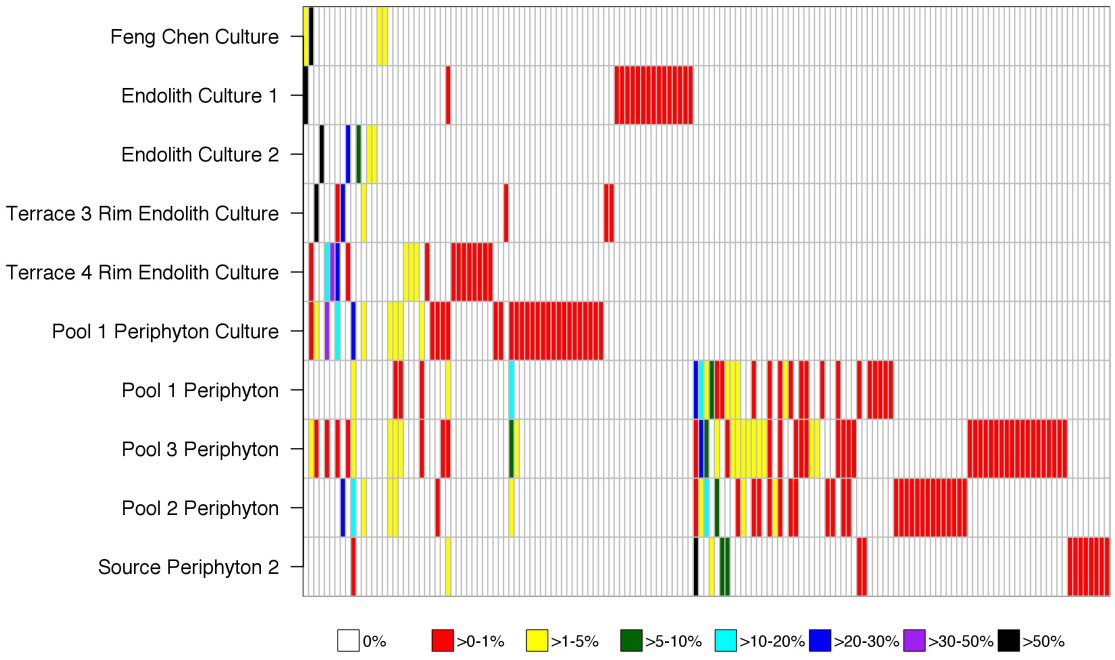


Figure 4.6: Cyanobacterial OTU distribution of cultures compared to periphyton communities at Troll. OTUs have been ordered according to the cultures, with OTUs shared between the cultures and periphyton on the left. Colors represent percentage contribution of each OTU to the whole cyanobacterial community in each sample.

The cultured communities cannot be identical to the natural periphyton communities because the culturing took place in a closed system. Because the culture flask does not permit organisms to enter the system, as verified experimentally by negative controls, only organisms that were present in the endolithic community could have been cultured. Cyanobacteria originally present

in the periphyton but extinct in endoliths could not appear in endolith cultures. Microorganisms that were minor or undetected in the endolith, and possibly a remainder from the periphyton, dominated the resulting culture. This resulted in large community distances between the endolith and their cultures in the nMDS and UniFrac plots. In contrast, the pool 1 periphyton culture clusters closer to its corresponding sample, pool 1 periphyton. However, their similarity is more phylogenetically based (UniFrac) than based on shared OTUs.

Culturing of endolithic samples resulted mostly in different cyanobacterial communities than are present in the endolithic community. In fact, the cultures are more similar to periphyton than to the endolith. Microorganisms that were not detected in the endolith grew in the aquatic setting, indicating survival of these organisms in the endolith below the detection limit. Similarly, Fenchel *et al*. (74) were able to culture 20 ciliate protozoans from lake sediments under native conditions, but when the sediments were tested under different environmental conditions an additional 115 organisms were cultured. The authors argued that the sediment contains organisms able to thrive under native environmental conditions, and also serves as a “seed bank” of other organisms that can flourish under different conditions. Similarly, I suggest that Cyanobacteria from the periphyton survived in the endolithic community and then flourished in the changed environmental conditions of the cultures.

4.2.4 Taxonomy

Figure 4.7 displays the phylogenetic composition of endolithic communities, pool 1 periphyton and cultured isolates. Pairs, such as endolith (E) and culture (C) or periphyton (P) and culture (C), are marked by black bars at the top. The cultures-only samples and endolith-only samples are marked with a green or orange bar, respectively.

The taxonomic comparison also shows that the cultured samples do not represent the endolithic samples. Interestingly, most OTUs in the cultured samples are unknown Cyanobacteria. The environmental endolithic samples show large similarities in their cyanobacterial composition, however, in each case their cultivated counterpart is very dissimilar. Additionally, although each culture originated from endolithic samples that are similar in composition (see Chapter 2), the cultured isolates are very dissimilar from one another. The taxonomic distribution is consistent with the separation of endolithic and cultured samples along the first PCoA axis, and also suggests why there is a large spread of the cultured samples along the second PCoA axis. While the unclassified Cyanobacteria would be better identified with full 16S sequences, the UniFrac results still clearly show that the cultured Cyanobacteria are not closely related to Cyanobacteria in the environmental samples.

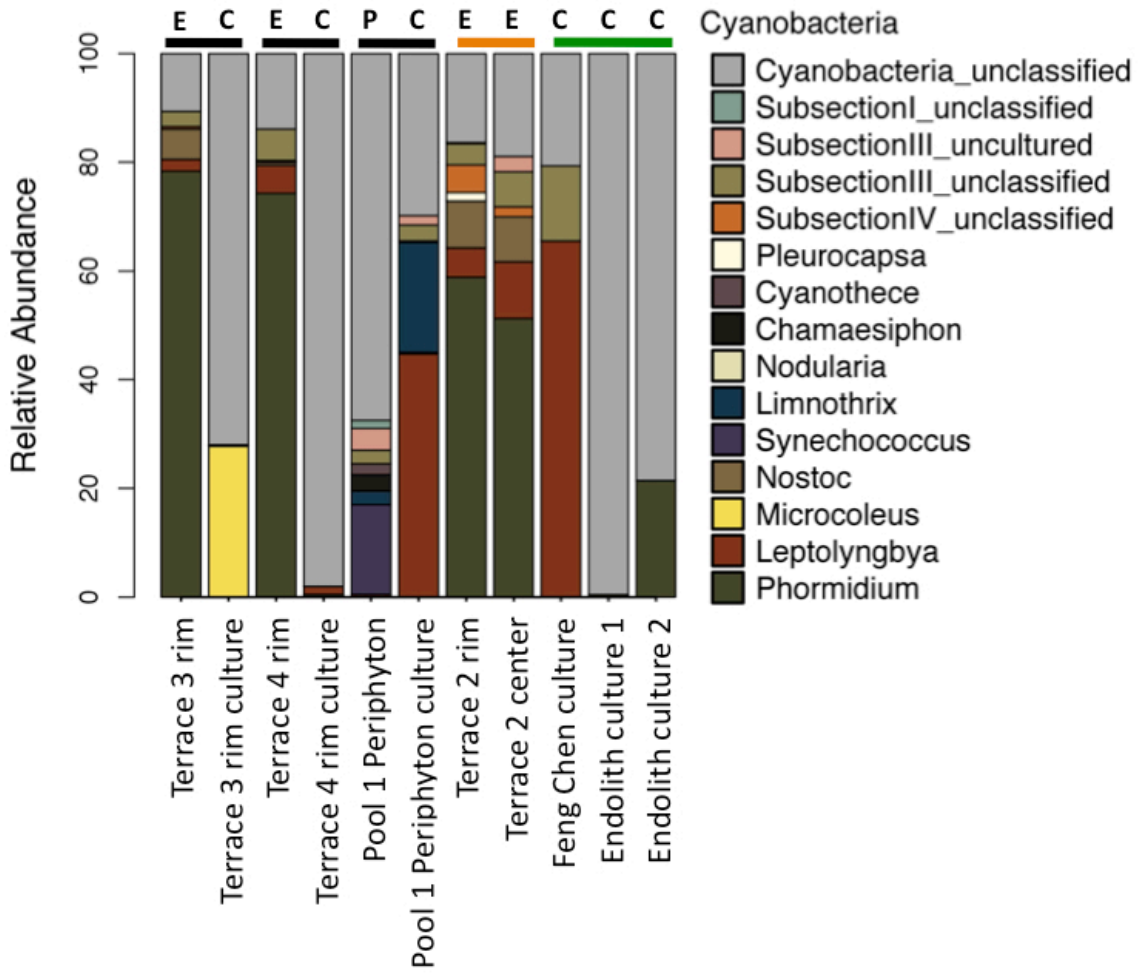


Figure 4.7: Cyanobacteria taxonomy for cultures in comparison to endolithic communities. The black bars indicate a pair of cultured isolates and the original environmental samples, the orange bar indicates two extra endolith communities for comparison and the green bar indicates the three cultured isolates shown in Figure 4.3. E = endolith, C = culture, P = periphyton.

Several studies have shown that there can be a low overlap between cultures and clone libraries. For example, Foster *et al*. (85) and Burns *et al*. (26) showed low similarities for Shark Bay stromatolites and other microbial diversity studies (64, 70, 180). Most authors argue that the limitations of both cultivation-based and cultivation-independent analyses of microbial communities are responsible for dissimilarities of cultures and environmental communities. It is possible that necessary environmental conditions do not persist in culture

enrichments, and community properties are lost under artificial growth conditions. Particularly for samples from Troll, where endolithic communities are exposed to dry, nutrient-poor, light-limited and cold environments, non-representative growth conditions may have existed in the laboratory, resulting in rapid growth of rare endolithic Cyanobacteria that are not a major part of the natural community. Therefore it is possible that culturing selects for isolates most adapted or able to grow under culture conditions, which may not necessarily represent the major composition of the original environmental samples.

Isolation and characterization of microorganisms usually involve standard culturing techniques and commercial or broad growth media. As mentioned above, culture-based techniques mostly limit the cultivation of observed environmental microorganisms to 1% of the community present (124). It is possible to circumvent some of the limitations of such media and maximize the cultivable fraction of microbial communities by mimicking the natural environment, including factors such as such as nutrients, temperature or pH. For example, the SN-media used in this study can be subsidized with seawater. Another example would be addition of soil. Therefore, it is possible that the SN medium was not ideal for the cultivation of the endolithic community from Troll Springs.

Culturing of soil samples has shown that Acidobacteria are difficult to enrich although these organisms constitute 20% on average of bacterial soil community (239). In contrast, Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria are cultured more easily due to their ability to grow under

laboratory conditions. Hugenholtz (124) argues that common culture methods misrepresent the natural community because the grown microorganisms rarely are abundant or functionally significant in the environment from which they were cultured. The author calls these microorganisms the “weeds” of the microbial world.

4.3 CONCLUSIONS

Because most bacteria can only be cultivated if their metabolic and physical requirements are reproduced in culture, the media and environmental conditions used here may not have represented the optimal conditions for growth and representation of the endolithic community. This effect perhaps led to cultivating Cyanobacteria that are just a minor fraction of the endolithic community.

Despite this finding, culturing is an important tool in characterizing microorganisms. Culture-base and culture-independent molecular techniques are not mutually exclusive and should be used to investigate the diversity, function, and ecology of microorganisms.

4.4 MATERIALS AND METHODS

4.4.1 Samples

Table 4.1: Samples used in the culture study

Environmental Sample		Cultured Isolate	Inoculation	DNA extraction	Comments
Terrace 3 rim endolith	E2	E2-Cul	8DEC08	2SEP09	Salinity 0%
Terrace 4 rim endolith	E4	E4-Cul	8DEC08	2SEP09	Salinity 0%
Pool 1 periphyton	Pool1-Peri	Pol1-Peri-Cul	5SEP08	14SEP09	Salinity 0%
Troll14-8-V7a*		End1-1-Cul	26FEB07	2SEP09	Salinity 0%
Troll14-8-V7a*		End1-2-Cul	26FEB07	2SEP09	Salinity 3%
Troll05-33SN30 10-12-05		Feng-Cul	26SEP07	2SEP09	Sub-culture, originally cultured by Feng Chen, Salinity 3%

* environmental samples were not sequenced

4.4.2 Culture conditions

Rock samples were broken apart in a laminar flow hood under aseptic conditions. Pieces of rock with a visible green line or samples of periphyton were added to the culturing media.

All the endolith isolates were grown in SN medium under different illumination and temperature conditions. The media recipe and direction were provided by Kui Wang (277). The cultures were grown in a flask and placed on the laboratory bench or in the refrigerator. Cultures grown in darkness were placed in a cardboard box and packed in aluminum foil to ensure that no light would come through.

Culture growth was documented for several months. A negative control (flask with media, without rock pieces) was placed at each culturing location to ensure that no random growth from the laboratory environment took place.

4.4.3 Sequencing and multivariate analysis

I used the same 454 sequencing, sequence processing, nMDS and UniFrac analyses described in Chapter 2.

CHAPTER 5: IMPLICATIONS AND FUTURE WORK

5.1 IMPLICATIONS: "EVERYTHING IS EVERYWHERE, BUT THE ENVIRONMENT SELECTS"

5.1.1 Introduction

Lourens Baas Becking, a Dutch microbiologist, stated in 1934 "Everything is everywhere, but the environment selects" (5). In this model, geographic barriers to dispersal are asserted to have no influence on microbial distribution, as they do for macro-organisms. This does not mean that microorganisms do not show biogeographic patterns, but rather that the environmental conditions at each location determine the distribution.

"Everything is everywhere, BUT the environment selects" is technically self-contradictory; if the environment truly selects than everything cannot be everywhere. The real point is that everything COULD BE everywhere if the environmental conditions were favorable. Dispersal of microbes by wind, water, and other agents can be highly effective, so the barriers of geography are less important to microbes than they are to macro-organisms. In the Baas Becking hypothesis, dispersal on a global scale provides a continuous supply of all types of microbes to all places. The microbes that encounter settings where the conditions suit them thrive in those settings – they are the ones that "the environment selects".

Microbial geographic distribution has been attributed by some authors primarily to environmental influences (114, 121, 154), in accordance with the Baas Becking hypothesis. Others have placed the main emphasis on historical events (*i.e*., geographic separation), independent of environmental factors (200, 283), while still others have invoked both (101, 294). Martiny *et al*. (178) suggest that the relative influence of environment and distance depends on spatial scale. They argue that geographic separation exerts the controlling influence over large distances (>3000 km), environmental conditions over small distances (<10 km), and that both are important over intermediate distances.

The situation is complicated by the relative ease with which single-celled organisms undergo mutations and horizontal gene transfer. Exchange of genes and changes in metabolic genomes are more likely in microbes than in complex organisms, allowing rapid adaptation to new environmental niches. Consequently, an organism's genome may change while their ribosomal genes remain the same. A biogeographic pattern determined using metabolic genes could therefore reveal the influence of local environmental conditions more readily than one determined using ribosomal genes. Using ribosomal genes could lead to a coarse pattern suggesting a cosmopolitan distribution, whereas use of metabolic genes could lead to finer scale pattern.

While it is possible in principle to examine biogeographic patterns by tracking the distributions of individual species, the species concept itself is clouded for microbes by horizontal gene transfer, especially if a genome is dependent on the environment. Microorganisms interact with each other, so it

may be more productive to use variations in community makeup as biogeographic indicators, rather than the distributions single microorganisms. Using communities instead of single organisms also allows leeway for minor underlying changes in situations where dominant organisms are the best biogeographic indicators. Minor taxa may tolerate or survive environmental conditions, but are not necessarily the drivers of that community. Nesbø *et al*. (187) specifically suggest that biogeography should be investigated on the basis of the global distribution of genes.

Issues of community stability and succession are also important. When we examine a community, are we seeing mostly ever-changing “weeds” in an environment, or are we looking at the steady “trees” in a forest? Weeds are capable of growing in many places very quickly (they are everywhere), but are also replaced quickly as the environment and the balance of competition change. Stable species are more permanent, but also more restricted to certain places and conditions. So is it more likely to find “weeds” everywhere but stable communities not? Are weeds everywhere and stable communities what the environment selects? Chapter 3 showed that endolithic communities at Troll are more fixed and stable than the periphyton in the pools, where environmental conditions change more erratically. Perhaps, then, it is examination of the endolithic communities at Troll that can most contribute to evaluation of the Baas Becking hypothesis.

5.1.2 Evidence From Troll Springs

The steep environmental gradients at Troll Springs are restricted to a small geographic setting, only a few hundred meters in size. Dispersal can be expected to be highly effective over such short distances, allowing the environment to select the microbial communities present. Indeed, that it what is observed. As shown in Chapter 2, microbial communities at Troll are governed primarily by temperature/pH in aquatic settings, and by water content in terrestrial settings. So within the confines of Troll Springs itself, it is changing environmental conditions that result in a particular pattern of communities.

Environmental conditions also select communities when samples from Troll are transported to the laboratory. Cultivation of cyanobacteria from endolithic communities (Chapter 4) led to a cyanobacterial community different form the endolith but more similar to the periphyton from aquatic environments. During cultivation, environmental conditions changed from terrestrial to aquatic (liquid media), from cold to warm (room temperature) and from dark to illuminated (overhead lights in the laboratory). The conditions in the cultures therefore resemble conditions in the pools, where periphyton are exposed to light, warm temperatures and water. Again, environmental conditions appear to select the community.

The most important contribution that my data make to evaluation of the Baas Becking hypothesis comes from comparison of endolithic communities at Troll Springs to those elsewhere. This comparison suggests that endolithic community makeup is driven more by environment than by location on global

scales. As discussed in Chapter 2, endolithic communities at Troll show strong similarities to those in alpine regions worldwide and elsewhere in the Arctic, even though the colonization process differs. The cool, moist environment at Troll is similar in many respects to that found in high alpine and other Arctic settings, but dramatically different from that of hot deserts, and even from the cold deserts of Antarctica. The similarity of Troll endolithic communities to alpine and other Arctic endoliths, and the contrast with others, therefore suggest that similar environmental conditions (*e.g.*, water content in the rock) select similar communities.

5.1.3 Discussion

As noted above, the debate about "everything is everywhere" involves both phylogeny and metabolism. While microorganisms may undergo rapid genomic changes driven by environmental conditions, that does not necessarily mean that their genetic fingerprint (*i.e.*, their ribosomal genes) will change. Therefore, it should not be assumed that the genetic relatedness between two microorganisms will be completely independent of geographic distance. Using phylogeny helps determine the distribution of microbes, but it does not give adequate insight into their metabolic processes.

We are comparing apples and oranges when we compare phylogeny and metabolism as applied to biogeography. Of course, both are important and not mutually exclusive, but each tells a different story. Where metabolic genes can change as a consequence of adaptation, ribosomal genes are less likely to. So it

is not surprising to find organisms with identical16S in similar environments, but that their metabolic genes show differences according to their geographic location. Local environmental conditions and competition from other organisms present will drive evolution and diversification of metabolic genes.

How do the results of this dissertation bear on the debate over the Baas Becking hypothesis? Everything could theoretically everywhere, but my results, particularly for endoliths, suggest that the environment is a driving selection factor. I don't think that microorganisms that make up the endolithic communities at Troll are ubiquitous everywhere, but they are cosmopolitan (ribosomal gene) in the range of environmental settings that are appropriate for them. Dispersal of these organisms is facilitated by their small size and perhaps their ability to survive long periods transport while dormant. So, my results support the idea that similar environments favor similar microbial communities. Whether or not they are metabolically the same, however, depends on finer underlying parameters of that environment.

5.2 FUTURE WORK: WHEN THE LIGHTS GO OUT AT TROLL

In the previous chapters, I described analysis of data collected at Troll Springs during the summer. However, polar regions experience extreme seasonal changes in illumination, with months of constant daylight in the summer and darkness during the winter. These changes, in particular the extended period of darkness, will have an influence on photosynthesis, and potentially on microbial community makeup and structure at Troll. Although I have not yet

collected samples in seasons other than summer, I discuss here possible hypotheses regarding annual changes at Troll. These hypotheses can help guide future work that will provide an even more complete picture of the microbial ecology of Troll.

5.2.1 Hypotheses

Hypothesis: ***The periphyton in the pools mostly die during the winter months, leaving behind organic matter as a potential food source. The pools are re-colonized by the same community in the spring.***

The microbial communities in the pools are subjected to large and rapid changes in environmental parameters like pH and temperature. It is therefore possible that organisms mostly die during the winter and then re-colonize the pools in the spring, locally restarting the process of succession (as described in Chapter 3). As described in Appendix A, free-floating filament samples collected in 2008 and periphyton samples collected in 2009 cluster together in their phylogeny for pools 1 and 2. This observation suggests that the communities in these pools were similar in those two successive summers, though it provides no information about community makeup in the intervening seasons. If this inference is correct, then if the periphyton died during the winter they became re-colonized the next year with a similar community. Similar repeated seasonal periodicity has been shown for chlorophytes and Cyanobacteria, especially *Phormidium*, in fellfield soil (59) and streams (110) of Signy Island in Antarctica. Both

environments showed rapid spring growth of filamentous chlorophytes which then later were replaced by cyanobacteria as snow and ice disappeared. Both papers suggest that inocula either overwinter or are deposited from the melting snow. Hawes (109) showed that stream chlorophytes can overwinter as single-celled vegetative propagules. The Antarctic freshwater Cyanobacteria are adapted to environmental conditions there and grow rapidly under ambient environmental conditions (111). Davey *et al*. (59) reported a period of winter growth of *Phormidium* in fellfield soil, perhaps indicating that these cells are viable during the dark and cold period. Significantly, algae and Cyanobacteria re-grow from small inocula each year, where the spring populations of chlorophytes are required to grow rapidly at low temperatures followed by Cyanobacteria. This periodicity suggests that such a community can rapidly recover to its summer state following a seasonal disturbance.

Hypothesis: ***The microbial community is able to adapt to constant darkness by changing metabolic preferences.***

Typically Cyanobacteria are described as photoautotrophic organisms, but certain genera, like *Prochlorococcus, Synechococcus, Anabaena, Nostoc, Pseudoanabaena* and *Planktothrix*, can take up organic compounds (35, 69, 198, 227). Microorganisms capable of photosynthesizing as well as taking up organic carbon as a food source are called mixotrophic. In marine and freshwater ecosystems of the polar regions in particular, mixotrophy has been proposed to be widespread (184). Cottrell *et al*. (47) showed that normally photoheterotrophic

microbes can also inhabit polar waters during extended periods of winter darkness. In particular, they found that *Synechococcus* abundances were equivalent in winter and summer, suggesting that mortality is balanced by metabolism supported by consumption of organic matter.

Hypothesis: ***The summer phototrophic microbial community is not able to adapt to winter conditions, leading to seasonal change of the dominant community from phototrophic to heterotrophic.***

With decreasing sunlight and photosynthesis in winter, phototrophic organisms may be replaced by heterotrophs, resulting in a change in the community. It is likely that heterotrophic organisms sustain their rates of production year round; it is less clear whether the complete phototrophic community would disappear. It is plausible that some photosynthetic biomass is preserved all year around. For example, photosynthetic eukaryotic algae in polar regions are able to survive long periods in the dark (25, 169), using heterotrophy or storage products and reduced metabolic rates to survive. McMinn *et al*. (182) showed that sea ice algae cells remain active and are adapted to their ambient light environment, followed by relatively high growth rates as the sun returned in spring. Interestingly, the authors suggest that there are algae that are acclimated to the lowest light levels, but not to higher illumination, resulting in different strategies selected to survive darkness. This would imply a possible autotrophic succession as illumination increases, rather than a complete switch to heterotrophy. In contrast, some studies in non-polar regions reported that

bacterial biomass in oligotrophic seawater can exceeds that of phytoplankton (36, 92), resulting in heterotrophic dominance in winter when little or no solar irradiation is available. It needs to be determined, however, if adaptation to the extreme illumination variations in polar regions takes place, perhaps resulting in an autotrophic-heterotrophic consortium during winter.

5.2.2 Discussion

Figure 5.1 shows processes involved in succession and seasonal changes at Troll. As discussed above, the periphyton in the pools may mostly die during the winter months, while the endolith community may more stable and overwinter.

The black arrows in Figure 5.1 represent non-seasonal changes in environmental conditions, for example driven by changing air temperature or shifting patterns of water flow, that take place over timescales long enough drive changes in community makeup. As I showed in Figure 2.17, changes in temperature drive corresponding pH changes; both may drive variations in community makeup. The microbial communities in the pools may therefore be in a near-constant state of succession, with changing primary production processes (eukaryotic and bacterial) and phylogenetic composition as environmental conditions change. As shown by the double-ended black arrows in Figure 5.1, conditions in the pools can change in complex and sometimes repeatable ways, cycling through a range of conditions and communities.

Strong seasonal changes in illumination and temperature could also cause major community changes on an annual basis (gray arrows). This succession could be repetitive from year to year, with pools returning to their previous state each summer. Similar seasonal patterns have been observed for bacterioplankton communities in several systems, in which communities reassemble year after year (52, 94, 144). The seasonal pattern at Troll would be particularly pronounced if a heterotrophic community replaces the autotrophic community in the winter. Mixotrophic changes in metabolic preferences could also come into play, with organisms changing to carbon uptake processes when illumination is low. The observations found from studying algae and Cyanobacteria seem more compatible with a mixotrophic approach than a complete switch to heterotrophy.

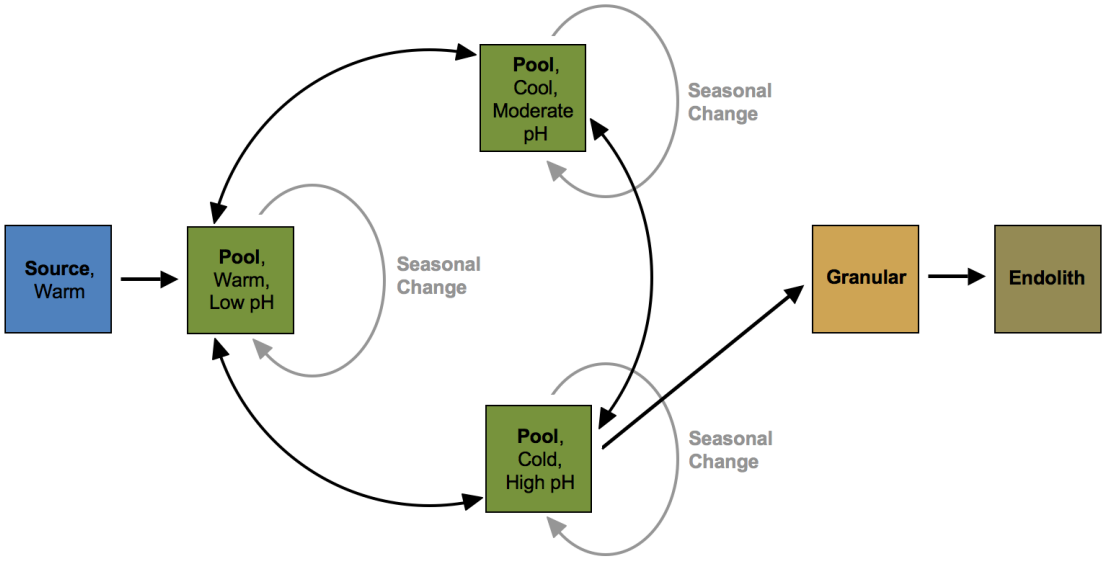


Figure 5.1: Processes involved in succession and seasonal changes at Troll. See text for details.

All of the endolithic communities sampled at Troll are similar in community makeup, despite being in rock of widely varying ages and having been collected in different years. This observation suggests that community makeup in the endoliths is relatively stable over time. Because the endolithic communities are apparently more stable than then pool communities, it is unlikely that undergo succession on a seasonal basis. And although it is possible for multiple successional cycles to take place in the aquatic communities, the end stage is always the endolithic community.

Vestal *et al.* (270) showed that endolithic communities in Antarctica are able to photosynthesize at temperatures below 0ºC at very low light levels, indicating that autotrophic and heterotrophic processes can continue during much of the Antarctic winter season. The endolithic communities are surround by dense EPS sheets, providing the possibility to store water, contributing to their ability to survive harsh winter conditions. Again, the protective environment of the endolithic community may make it possible for well adapted organisms there metabolize when water-based organisms are already frozen.

5.2.3 Summary

Any of these hypotheses could be at work during the winter months at Troll, and they are not mutually exclusive. A daunting but important challenge for future work will be to visit Troll during the cold and dark winter months, and obtain

samples that can help complete the picture of microbial in this unique Arctic environment.

CHAPTER 6: PEAK HEIGHT THRESHOLD SELECTION FOR ARISA DATA PROCESSING

6.1 INTRODUCTION

DNA profiling or fingerprinting techniques are an essential part of microbial ecology. One of the most widely used such techniques is automated rRNA intergenic spacer analysis, or ARISA (82). ARISA depends on automated capillary electrophoresis of fluorescent labeled DNA fragments, using it to discriminate intergenic fragment sizes present in a sample.

When analyzing electropherogram profiles, two types of noise are typically present, which I refer to here as “instrument noise” and “sample noise”.

Instrument noise refers to artifacts produced by the instrument because of issues such as the polymer and capillaries used or cleanliness and alignment. It can differ among instruments, as well as among samples and even between sample replicates. Instrument noise is usually removed by applying a conservative minimum peak height threshold to the raw electropherograms. The goal is to set this threshold as low as possible, eliminating noise from the data but preserving all peaks that rise above the instrumental noise level. The appropriate background value can be determined by observing background levels in the absence of a signal. Such a threshold is necessary for virtually all ARISA data. Typical values used have been in the range of 50-250 fluorescent units (18, 93, 94, 146, 168, 218, 291, 295).

Sample noise, in contrast, refers to false peaks resulting from PCR artifacts or sample contaminants. Sample noise is normally dealt with by applying an additional threshold that is based on the percentage of total amplified DNA. It is particularly important to set such thresholds carefully when techniques that are based solely on the presence or absence of peaks are to be used in the subsequent analyses (113, 159, 294, 295), since presence/absence analyses disproportionately weight weak peaks. In past work, such thresholds have typically been applied across a whole sample set, treating all samples identically. Values used by various research groups have included 0.09% (94, 114, 226), 0.1% (218), 0.5% (113) and 1% (23, 30) of the total fluorescence.

A more sophisticated approach was that of Luna *et al*. (168), who developed a method that dynamically determines the threshold depending on the characteristics of all profiles in a data set. Their method takes into account the maximum number of peaks that can be observed in a profile, and selects a threshold based on the conservative assumption that that many peaks each contribute equally to the total fluorescence – *i.e*., the threshold is 100% divided by the maximum observable number of peaks. Peak height thresholds calculated in this fashion depend on the data set, with reported values of 0.32% (168), 0.24% (29) and 0.11% (58). The calculation of this threshold is a theoretical value for the highest number of operational taxonomic units (OTUs) that can be detected using that technique.

Based on results obtained in Chapters 2 and 3, I argue that the threshold used to eliminate sample noise should in fact be sample dependent. The reason

is that species richness variations among samples can affect standardized peak heights; *i.e.*, the percentage contribution to the total fluorescence will decrease for each peak the more rich in species a sample is. Applying a uniform threshold across a complete sample set can therefore result in deletion of desired peaks in a species-rich sample. I argue instead that each sample replicate pair should be treated individually.

The question addressed in this chapter is how to find the best value for ARISA peak height thresholds.

6.2 BACKGROUND

6.2.1 Data standardization and the effect of varying richness

The normal procedure in ARISA data reduction is to standardize data, allowing for more direct comparison of samples to one another. Standardization is performed by summing the all the counts in a profile, dividing each peak height by that sum, and converting to a percentage. Each standardized peak height therefore gives the percentage of the total counts for that sample that lie in that peak. This procedure is particularly important when ARISA runs differ in their peak intensity. As noted above, after standardization, a common threshold is typically applied to all samples.

The problem arises when one attempts to cross-compare standardized samples to which a common threshold has been applied. Figure 6.1 illustrates two samples with different richness, where sample 1 has 20 peaks and sample 2

has 80 peaks with a similar distribution of heights. As noted above, standardization to 100% takes *all* peaks into account. So if we compare two samples that have similar peak heights, such as 1000 and 500 counts in sample 1 and the same values in sample 2, then these peaks will be standardized to dramatically different percentages because sample 2 is much richer than sample 1, and therefore contains many more total counts.

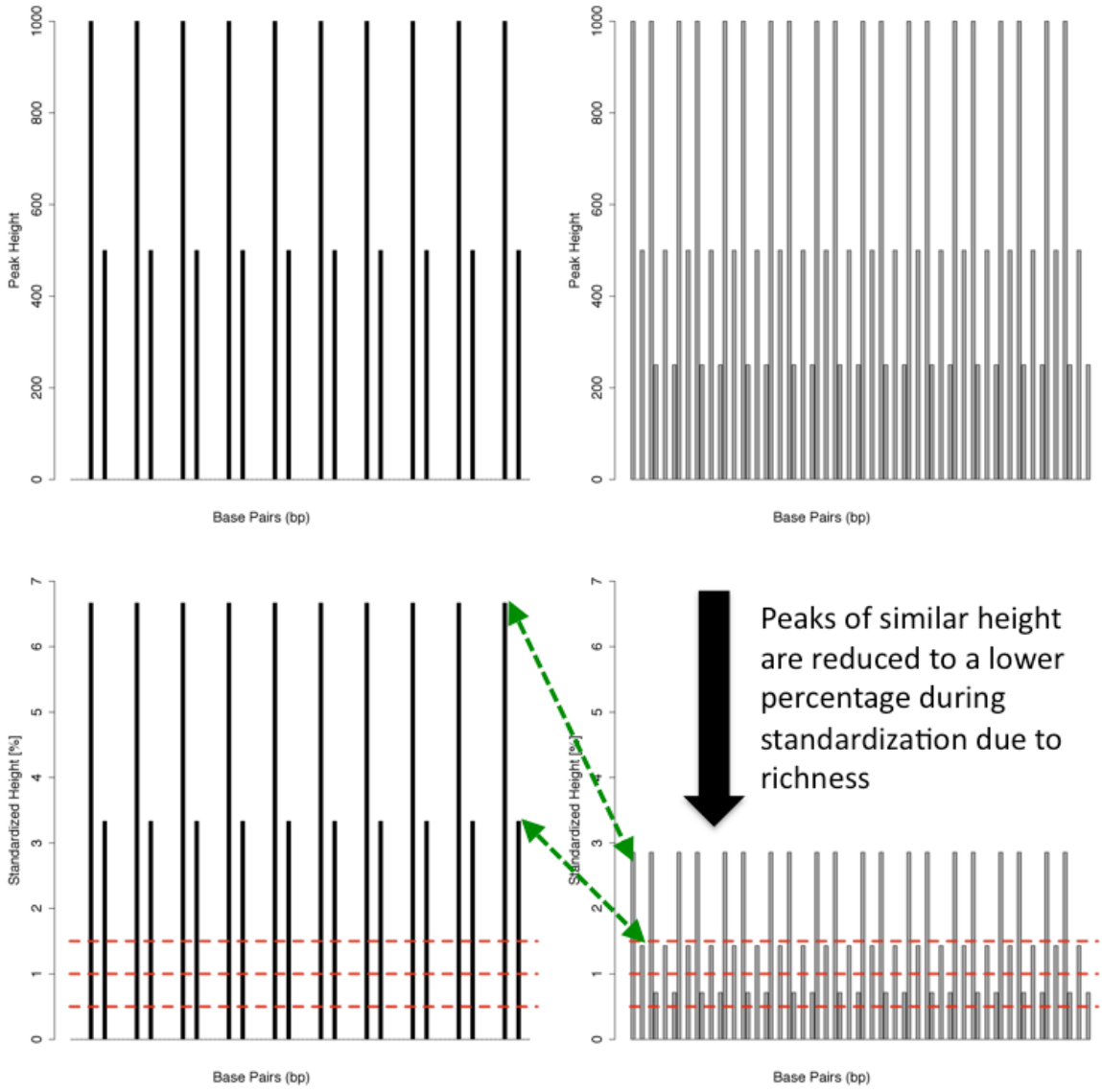


Figure 6.1: Comparison of samples analyzed with ARISA. Sample 1 on left; sample 2 on right. The upper row shows the original counts, while the lower row shows the data after standardization to 100%. Threshold lines in the lower row are drawn at 0.5%, 1% and 1.5%. Use of a common threshold for both samples can eliminate many valid peaks in the richer sample.

After standardization, originally equivalent 1000-count peaks have been standardized to 6.6% in sample 1 but just 2.8% in sample 2, reducing their effective height by more than half although they originally had the same height.

This potentially can put valuable data peaks below a threshold line for a rich sample, resulting in data loss if that threshold is used.

Instead of using a common threshold across all samples, I conclude that it is important to select thresholds that are sample dependent and take richness into account.

6.2.2 Comparison of replicate pairs

A similar problem can arise when comparing two replicates in a pair. Figure 6.2 shows a replicate with the same number of peaks in each, but different peak intensities (peak heights). Also shown are noise peaks of comparable height present in both replicates (red). Replicate 1 is identical to replicate 2 in data peak distribution and noise level, except that the peaks in replicate 2 are half the intensity of those in replicate 1.

Standardization will equalize both samples. However, although the noise is the same in both replicates, it will gain weight in replicate 2 as soon as the replicates are standardized (Fig. 6.2 lower right). Some of these noise peaks could then be counted as data peaks if not thresholded properly.

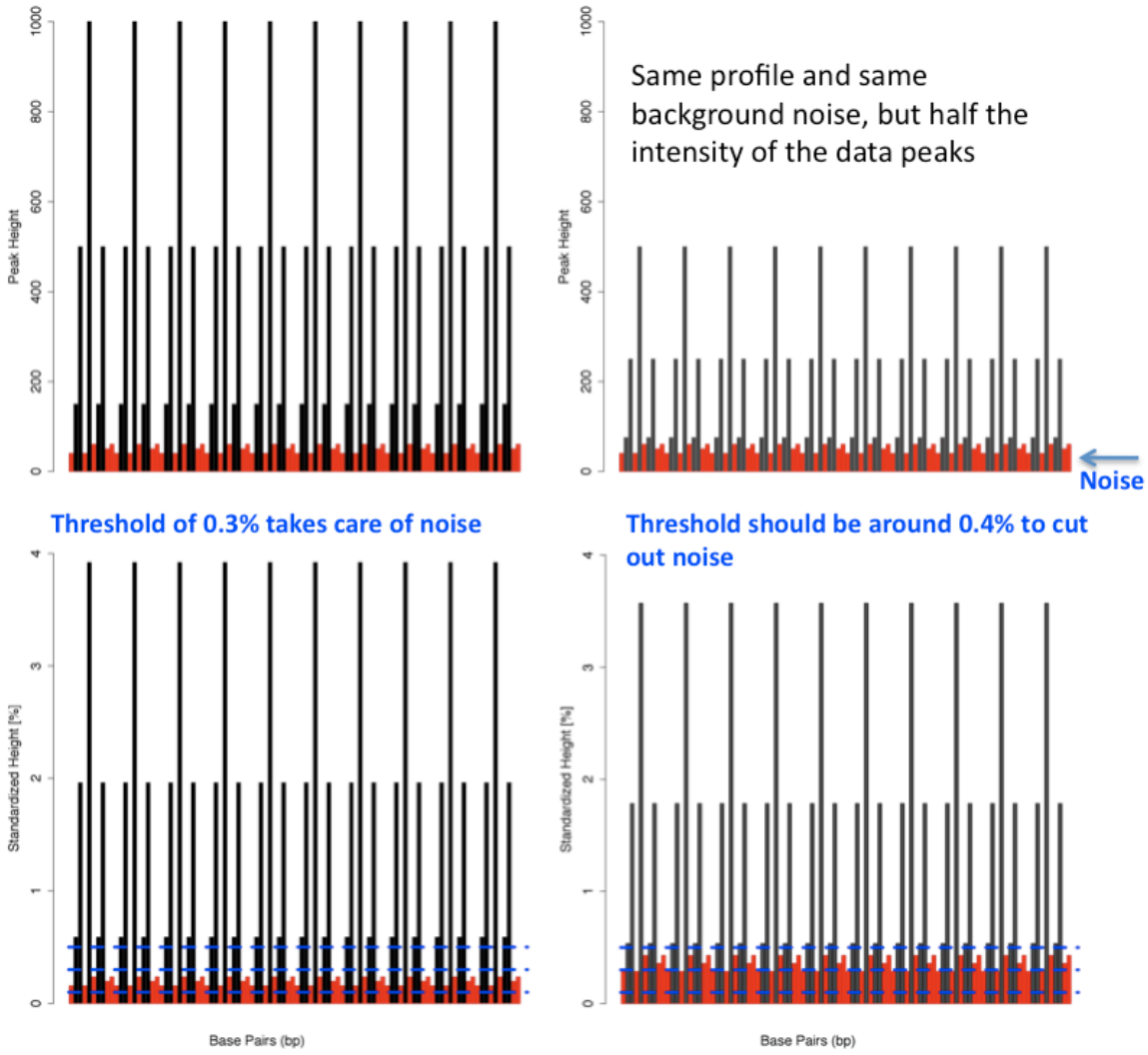


Figure 6.2: Comparison of replicates analyzed with ARISA. Replicate 1 on left; replicate 2 on right. Upper row shows original counts before standardization; lower row shows the standardized data. Red peaks are noise, blue dashed lines are thresholds drawn at 0.1%, 0.3% and 0.5%.

Thresholding of the replicates will cut out the noise. However, if the same threshold is applied to both replicates then either peaks will be lost or noise will be interpreted as data. For example, if a threshold of 0.3% were applied to replicate 1 then it would eliminate the noise, but it would not be appropriate for replicate 2 because some noise would be kept. Again, it is most appropriate to apply a properly chosen threshold to each replicate separately.

6.2.3 Sources of sample noise

ARISA is based on PCR amplification of the 16S ribosomal gene. When using environmental samples, the PCR template is a mixture of different 16S rRNA genes (depending on the taxa) with different ratios, potentially leading to biases during amplification. PCR can show two types of error categories: sequence artifacts (leading to false positives) and unequal amplification or efficiency (leading to false negatives). Sequence artifacts can arise due to the formation chimeras (19, 125, 151), polymerase errors or heteroduplex formation (221, 233, 249), and are considered false positives because PCR results in a signal not represented by an OTU. In addition, PCR biases can result in skewing the distribution of PCR products due to the amplification efficiency of the template and amplification inhibitions by most abundant templates.

PCR bias can lead to incorrect product-to-template ratios, resulting in different replicate profiles. Two sources for these biases are PCR selection and PCR drift (272). PCR selection refers to a preferred amplification of genes due to properties of the gene or its genome. Polz and Cavanaugh (215) showed that the signal ratios of tested genomic templates never corresponded to the ratios of the PCR products. Additionally, replicates showed variation. The authors concluded that PCR selection is the driving force for unequal amplification of templates. Low template concentration can lead to stochastic fluctuations in the early cycles of PCR, such as primer annealing to the genomic template. The authors suggest combining several replicate PCR amplifications to minimize PCR drift and to increase reproducibility.

PCR drift is caused by stochastic variations in the early stages of amplification from the genomic template that are not repeatable in replicate reactions (272). The genomic template might be associated with proteins or form second-order structures, consequently interfering with primer annealing. Wagner *et al*. (272) showed that PCR drift increases for decreasing numbers of initial molecules. Consequently, rare OTUs could cause PCR drift in a sample resulting in single peaks that are not repeatable in a replicate.

6.2.4 Other steps in ARISA data processing

Once a peak height threshold has been applied to the raw electropherograms to account for instrument noise, three other steps can be performed in addition to thresholding to eliminate sample noise: binning, additional peak rejection, and outlier rejection. Here I assume that the researcher has used the standard practice of obtaining two ARISA measurements (a replicate pair) for each sample in a sample set.

Binning, *i.e.*, combining ARISA peaks that lie close to one another into a single OTU, is typically performed in order to account for variability in peak calling, migration and run-to-run variations. Several different binning approaches have been used, including a fixed binning window (226), a changing binning window depending on the fragment size (94) and binning to the nearest integer (113).

Peak rejection is the process of eliminating peaks believed to be false positives. Some researchers reject single peaks that are not present in both replicates for a given sample (58, 152, 168, 218). However, as noted above, sometimes it cannot be determined if single strong peaks are artifacts or real peaks. Additionally, peaks can shift by several base pairs in one replicate due to instrument variability, and consequently can be rejected as false peaks unless some accommodation for this variability is made.

Outlier rejection is the process of removing from the data set any replicate pairs that are so different from one another that they cannot be considered useful replicates. One quantitative approach to this problem was that of Ruan *et al*. (232), who computed pairwise binary dissimilarities for unbinned presence/absence transformed profiles, and rejected as outliers any replicate pairs whose dissimilarity was not within 1.96 standard deviations (95^{th} percentile) of the mean dissimilarity for all samples in the set.

6.3 APPROACH

My approach to setting thresholds that eliminate sample noise is based on comparing two replicate profiles for each sample in a suite. Thresholds are calculated individually for each replicate in a pair, and separately for each sample. The thresholds are selected to be the lowest ones that acceptably minimize the dissimilarity between the replicates after thresholding. If a choice of threshold results in the two replicates in a pair failing some quantitative test of similarity, either that threshold or that sample must be rejected. An appropriate

test of similarity can be used both to set thresholds and to recognize “outlier” samples that should not be used in the analysis.

The test of similarity must be chosen with care. ARISA data are usually analyzed by noting just the presence or absence of peaks, rather than using their heights. Popular measures of similarity that are based on presence/absence alone include Sørensen’s index (which is equal to one minus Bray-Curtis dissimilarity) (20), and Jaccard’s index (132). If peak heights were to be considered, then standard statistical measures of similarity like Pearson’s product-moment correlation coefficient (Pearson’s *r*) could be used.

The measure of similarity used to select the thresholds should be compatible with the analysis techniques that will be used subsequently. If presence/absence-based techniques will be used, then a measure of similarity based on presence/absence is called for. Again, this is because weak peaks become disproportionately important when presence/absence is used. I have therefore chosen Sørensen’s index. In practice, what I calculate is Bray-Curtis dissimilarity, which is one minus Sørensen’s index. I compare the Bray-Curtis dissimilarities for each thresholded replicate pair to one another, and declare any that are more than a chosen number of standard deviations above the median to be outliers.

6.4 IMPLEMENTATION

In implementing the approach described above, steps are performed in the following order:

Step 1: Application of a minimal threshold, in fluorescent units, to all profiles, to eliminate instrument noise.

Step 2: Standardization of all profiles to 100%.

Step 3: Application of a range of possible thresholds to both replicates in each pair.

Step 4: Fixed-width binning of both replicates in each pair for all possible threshold combinations.

Step 5: Presence/absence transformation, a conservative way of circumventing PCR biases.

Step 6: Computation of Bray-Curtis dissimilarity for all possible threshold combinations.

Step 7: Selection of the optimal thresholds for each replicate in each pair.

Step 8: Identification of outlier samples.

Steps 1 and 2 have been described already. The additional steps are described below.

Step 3: The goal of the algorithm is to assess all possible combinations of reasonable thresholds, selecting the two thresholds for each pair that yield the best result. As noted in the Introduction, threshold values used in the literature have spanned the range from 0.09 to 1.0% of total standardized fluorescence. I therefore allow the choice of threshold for each replicate to vary from 0.0 to 1.0%

in steps of 0.01%, making a total of 101x101 = 10,201 possible combinations. For all combinations, the thresholds are applied to each pair by eliminating all peaks with a standardized peak height lower than the threshold.

Step 4: Due to the nature of fragment analysis, there is some uncertainty in the estimates of ARISA fragment length, which can be circumvent by binning OTUs as described by Fuhrman *et al*. (94). After thresholding, the standardized ARISA data are binned using the fixed binning capability of dpbin (232).

ARISA fragments are analyzed using genetic analyzers that are typically less accurate as fragment size increases (22). It is therefore usual to use a "fixed window" bin size with a width that is adjusted depending on fragment size. Several studies have used different bin size strategies. Hewson and Fuhrman (114) used 3 bp for fragment lengths up to 500 bp and 7 bp for fragments larger than 500 bp. Fisher and Triplett (82) used 1-2 bp for fragment sizes below 1000 bp, 3-5 bp for fragments up to 1150 bp and 13 bp for the largest fragments. Brown *et al*. (22) used 3 bp for fragments ranging from 400 to 700 bp, 5 bp from 700 to 1000 bp and 10 bp for fragments from 1000 to 1200 bp. For this work, I used bin sizes of 3 bp for fragments ranging from 300 to 500 bp, 5 bp from 500 to 1000 bp and 9 bp for fragments from 1000 to 1200 bp, consistent with the work of Ruan *et al*. (232).

Thresholding takes places before binning because unwanted peaks could otherwise influence binning results.

Step 5: After binning, each profile is transformed to presence and absence, equalizing all peak heights as is typically performed for ARISA data.

Step 6: After presence/absence transformation, Bray-Curtis dissimilarity is calculated for each replicate pair for all combinations of the pair's two thresholds. The result is a 101 x 101 matrix of Bray-Curtis dissimilarity values, as shown schematically in Figure 6.3 A.

Step 7: The important next step is selection of the two thresholds that yield the best result. This step is performed as follows:

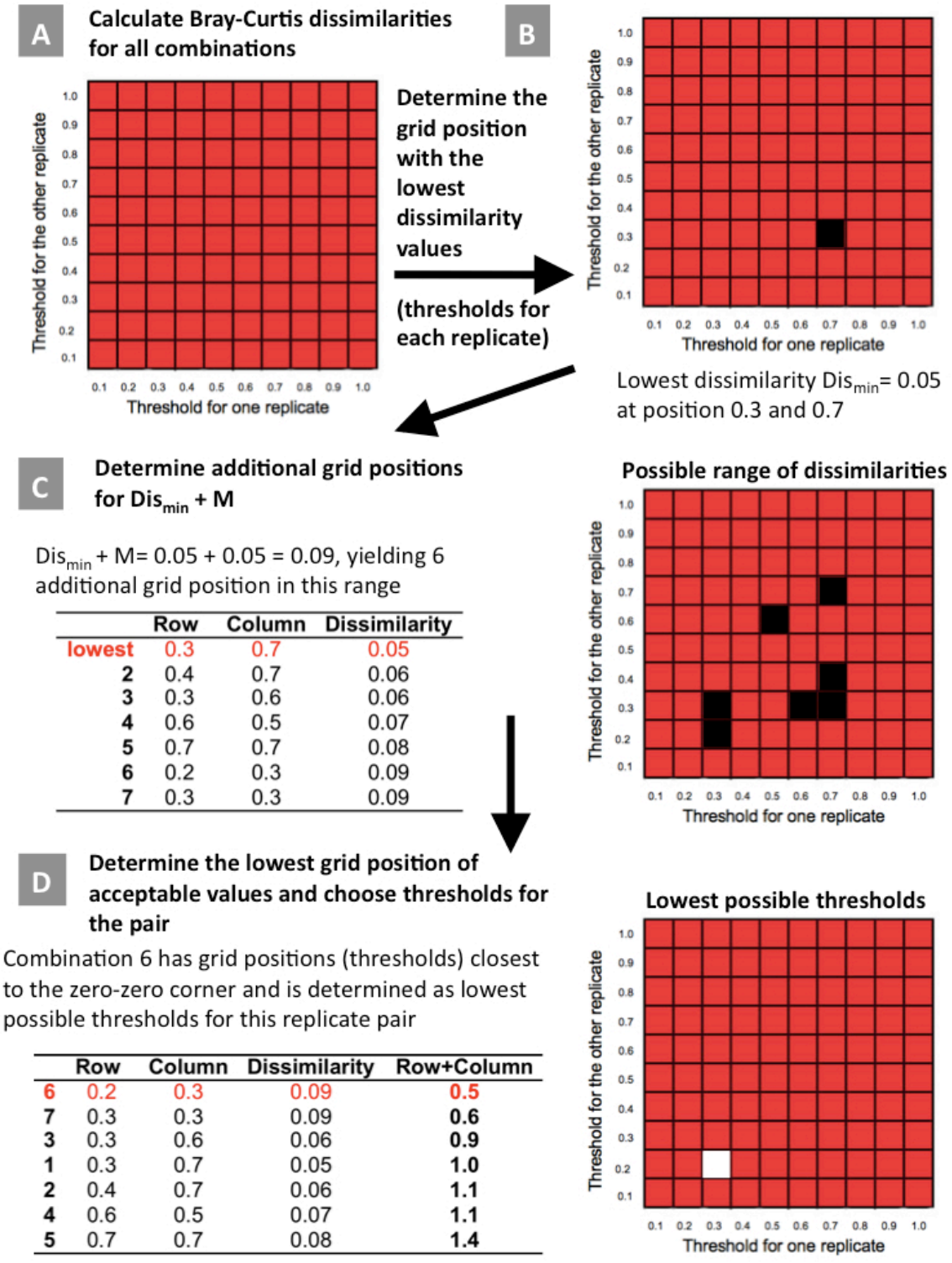
7.1. Find the matrix location with lowest dissimilarity, Dis_{min}. In Figure 6.3 B, Dis_{min} (0.05) is at threshold positions 0.3 and 0.7.

7.2. Select an amount M by which it will be considered acceptable for the dissimilarity yielded by the chosen thresholds to exceed Dis_{min}.

7.3. Find all combinations of Threshold 1 and Threshold 2 that yield a dissimilarity that is less than or equal to Dis_{min} + M. In Figure 6.3 C, M = 0.04, meaning that any dissimilarity values from 0.05 and 0.09 and their associated thresholds (black squares) are considered to be in the acceptable range.

7.4. Sum the row and column values (Threshold 1 + Threshold 2) for all acceptable threshold pairs.

7.5. Select the threshold pair with the lowest summed value of (Threshold 1 + Threshold 2). In Figure 6.3 D, of the seven possible combinations, number 6 (Threshold 1 = 0.2 and Threshold 2 = 0.3) is the one selected for this sample pair.



	Row	Column	Dissimilarity
lowest	0.3	0.7	0.05
2	0.4	0.7	0.06
3	0.3	0.6	0.06
4	0.6	0.5	0.07
5	0.7	0.7	0.08
6	0.2	0.3	0.09
7	0.3	0.3	0.09

	Row	Column	Dissimilarity	Row+Column
6	0.2	0.3	0.09	**0.5**
7	0.3	0.3	0.09	**0.6**
3	0.3	0.6	0.06	**0.9**
1	0.3	0.7	0.05	**1.0**
2	0.4	0.7	0.06	**1.1**
4	0.6	0.5	0.07	**1.1**
5	0.7	0.7	0.08	**1.4**

Figure 6.3: Schematic depiction of the algorithm for threshold selection. See text for details.

The parameter M is intended to have a "common sense" value that allows dissimilarities that are “close enough” to Dis_{min} to be considered. For example, if two thresholds of 0.9 and 0.8 led to a dissimilarity of 0.06 and two thresholds of 0.4 and 0.6 to a dissimilarity of 0.07, then it might be reasonable to choose the lower threshold values, although their dissimilarity is slightly higher, to include more peaks in further analysis. Therefore, M should be chosen to provide the desired definition of what "close enough" means.

Step 8: Once thresholds have been selected for all replicates in all pairs, the final step is to identify any replicate pairs that should be rejected as outliers. This step is performed as follows:

8.1. Determine the Bray-Curtis dissimilarity value (replicate 1 vs. replicate 2) for the chosen thresholds

8.2. Determine the median and standard deviation of all dissimilarity values for all replicate pairs. The median is less influenced by outliers and therefore more appropriate than the mean.

8.3. Reject any replicate pairs that have a dissimilarity value more than N standard deviations above the mean.

8.4. Remove these replicate pairs and recalculate the median and standard deviation without the influence of outliers.

The choice of N here is important, and also a matter of personal taste. I recommend use of N = 2.

6.5 RESULTS

6.5.1 Dissimilarities of evenly and unevenly distributed replicates

Figure 6.4 shows two examples of the 101 x 101 dissimilarity matrix, for the terrace 2 center endolith sample (left side) and the terrace 4 rim endolith sample (right side). The calculated dissimilarities are displayed in the top row as color contour maps, showing dissimilarity as a function of the thresholds for each replicate in the pair. 3D surface representations of the contour maps are shown in the middle row of the figure. The bottom row shows the standardized ARISA profiles from which the data were generated, for both replicates in each pair.

Ideally, the lowest values of dissimilarity should lie along the diagonal, drawn as a dotted line on the contour maps in the top row of Figure 6.4. The sample on the left has two replicates with similar peak amounts and distributions. The bottom row shows how similar the replicates in this pair are to each other. When thresholds are applied to this sample, the lowest values of dissimilarity are indeed found close to the diagonal.

However, if two replicates are unequal in their distribution or number of peaks (*e.g.* due to noise) then the lowest dissimilarity may lie off the diagonal. Indeed, this possible asymmetry is the reason that each replicate in the pair

requires its own threshold. For example, the sample on the right shows initial peak counts of 105 and 53 for the two replicates, resulting in a markedly uneven distribution of peaks (highlighted by arrows in Figure 6.4, bottom row, right). The goal of the algorithm, as in all cases, is to eliminate only as many peaks as necessary in each replicate to achieve the closest match between them. The greater richness in one replicate means that peak heights are reduced by standardization, as shown in Figure 6.1. A common threshold applied to both replicates would result in unbalanced treatment of the standardized peak values. So the algorithm will suggest threshold values that lie off the diagonal, accounting for the different initial richness in each replicate of the pair.

The symmetry of the left sample and the asymmetry of the right sample are shown very clearly by the 3D surface in the middle row of Figure 6.4.

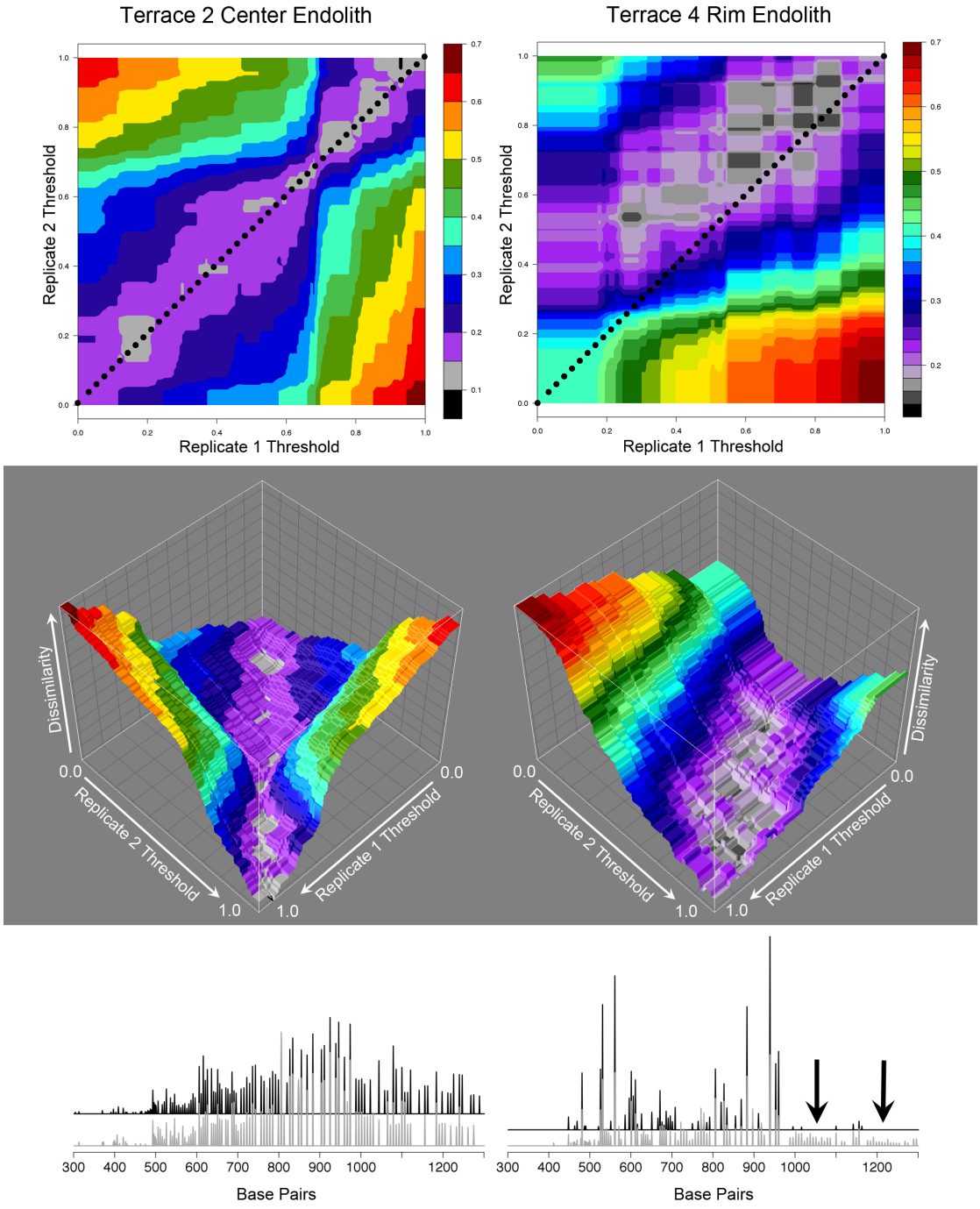


Figure 6.4: Calculated 101 x 101 dissimilarity matrices for two samples, displayed as contour maps (top row) and 3D surface maps (middle row). The bottom row shows the standardized ARISA profiles. Similar profiles lead to a relatively symmetric dissimilarity matrix (left sample), while major differences (highlighted by arrows) can lead to an asymmetric dissimilarity matrix (right sample).

6.5.2 Selecting the thresholds

Although Dis_{min} is the lowest possible dissimilarity, as noted above it might be reasonable to consider thresholds that the researcher considers close enough to Dis_{min}. The parameter M identifies the acceptable additional dissimilarity above Dis_{min}. Figure 6.5 shows contour plots illustrating use of three different values of M, for the same samples that were shown in Figure 6.4. In each panel of Figure 6.5, the area where $Dis \leq Dis_{min} + M$ (that is, the acceptable range defined by M) is shown in white.

For M = 0.0 (first column of Figure 6.5) no thresholds other than ones leading to Dis_{min} are allowed. Picking the acceptable thresholds that yield the lowest value of (Threshold 1 + Threshold 2), the chosen thresholds are relatively high, (0.97, 0.93) and (0.92, 0.74) (yellow circles, first column). While these thresholds produce the best possible similarity between the samples, they cut out many small peaks, potentially eliminating important minor OTUs. Note that the thresholds that make both replicates most similar to each other lie close the diagonal for terrace 2 center endolith (upper row), but farther away from it for terrace 4 rim endolith (bottom row).

Using a non-zero value for M widens the range of possible thresholds, at the cost of some reduced similarity in replicates. The second and third columns show examples for M = 0.025 and 0.05. Use of M = 0.025 results in a dissimilarity range of 0.09-0.115 for terrace 2 center endolith (upper row, middle column) and 0.137-0.162 for terrace 4 rim endolith (bottom row, middle column). For M = 0.05 results are 0.09-0.14 and 0.137-0.187, respectively. The selected

thresholds move to lower values, increasing the number of peaks available for analysis.

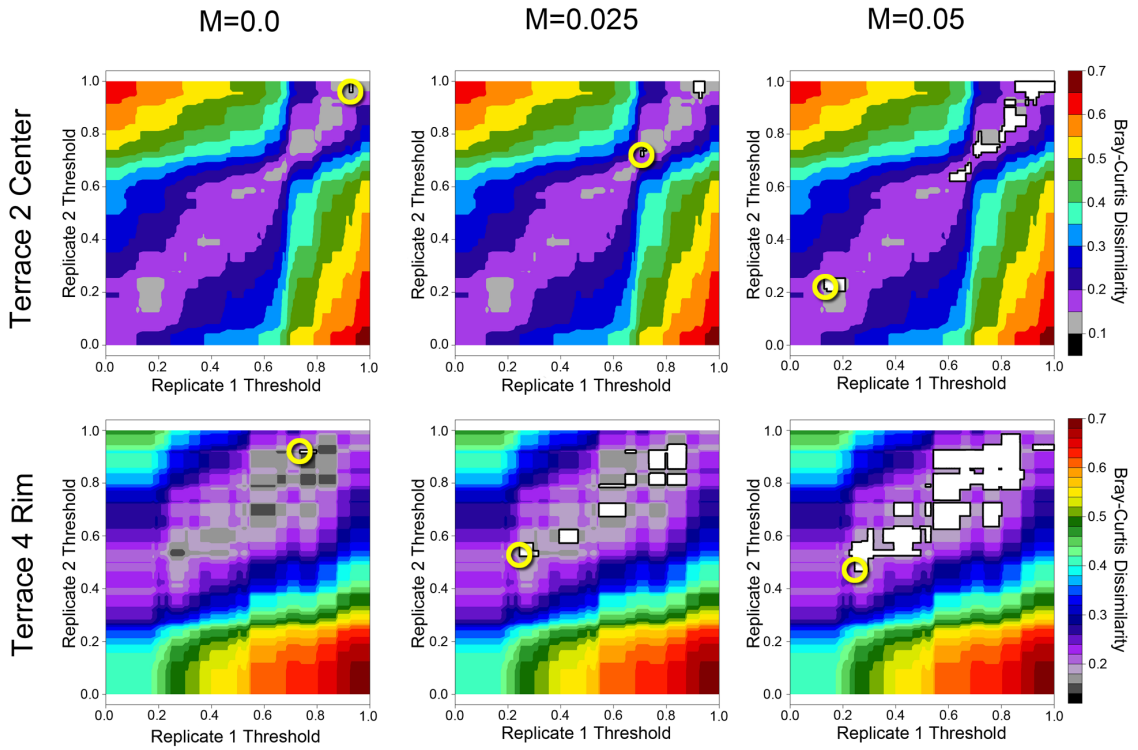


Figure 6.5: Contour maps of calculated 101x101 dissimilarity matrices for the same two samples shown in Figure 6.4. White areas show the acceptable values of dissimilarity for three different values of M. Yellow circles show the thresholds chosen by the algorithm.

6.5.3 Outlier rejection and the consequences of independent threshold selection

Figure 6.6 illustrates the consequences of selecting thresholds independently for each replicate using the algorithm described above. Results are shown for three different samples. For each sample I show the thresholds chosen by the algorithm for three different values of M (yellow circles on the

contour plots), as well as the positions of three possible common thresholds (0.1, 0.5, and 0.9%).

The consequences of these choices are shown in the middle and lower rows of Figure 6.6. In the middle row, the Bray-Curtis dissimilarities for the three different common thresholds are displayed. In the lower row, Bray-Curtis dissimilarities for thresholds selected using my algorithm are shown. The three colored circles on each plot show the points corresponding to the three contour plots in the top row. The other points are for other samples from Troll. The solid red line in each plot shows the median for all samples, and the dashed red lines lie one and two standard deviations above the median after outlier removal has been performed.

Three observations stand out. First, the median dissimilarities using my algorithm are always lower than those for any of the common thresholds. Second, the standard deviations are also smaller, with the results for most samples tightly clustered together. Both of these characteristics are highly desirable; they mean that the chosen thresholds have succeeded in making two replicates that are ideally identical as similar to one another as possible. The third observation is that the general pattern of Bray-Curtis dissimilarities is similar for all samples using my algorithm, but variable using different common thresholds. So choosing a common threshold for all samples not only treats the samples non-optimally, it also treats them unequally.

One of my samples, designated G-out on Figure 6.6, has a Bray-Curtis dissimilarity substantially greater than all the others. Using the outlier rejection

criterion of two standard deviations above the median (*i.e.*, N = 2), this one sample is rejected as an outlier. All the others are retained. Note, however, that use of common thresholds and the same outlier rejection criterion would also lead to rejecting sample M for a common threshold of 0.1%, and rejecting sample H for a common threshold of 0.9%. Choosing thresholds independently, both of these samples can be retained.

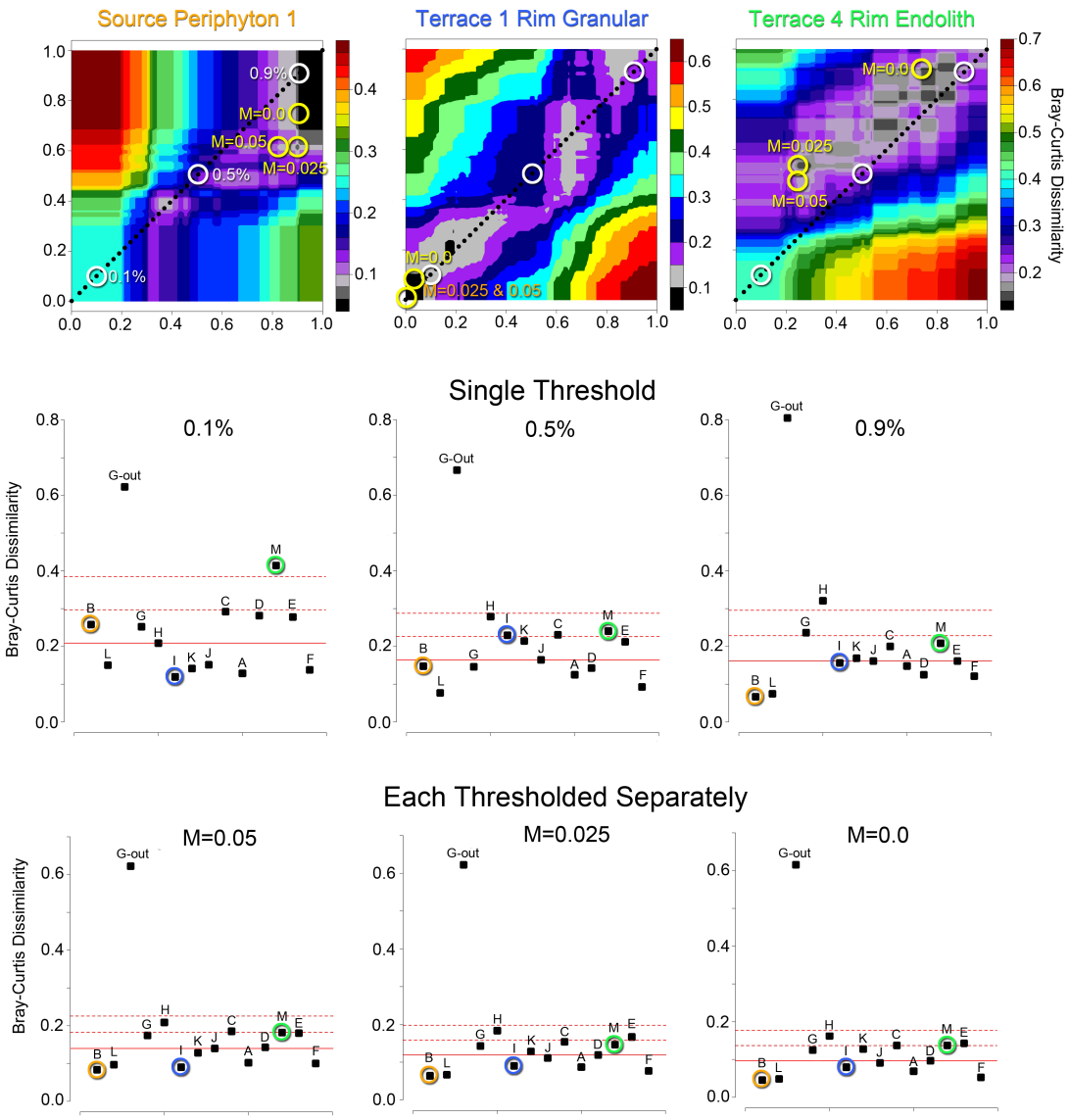


Figure 6.6: ARISA thresholding. Top row: Contour maps of calculated 101x101 dissimilarity matrices for three samples. Yellow circles show thresholds selected by the algorithm for three different values of M. White circles show three possible common thresholds. Middle row: Bray-Curtis dissimilarity for all samples using the three different common thresholds. Colored circles correspond to the samples shown in the top row. Bottom row: Bray-Curtis dissimilarity for all samples using independently-selected thresholds and three different values of M. Solid red lines in the middle and lower rows show the median value, and dashed lines lie one and two standard deviations above the median after outlier removal has been performed.

6.6 SUMMARY AND CONCLUSIONS

Despite the availability of high-throughput sequencing techniques, ARISA remains one of the fastest, easiest and most effective means of assessing variations in microbial community structure. It is particularly well suited to comparing large numbers of samples, where the focus is on recognizing differences in community structure rather than identifying specific taxa. But as my results from Chapters 2 and 3 show, microbial samples, even from closely related communities like those at Troll Springs, can exhibit large variations in richness. And as my results in this chapter show, richness variations require that the essential thresholding necessary for ARISA data analysis be performed on a sample-by-sample basis.

This chapter presents a technique for sample-by-sample threshold selection, based on the simple idea that the best thresholds are the ones that make two replicates from the same sample most similar to one another. The thresholding technique also lends itself readily to identification and elimination of outlier samples. My results above show that the technique can be readily applied to ARISA data from a range of environmental samples. I have implemented the algorithm in R, and the code can be made generally available to the ARISA research community.

APPENDIX A: THE STORY OF THE PLANKTONIC CELLS, OR HOW THE PERIPHYTON WON THE RACE

A.1 INTRODUCTION

At Troll Springs, warm water from the spring source flows downhill to partially fill a number of travertine terraces, forming shallow pools. These pools are interconnected, with water that spills from one pool filling the next below it. Ultimately the pools dry up, and endolithic communities are formed. Because flowing water can easily transport planktonic cells that are suspended in it, one initial hypothesis for formation of the endoliths at Troll was that the planktonic cells of the pools are related to the endolithic communities.

In order to test this hypothesis, in 2008 I collected water samples from the source, pool 1, and pool 2. Large volumes of water were passed through a series of filters of decreasing pore size, first separating out large filamentous organisms suspended in the water. Subsequent filtering through smaller pore sizes further separated out individual planktonic bacterial cells (Fig. A.1). I extracted DNA from the filamentous and planktonic samples, comparing community makeup to those of periphyton from the same pools, and to endoliths. For all samples I used parallel-tagged 454 pyrosequencing for partial 16S analyses.

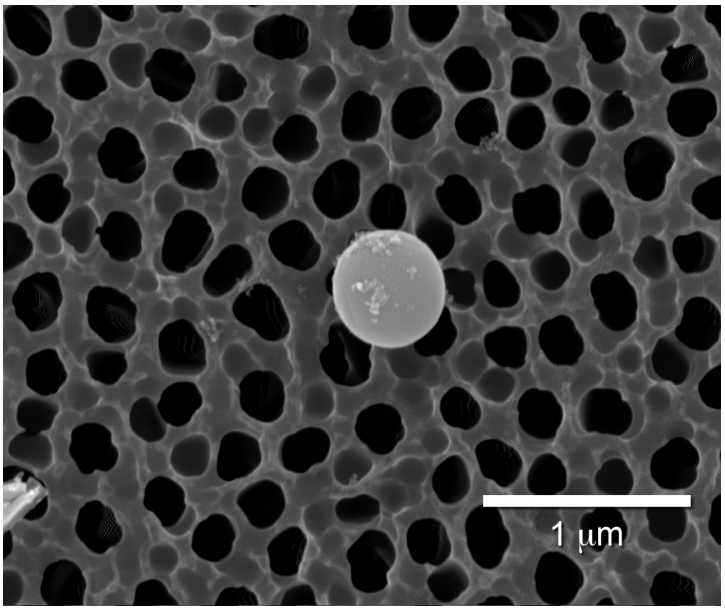


Figure A.1: SEM image of a planktonic bacterial cell isolated from pool 2 using a 0.2-μm filter.

A.2 RESULTS AND DISCUSSION

Even the earliest results suggested that the planktonic communities in the pools have little in common with endolithic communities. Figure A.2 shows initial results of 454 sequencing for planktonic cells in pool 1 and the terrace 4 rim endolith. OTUs are defined at the 97% and 80% similarity levels in this figure. At the 97% level, only 14 OTUs (out of a total of 2948) are common to both the pool and the endolith. Even at the 80% level, this number increases to only 25 out of 1992. The difference is displayed vividly on the right side of Figure A.2, showing pool and endolith heatmaps that are almost negative images of one another. So even this first look suggested that the endolithic communities at Troll are probably not related to the planktonic cells in the pools.

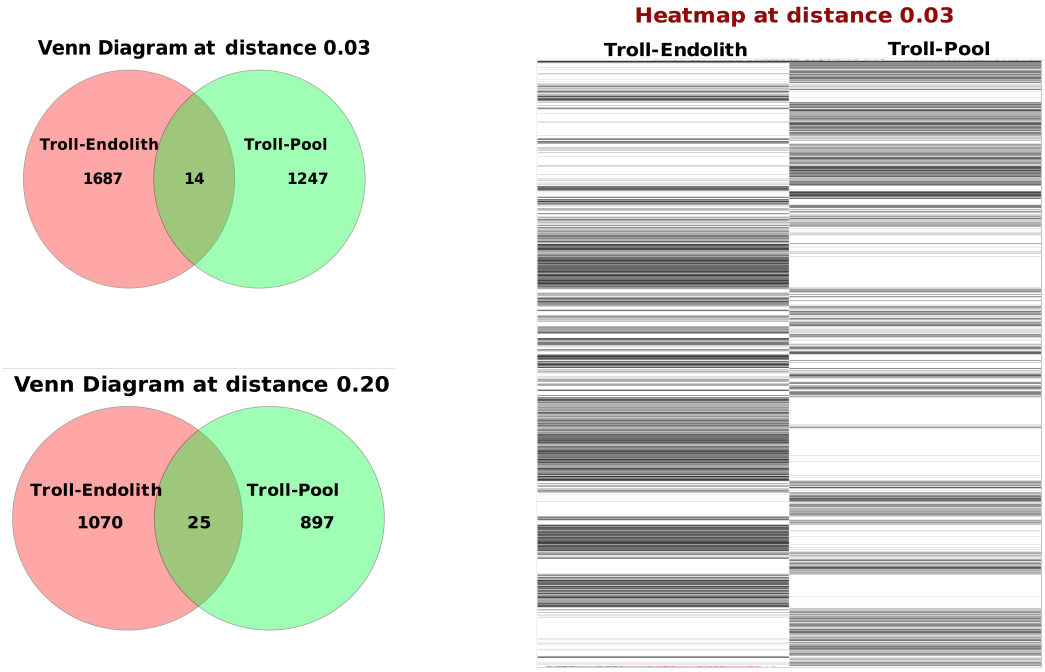


Figure A.2: Venn diagrams and heatmaps for pool 1 planktonic cells and the terrace 4 rim endolith.

The same result is found when only Cyanobacteria are considered (Fig. A.3). Just 4 Cyanobacteria OTUs are common to both the pool and the endolith. Also, Cyanobacteria richness is very low in the pool, with only 16 Cyanobacteria OTUs identified at the 97% similarity level. As shown in Chapter 2, Cyanobacteria are the main primary producers in the endoliths, but clearly there are not many planktonic Cyanobacteria taxa in the pools. These observations provided the first suggestion that the endolithic communities at Troll may be primarily derived from periphyton, rather than from planktonic organisms.

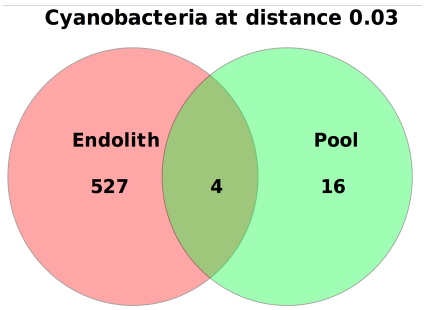


Figure A.3: Venn diagram of pool 1 planktonic Cyanobacteria cells and the terrace 4 rim endolith cyanobacteria.

Because of these results, in subsequent years I shifted my attention to collection and analysis of periphyton samples. Once that work was completed, I explored the relationships among all of my samples using non-metric multidimensional scaling. Figure A.4 includes nMDS results from most of the Troll samples discussed in Chapters 2 and 3. This figure also includes the earlier water samples that contained planktonic bacteria, and filter samples that contained larger, mainly filamentous biomaterials that were floating in the water. The data used to create this figure were not presence/absence transformed, and do not include chloroplast sequences.

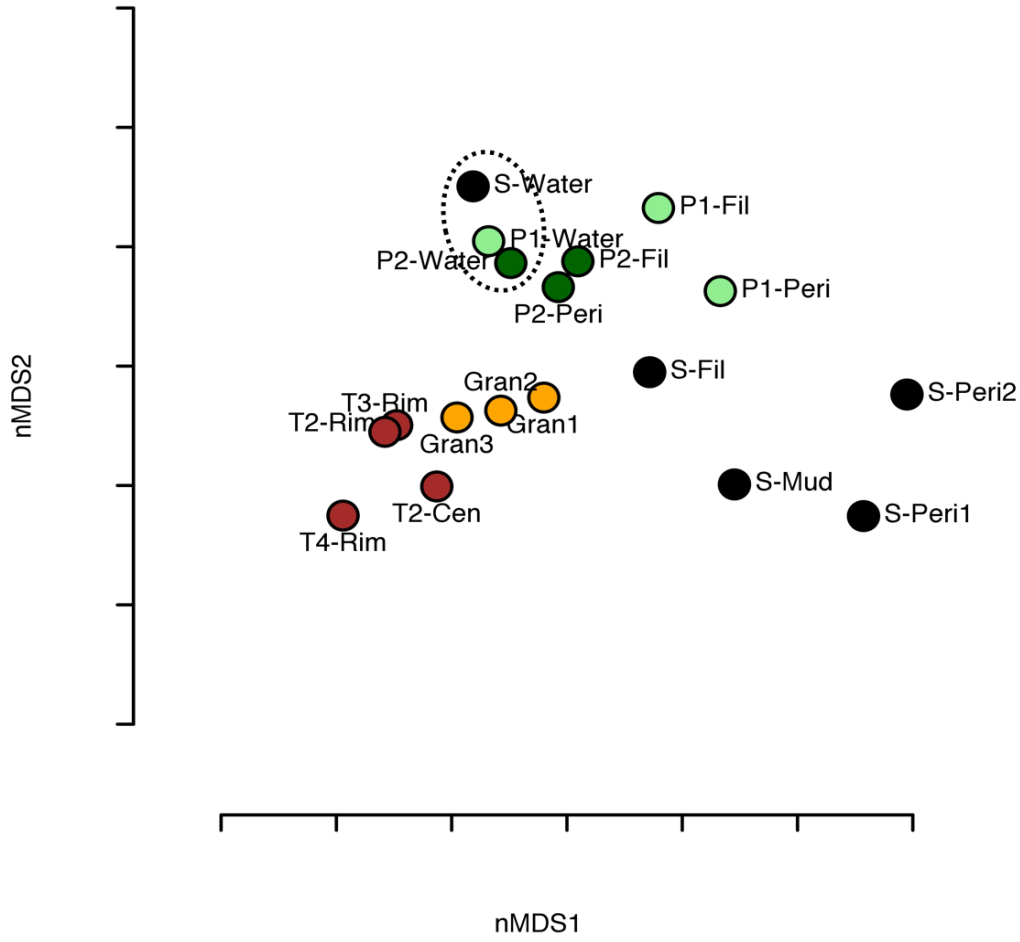


Figure A.4: nMDS results for Troll samples from Chapters 2 and 3, plus filter samples containing filamentous biomaterials (labeled “Fil”) and water samples containing planktonic bacteria (labeled “Water”).

Several features of this nMDS plot are noteworthy. First, the general structure reported in Chapter 2, with endoliths, granular samples, pools, and the source all separated from one another is preserved. Second, the filtered samples that contain filamentous biomaterials plot close to the periphyton samples from the same pools (*i.e.*, P1-Fil plots close to P1-Peri, P2-Fil close to P2-Peri). This observation suggests that the filamentous materials are detached local periphyton fragments that are floating freely in the water. And because water

flows readily from one pool to another, this means that periphyton materials may be transported from one pool to another that is downhill from it.

A third important observation is that the tangential flow filtered (see section A.4.1) water samples all cluster together (dashed oval in Fig. A.4), far from the corresponding filamentous and periphyton samples. So while the filamentous community in each pool is closely related to the local periphyton, the planktonic bacterial community is distinct from local periphyton and filaments, but common to both of the pools sampled and to the source.

A final observation is that the source filaments plot far from the source periphyton, and closer to the pool 1 and 2 periphyton. This observation suggests that these source filaments may not be directly derived from the source periphyton, or may come from just a portion of it. (As shown in Chapter 2, the source periphyton differs strongly from other periphyton at Troll, being dominated by eukaryotic algae.) It also suggests that filaments from the source may have been transported to the pools, taking hold in the filament and periphyton communities there.

Figure A.5 shows a weighted UniFrac principal coordinates analysis for the same samples. The same trends observed in Figure A.4 are seen. The endolith and granular samples are separated from one another and from the aquatic samples along PCoA1. The aquatic samples are well separated from one another by PCoA2. The three water samples all cluster very close together, at high values of PCoA1 and PCoA2. Each pool filament sample is very close to its corresponding periphyton sample, and the source filament sample is well

separated from the source periphyton. So using phylogenetic information, I reach the same conclusions reached using nMDS analysis of OTU composition.

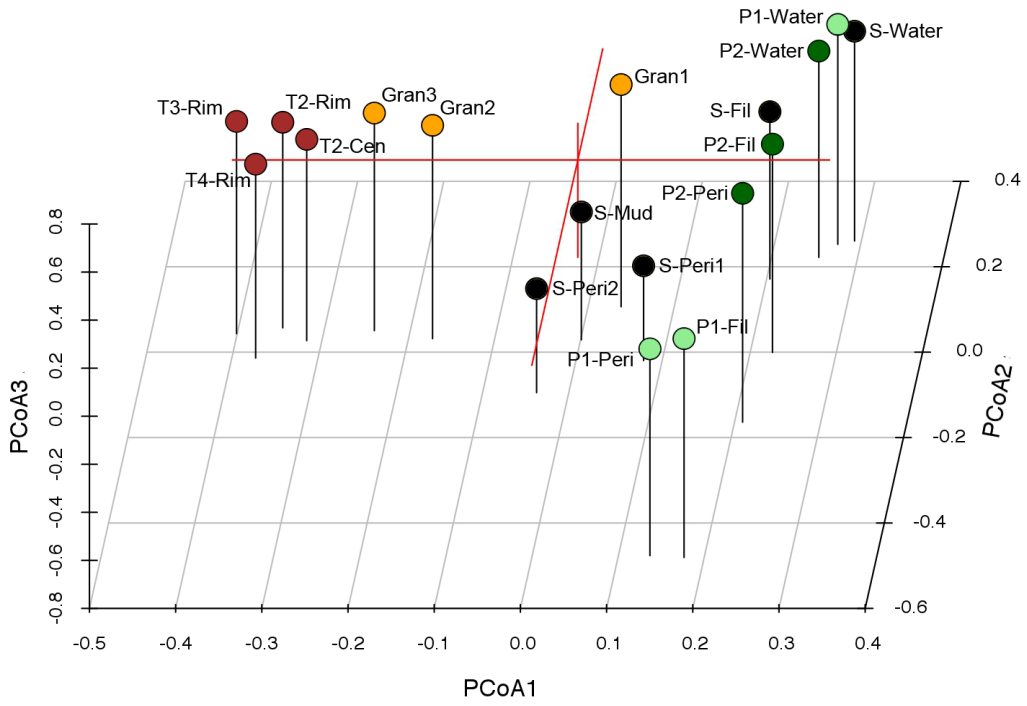


Figure A.5: UniFrac PCoA results for Troll samples from Chapters 2 and 3, plus filter samples containing filamentous biomaterials (labeled “Fil”) and water samples containing planktonic bacteria (labeled “Water”).

The close relationship between each pool periphyton sample and its corresponding filamentous sample is noteworthy, because the samples were collected in different years. Year-to-year variability of microbial community makeup at Troll remains to be characterized. As I showed in Chapter 2, pool-to-pool variations in community makeup are significant, particularly in the major OTUs. For at least 2008 and 2009, however, the similarities between periphyton

and filamentous samples hint that major temporal changes may not have occurred for pools 1 and 2 and similar communities were present these years.

As I noted in Chapter 3, community richness and evenness are strongly correlated at Troll Springs. Interestingly, the filamentous and planktonic samples from 2008 lie on the same trend. Figure A.6 shows the same relationship between richness and evenness that was displayed in Figure 3.4, with filter and water samples added. As I noted in Chapter 3, a positive correlation between richness and diversity generally means that as new taxa are recruited, their numbers increase more sharply than those of taxa already present. Apparently this trend at Troll applies to the planktonic and filamentous communities as well as it does to the others studied. It also applies across multiple years of sample collection.

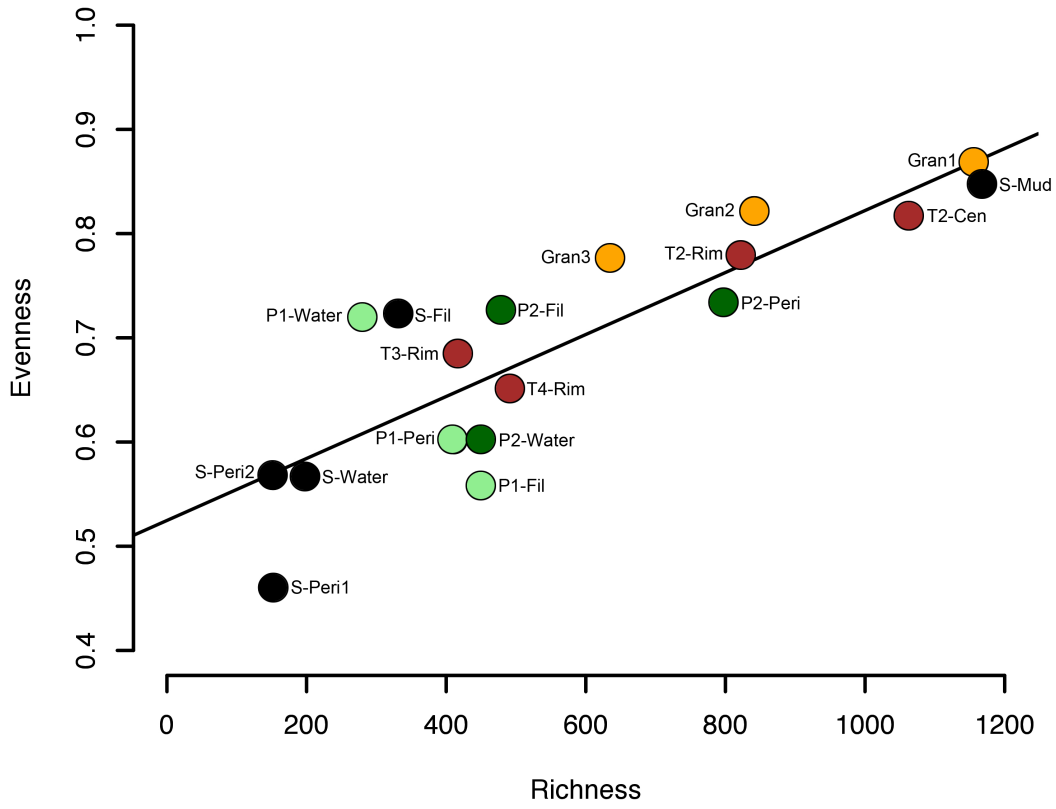


Figure A.6: Community evenness *vs*. richness at Troll Springs, including filter samples containing filamentous biomaterials (labeled "Fil") and water samples containing planktonic bacteria (labeled "Water"). A regression line is shown, with a correlation coefficient of r=0.85.

A.3: CONCLUSIONS

The work reported in this appendix was chronologically some of the earliest in the course of my dissertation research. Its goal was to test the hypothesis that endolithic communities at Troll are related to planktonic cells carried from the spring source and pools to endolithic sites by flowing water.

As the results above show, this hypothesis was incorrect. The aquatic environments at Troll do indeed have a community of planktonic bacteria. But that community is specific to the water of the source and pools, is common

among those water bodies, and is unlike communities elsewhere at Troll. Planktonic bacteria are not a precursor to the endolithic communities. It was this realization that made me turn instead to the periphyton in the pools as a precursor to the endolith, and that led to the results reported in Chapters 2 and 3.

This work did, however, reveal some interesting clues related to periphyton. The filamentous biomaterials in the water at Troll are unique to each water body, and in the pools are closely related to the local periphyton. It was the periphyton that won the race, but the results here may point to filamentous material carried by flowing water as the mechanism by which periphyton-forming organisms are transported among pools at Troll.

A.4: MATERIALS AND METHODS

A.4.1 Sample collection

In August of 2008 I collected water samples from Troll Springs. Samples were collected either directly (Fig. A.6a) or via siphoning (Fig. A.6b).

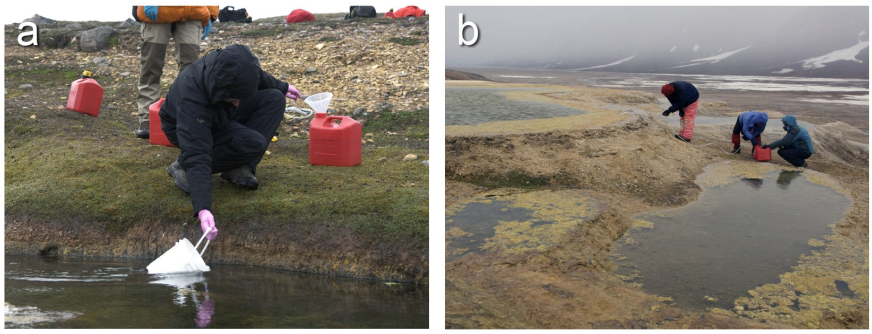


Figure A.6: Direct water sampling from the source (a) and siphoning from the pools (b).

The sampling and filtering strategy I used for these water samples is outlined in Figure A.7. I collected 40 l of water each from the source, pool 1, and pool 2. Subsequent processing of water samples consisted of pre-filtration to remove large particles, followed by tangential flow filtration (TFF, (97)). The 2 l of concentrate that resulted from TFF was filtered through membranes to separate out bacteria. 5 liters of the original sample was set aside for membrane filtration, which employed membranes of several sizes. The 2 µm membrane separated filamentous material from the planktonic cells (representing a stage between planktonic and periphyton), which where used for DNA extractions.

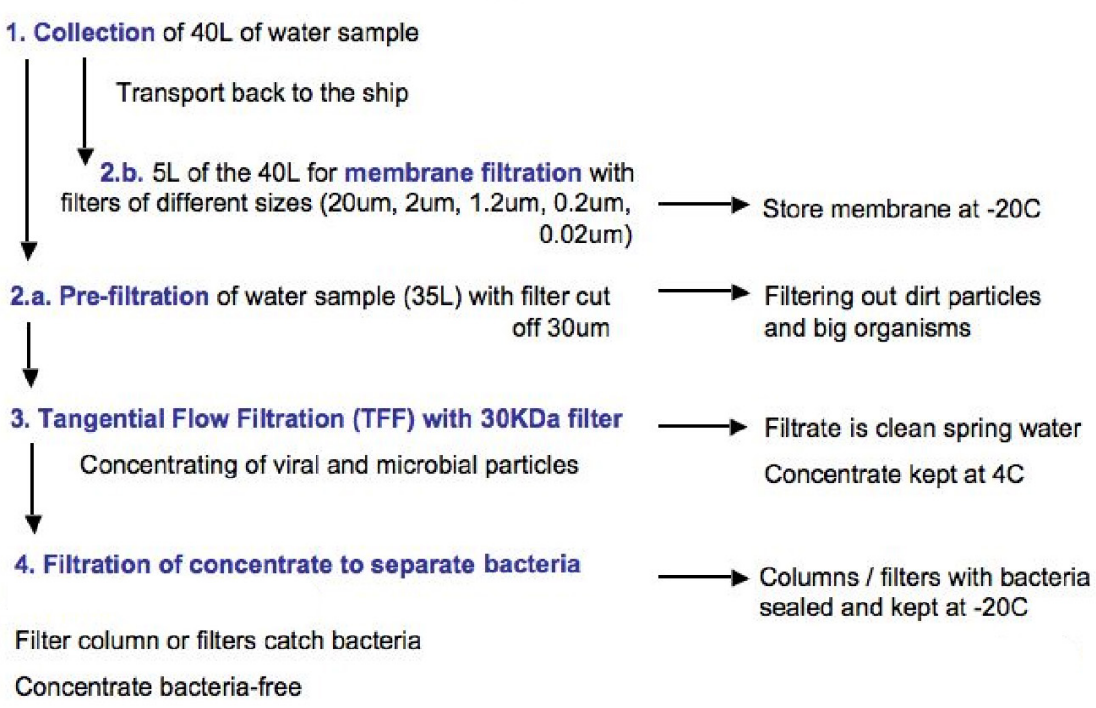


Figure A.7: Sampling and processing strategy for water samples collected in 2008.

Running the TFF unit took about 4-5 hours per sample. A TFF run includes cleaning of the filter column after storage, a water permeability test,

sample filtration, cleaning of the filter column, another water permeability test and inserting storage solution. Additional preparations for filtration included cleaning of the sample containers that were used for the filtration and the final concentrate.

After TFF processing, the concentrate was filtered through Sterivex columns and filter membranes for separation of bacteria, which took approximately 2-3 hours per sample. A separate membrane filtration procedure was performed using the original water sample and filters with different pore size, which also took about 2-3 hours per sample.

A.4.2 Sequencing and multivariate analysis

I used the same 454 sequencing, sequence processing, nMDS and UniFrac analysis techniques described in Chapter 2.

APPENDIX B: APPLICATION OF AN ECOLOGICAL MODEL, OR HOW DISTURBED PRODUCTIVITY LEADS TO VARIETY

In Chapter 3, I showed how the observed trends in richness, evenness, and diversity at Troll can be explained by variations in the properties of the environment, with community evenness affected mostly by the balance of competition, and richness by the availability of physical niches. It is also useful to examine the same trends in light of other published ecological models.

A number of models have been developed to explain how environmental factors influence community makeup in an ecosystem (131, 237, 261, 293). Kondoh (148) has developed a model based on the competitive exclusion scenario that unifies the relationships of richness to productivity and disturbance. The model predicts that a balance between productivity and disturbance yields the highest richness, with lower values at either extreme. The model is general, and in principle can be applied to any ecosystem.

Kondoh's model is based on the competitive exclusion scenario. Productivity is defined as the total rate of production of biomass in the ecosystem. A disturbance is a change in an environmental condition that can cause a change in the ecosystem. The model assumes that the environment consists of a large number of discrete patches where each patch is empty or occupied by species. Inter-patch colonization and within-patch extinctions determine the proportion of each patch occupied by a given species. The model assumes that an increase in productivity enhances the colonization rate of all

species, and that an increase in disturbance enhances the extinction rate of all species.

Species are characterized by a colonization rate, an extinction rate, and their ability to survive during competition. In the model, some organisms are assumed to be superior competitors/inferior colonizers, and others to be inferior competitors/superior colonizers. Superior competitors are assumed to have a lower colonization rate or a higher extinction rate (108, 259). The model further assumes that an increase in productivity enhances the colonization rate of all species, and that an increase in disturbance enhances the extinction rate of all species. The fundamental result is that a balance between productivity and disturbance yields the highest species richness, with lower values at either extreme.

Figure B.1 is modified from Kondoh (148) to show schematically how this result might be applied to Troll Springs. Sample environments are placed on the plot based on variations in water content for the terrestrial samples (as a proxy for nutrient flux and productivity, as well as disturbance via desiccation), temperature variability for the aquatic samples (as another proxy for disturbance), and richness.

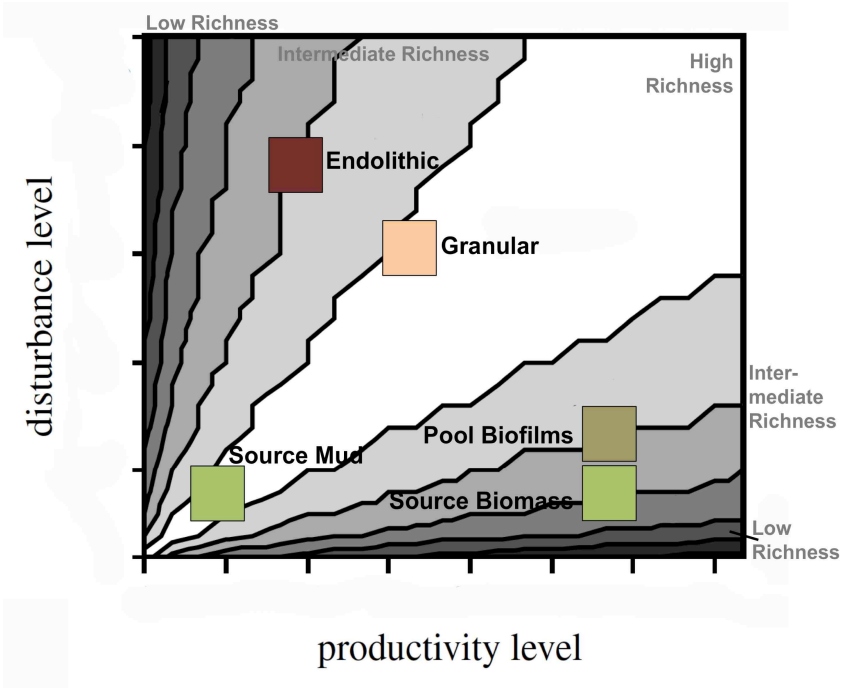


Figure B.1: Microbial community diversity at Troll Springs as a function of primary productivity and environmental disturbance, modified after Kondoh (148), used by permission. The shaded zones represent diversity as predicted by Kondoh's model. Samples are projected into the parameter space according to their interpreted levels of productivity and environmental disturbance.

Visibly large quantities of biological materials in the source and pools are an indicator of high productivity there. High biomass tends to indicate high productivity in many settings; for example, Pommier (217) showed that higher production correlated with greater biomass in marine bacteria in the Mediterranean, and that low richness was a consequence. In the aquatic environments at Troll, OTUs are distributed more unevenly than in the terrestrial environments. High productivity leads to high colonization rates, favoring inferior colonizers while disfavoring inferior competitors by increasing competitive exclusion. Cytophagaceae, Cyanobacteria (GpIIb), unclassified Proteobacteria and Peptococcaceae are dominant in the source periphyton 1 and 2, pool 1 and pool 3 periphyton, respectively (Fig. 2.7). I suggest that these are superior

competitors that limit the abilities of other organisms to colonize the same space. However, they appear to be vulnerable to disturbances, such as fluctuations in temperature and water availability. Source and pool periphyton are immersed in water year round, leading to small temperature fluctuations and low thermal disturbance relative to terrestrial samples, particularly for the source. The consequence of high productivity and very low disturbance according to Kondoh's model is low richness. This is consistent with the data, as richness is lowest for the source periphyton, and slightly higher for the pool 1-3 periphyton. Precipitation of calcite in these pools, particularly pools 2 and 3, might be a disturbance that creates spatial niches that increase the prospects for coexistence of OTUs, increasing richness and diversity. Interestingly, the periphyton in pool 2, which demonstrates significant carbonate precipitation, shows OTUs, such as Rhodobacteraceae and Caulobacteraceae, that become more dominant in the endolithic environment. Possible wintertime freezing (which does not occur at the source) could also increase disturbance.

The model predicts that richness is maximized at intermediate productivity and disturbance because those conditions allow for maximal coexistence of superior competitors and superior colonizers. Granular samples are wetter than endoliths but drier than pools, experiencing intermediate productivity and disturbance. These conditions favor species coexistence, and yield some of the highest richness and diversity at Troll. The situation is similar to soils, where diversity is high (54, 229) because pore spaces provide niches that harbor weaker species (31).

The source mud sample is unusual, with high water content that assures low disturbance, but burial in opaque sediments that inhibits photosynthesis, reducing productivity. Additionally, the mud creates niches, similar to those in the granular samples, for microbes to colonize. As in the granular samples, these conditions maximize richness and diversity.

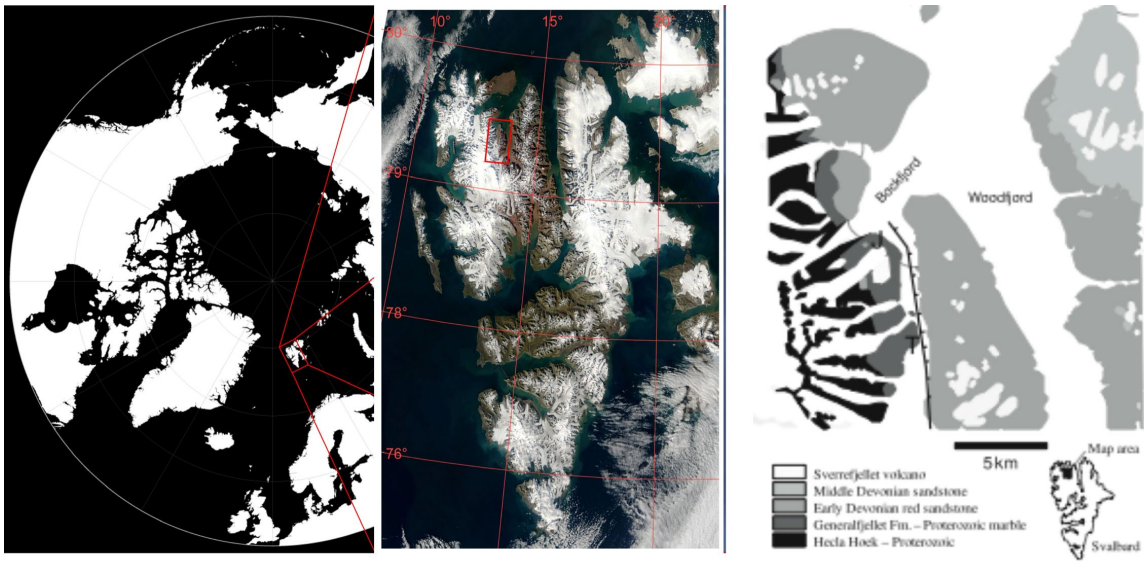
In the dry endolith samples, environmental disturbances enable proliferation of organisms that have higher colonization rates or lower extinction rates. These superior colonizers include Rhodobacteraceae, Caulobacteraceae, Xanthomonadaceae, Sphingomonodaceae. Chitinophagaceae, Acetobacteraceae, and (in the driest endolith) Truperaceae, all of which appear to gain an advantage via water loss. Cytophagaceae are also present in endoliths, although at reduced counts relative to the granular samples, suggesting reduction in their ability to compete under the driest conditions. Several Cyanobacteria groups also appear to be superior colonizers, not restricting the distribution of other OTUs in the pools, and coexisting with other superior colonizers in the endoliths. The driest samples at Troll are sites of moderate to high thermal disturbances, favoring colonization by organisms with faster intrinsic growth rates (inferior competitors), outcompeting the superior competitors (reducing competitive exclusion). Endoliths at Troll also have very low water content, and therefore very low nutrient fluxes and productivity. The net result of very low productivity and moderately high disturbance is richness and diversity higher than in the source, comparable to in the pools, but lower than the granular samples.

While my preferred explanation for the richness variations at Troll is the one presented in Chapter 3, it is interesting that the general model of Kondoh (148) can also be applied in a plausible if speculative manner to my data.

APPENDIX C: SEAFLOOR VOLCANISM ON THE KNIPOVICH RIDGE, OR HOW TROLL SPRINGS GOT ITS HEAT PUMP

C.1 INTRODUCTION

Troll and Jotun Springs (Fig. C.1) are geothermal springs in the high Arctic, and are warm and active year around. Their heat source and the spring setting enable microorganisms to thrive in this extreme environment. The springs are supplied by glacial meltwater enriched in Ca and CO_2 derived from Proterozoic marbles (135). At Troll Springs the carbonates precipitated from these waters form meters-scale terraces; at Jotun the terraces are considerably smaller. In this appendix, I consider the origin of both springs, and their relationship to volcanism and seafloor spreading in the north Atlantic and the Arctic Ocean.



Troll spring **Jotun spring**

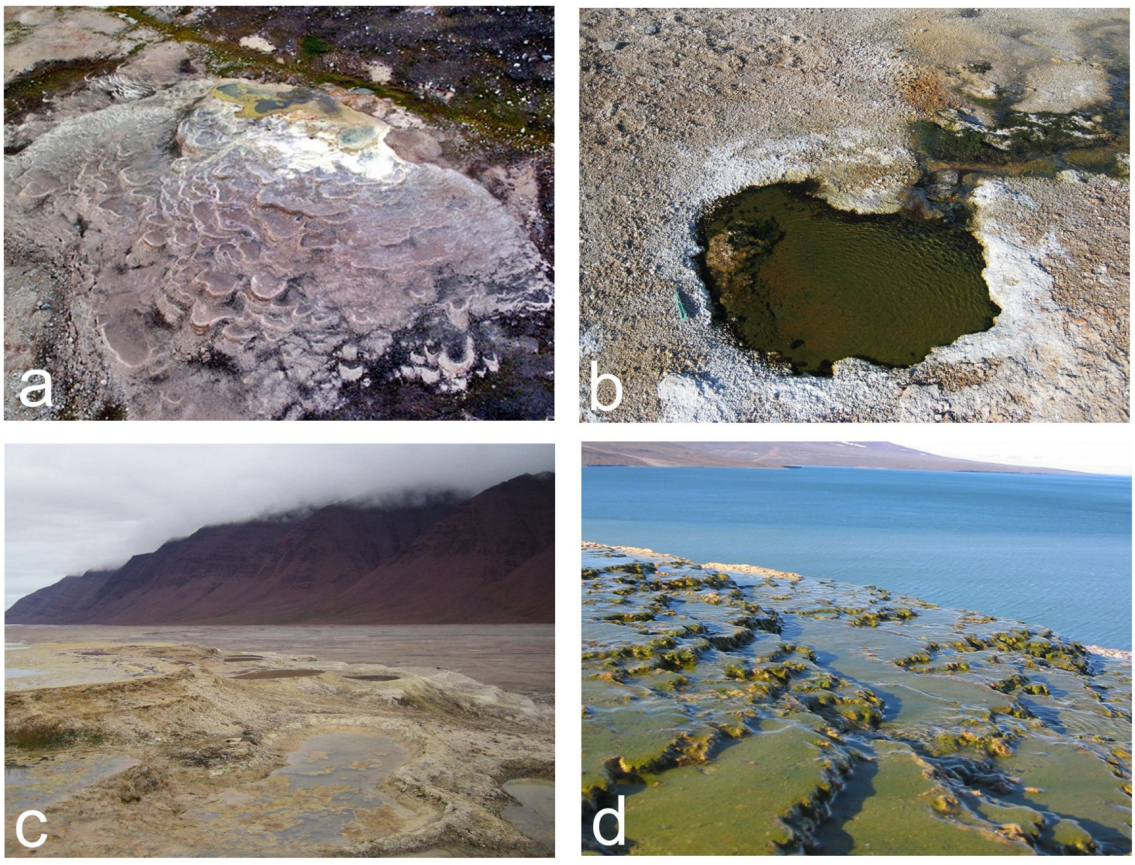


Figure C.1: Troll and Jotun Springs. Upper row shows their geographic location on Svalbard (map from Jamtveit *et al.* (135), used by permission). **a**: Troll Springs (picture left to right 300m across), **b**: Jotun Springs (picture about 3m across), **c**: Troll terraces (picture left to right 2m across), **d**: Jotun terraces (picture 0.3m across). Map: T = Troll Springs, J = Jotun Springs

Seafloor volcanism in the north Atlantic is most prominent along the Mid-Atlantic Ridge (MAR). Svalbard does not lie on the MAR, because the MAR by definition ends at Iceland. However, a segmented system of spreading ridges does extend northward from Iceland. From south to north, these ridge segments are called the Kolbeinsey Ridge, the Mohns Ridge, the Knipovich Ridge and the Gakkel Ridge (Fig. C.2). Together they effectively form an extension of the MAR. The Knipovich ridge lies closest to Svalbard, immediately to the southwest.

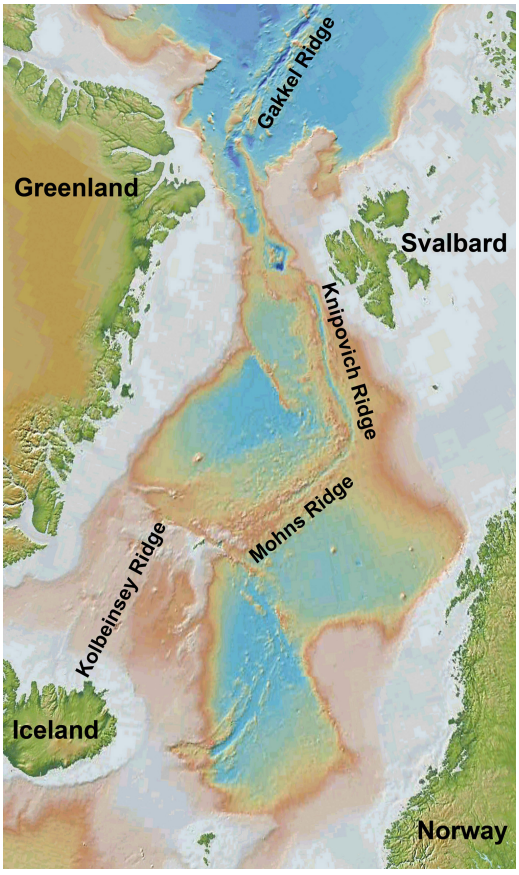


Figure C.2: Mid-ocean ridge system north of Iceland. The extension of the MAR consists of the Kolbeinsey Ridge, followed by the Mohns, Knipovich and Gakkel Ridges. The Knipovich Ridge is closest to Svalbard. Map was assembled using ocean bathymetry data from the NOAA National Geophysical Data Center (188).

C.2 DISCUSSION

Troll and Jotun Springs lie adjacent to Sverrefjellet, a Quaternary stratovolcano. The question of the possible relationship between the springs and the Knipovich Ridge can therefore be considered in two parts: Is the Sverrefjellet volcanism related to the Knipovich Ridge? And is the geothermal activity at Troll and Jotun related to the Sverefjellet volcanism?

Figure C.3 (254) shows the major tectonic features in the Svalbard region, and Figure C.4 (50) shows seafloor heat flow near Svalbard. These figures suggest that there potentially is a relationship between seafloor tectonics west of Svalbard (the Knipovich Ridge in particular) and volcanism on Svalbard.

Sushchevskaya *et al*. (252, 254) argue that magmatism on Spitsbsergen Island took place when the Knipovich Ridge developed near its margins (Fig. C.3). This evolution could have been associated with shifts in the spreading axis affecting the tectonics of the western margin of the island (50). Those tectonic motions were accompanied by magmatic activity of three volcanoes (Bockfjorden Volcanic Complex), which are aligned along the N-S trending Breibogen fault (255). Sverrefjellet is the most northern stratovolcano, followed by the diatremes (breccia-filed volcanic pipes) Halvdanpiggen and Sigurdfjellet. The age of Sigurdfjellet is 2.7 Ma, Halvdanpiggen is 2 Ma, and the most recent activity on Sverrefjellet took place between 10 and 6 thousand years ago. Quaternary volcanism progressed from the south to north, which happened together with tectonic opening of the Norwegian-Greenland basin (255). It may be significant that the Breibogen fault lies on approximately the same structural trend as the

Hornsund Fracture Zone and the Knipovich Ridge.

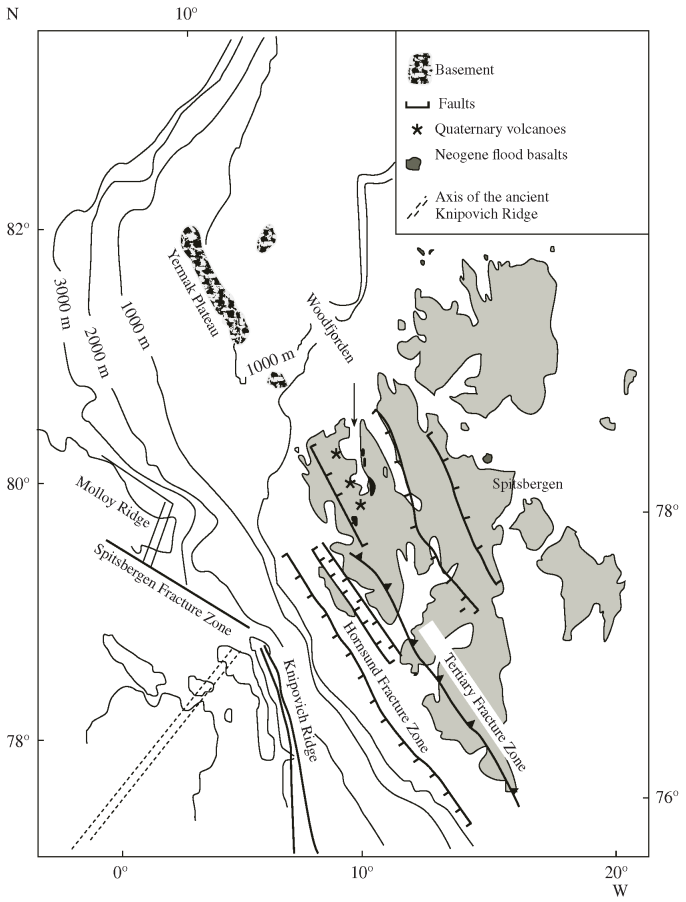


Figure C.3: Schematic geologic map of Svalbard and the Atlantic-Arctic region from Sushchevskaya *et al*. (254), used by permission. The map shows major tectonic features in this area. The three stars in the map represent the three volcanoes, starting from the north: Sverrefjellet, Halvdanpiggen and Sigurdfjellet. The arrow points to the Bockfjord/Wallenbergfjord area.

Crane *et al*. (50) describe the sea floor heat flow in the Svalbard region (Fig. C.4), which is asymmetric across the Knipovich Ridge, with higher flow on the side toward Svalbard. An area of high heat flow is aligned along a structural extension of the Woodfjord area on Svalbard known for its volcanic activity, where Sverrefjellet lies. This band also lies on the same structural trend as the Breibogen fault. These observations suggest that the Bockfjord Volcanic

Complex, including Sverrefjellet, may be off-axis volcanism associated with sea floor spreading immediately west of Svalbard.

Crane *et al*. (50) point out that the ridges bend in the Svalbard area, and argue that those bends create stresses that promote volcanic activity on the eastern side of the Knipovich ridge crest, resulting in off-axis volcanism. The authors conclude that those stresses led to the migration of the Knipovich Ridge, multiple zones of magma intrusion, and possibly off-axis volcanism on the ridge's eastern side. However geologists familiar with this area have pointed out to me that the deep-rooted Breibogen fault is older than the Knipovich Ridge, and probably also plays a role in the location of the volcanoes (Ivar Midtkandal, personal correspondence).

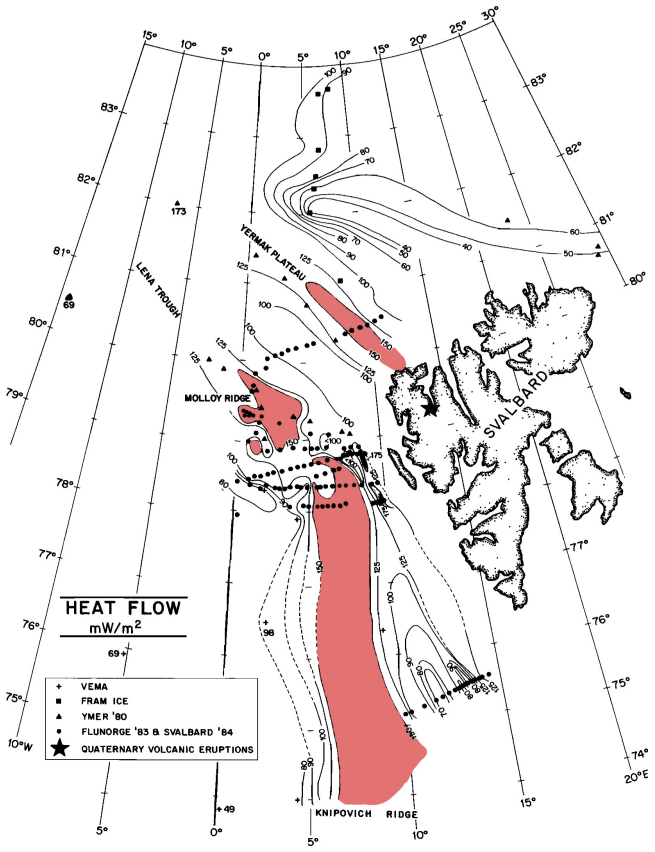


Figure C.4: Seafloor heat flow near Svalbard from Crane *et al*. (50), used by permission. Areas shaded in red have high heat flow. Note also the band of high heat flow extending from the Yermak Plateau toward the Bockfjorden region of Svalbard.

Geochemical observations support a relationship between the Sverrefjellet volcanism and the Knipovich Ridge. Sushchevskaya *et al*. (252, 254) showed that the lavas at Sverrefjellet are geochemically similar to lavas from the Knipovich Ridge. The similarities are primarily in radiogenic isotopes, and suggest that the deep magma sources for Sverrefjellet and the Knipovich Ridge are related. Additionally, the magma source for the Knipovich Ridge is different from the magma source(s) for the nearby Kolbeinsey and Mohns ridges (253, 254).

Regarding the relationship of Sverrefjellet to Jotun and Troll Springs,

Jamtveit *et al*. (135) argue that the heat source for the two springs does not derive from the Sverrefjellet volcano. They do not suggest an alternative. The only two thermal springs in the area lie very close to the only recent volcano, and to the Breibogen fault line. Although the thermal source for the springs may not stem directly from the volcano, which has been inactive for ~10^4 years, it remains likely in my opinion that the heat source for the volcano is the same as for the springs. Other workers have reached the same conclusion. Referring to the Sverrefjellet volcanism, a Norwegian Polar Institute website (56) states: “The thermal springs by Bockfjorden indicate that the geothermal gradient… is still high in the area in the aftermath of this volcanic activity”

C.3 CONCLUSIONS

Svalbard does not lie on the Mid-Atlantic Ridge or another spreading ridge. However, it is in proximity to the Knipovich Ridge, and this proximity may have influenced geological events on Svalbard. Those events are probably connected to the volcanism in the Bockfjord area and consequently also a source for the spring activities. The recent volcanism that formed Sverrefjellet can be interpreted as off-axis volcanism associated with the nearby Knipovich Ridge. Both the timing of the volcanism and the similarities in lava chemistry point to a related magma source. And despite a counter-comment in the paper by Jamtveit *et al*. (135), it is likely that the decaying stages of the heat pulse that produced Sverrefjellet are also the energy source for Troll and Jotun springs.

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