

Abstract

MICROBIAL DIVERSITY AND BIOGEOGRAPHY IN A SERPENTINITE-HOSTED ECOSYSTEM

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The goal of this project was to characterize the taxonomic and functional diversity and document biogeographic patterns of microbial communities in an ecosystem influenced by active serpentinization. Serpentinization is a geochemical process which occurs when ultramafic rocks characteristic of the Earth's mantle undergo aqueous alteration to produce altered mineral compositions (referred to as serpentines). This reaction results in highly-reduced, high pH fluids enriched in hydrogen, methane, and small organic acids, and depleted in dissolved carbonate. Serpentinization is common in environments such as slow-spreading mid-ocean ridges, where tectonic activity on the seafloor results in exposure of ultramafic rocks, and in ophiolites (sites where oceanic crust has been displaced onto continental margins). Environments associated with pervasive serpentinization tend to be anaerobic and highly alkaline (pH 10-12) and creates a range of unique niches for microbial colonization and diversification. While the process of serpentinization provides abundant fuels to support the growth of chemotrophic microorganisms, the highly alkaline, highly reducing environmental conditions challenge the limits of microbial physiology.

This work focuses upon the analysis of microbial communities that inhabit diverse niches within the Tablelands Ophiolite Complex, a serpentinizing environment

located within Gros Morne National Park in western Newfoundland, Canada. A key element of this research was to assess the overall diversity of prokaryotic populations in a terrestrial serpentinite-hosted ecosystem and to evaluate how this diversity varies in relation to habitat characteristics. These analyses provided insight into the range of microorganisms that are able to exist in these settings, and provide a basis to explore their physiological and metabolic adaptations. A widely used method to assess the taxonomic diversity of microbial communities is through the analyses of the 16S ribosomal RNA (16S rRNA) gene. The 16S rRNA gene is an ideal target because it is functionally constant, of a sufficiently length to permit detailed phylogenetic analyses, and distributed throughout all domains of life. In addition to an assessment of microbial taxonomic diversity, this project was also concerned with discerning the spatial distribution of microbial communities, and how these populations differ based on their proximity to the alkaline seeps created by serpentinization.

Finally, all of these genetic assays were conducted against a backdrop of contextual data including total microbial cell counts determined by epifluorescence microscopy, viable cell counts by dilution plating on high pH media, and geochemical data. Taken together, this information provided a thorough report of how the abundance and the composition of microbial communities vary in relation to point sources of alkaline seepage.

Fluid and rock samples were collected in July 2009 and used to complete the analyses described above. Microscopic analyses found relatively low total cell concentrations ($< 10^5$ cells ml⁻¹) with no discernable differences between sites. Phylogenetic analyses of bacterial 16S rRNA showed overall taxonomic diversity within

the site ranging from Alpha Proteobacteria to Firmicutes. However, site to site diversity varied significantly, with minimal species overlap between communities.

This study represents one of the first detailed microbiological studies of a terrestrial serpentinite ecosystem. Furthermore, by mapping the diversity and abundance of organisms in relation to the point sources of seep fluids, we have begun to generate an idea of which organisms are serpentinite-influenced, and which are serpentinite-adapted, which will aid in the exploration of the serpentinite hosted biosphere.

Analysis of these samples indicated low diversity and low abundances compared to more moderate ecosystems, likely due to the challenges of living in highly basic environments such as maintaining membrane integrity and preventing cell lysis, generating a proton motive force across the cell membrane, and limited availability of exogenous terminal electron acceptors. In terms of diversity and species present, distinct patterns emerged concerning the spatial arrangement of microbial communities in relation to active serpentinization sites. Communities directly exposed to active alkaline seepage were subjected to high pHs and highly reducing conditions and were more limited in diversity, compared to more moderate sites. Overall, microbial diversity appeared to increase away from the point sources of alkaline seepage. This indicates that the taxonomic diversity and abundance of microbes is inversely affected by the alkaline, reduced conditions associated with serpentinization. The data supports a trend that in general, the more extreme the pH, the lower the microbial diversity. The range of species identified and diversity levels are similar to findings from other microbiological assessments of serpentinite ecosystems. Low diversity and species of *Firmicutes* and *Alpha-proteobacteria* have also been reported at sites such as a non-saline alkaline spring

in Portugal and a slow-spreading mid-ocean ridge in the deep sea known as the Lost City Hydrothermal Field. The parallels between these sites suggest that serpentinization supports growth and activity of a narrow range of species, and that the extreme conditions associated with serpentinization will consistently limit microbial diversity.

MICROBIAL DIVERSITY AND BIOGEOGRAPHY IN A
SERPENTINITE-HOSTED ECOSYSTEM

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SERPENTINITE-HOSTED ECOSYSTEM

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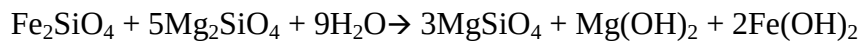
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Chapter 1: Background

Serpentinization is the hydration and alteration of ultramafic rock characteristic of the Earth's mantle (the region of the Earth from 10 km to 2890 km depth). The minerals olivine and pyroxene undergo compositional alterations upon exposure to water, converting them to serpentinite minerals such as lizardite, chrysotile, antigorite, brucite, and iron hydroxide (Schulte et al, 2006). The following reaction illustrates the process of serpentinization:

Reaction 1:



The iron hydroxide that is produced can undergo a subsequent reaction:

Reaction 2:



Reaction 2 generates molecular hydrogen, magnetite, and water, which under the appropriate conditions has been shown to through experiment, observation, and theory to facilitate the abiotic production of hydrocarbons such as methane and small alkanes (Schulte et al, 2006)

Minerals such as olivine and pyroxene are thermodynamically stable at the high pressures and temperatures typically observed in subsurface regions such as the lithosphere, which comprises the crust and the upper mantle. However, in certain circumstances tectonic forces can act upon these rocks to thrust them to the surface where they interact with seawater. Once water infiltrates the pores and channels within these rocks, the volumes of the materials expands and generates enough buoyancy to further fracture the ultramafic rocks and create fresh reactive surfaces. When a mafic mineral, such as olivine, meets the oxic water, it becomes thermodynamically unstable. The minerals will promptly react with the water and undergo serpentinization, yielding altered mineral compositions.

In addition to the serpentine minerals produced, these systems release highly reduced fluids containing high quantities of molecular hydrogen, as well as other molecules such as methane and small alkanes (Sherwood-Lollar et al, 2002). When the reduced fluids meet oxic seawater in marine serpentinites, it creates an interface that can be a source of free energy in the form of chemical disequilibria for various biochemical processes (McCollom et al 2007). The hydrogen is critical in this aspect because it is a universal electron donor, and can supply protons to electron acceptors involved in catabolic reactions (Fruh-Green et al, 2004). For example, microorganisms can catalyze the oxidation of hydrogen with carbon dioxide to yield methane and water in a process known as methanogenesis (Nealson et al, 2005). Moreover, the disequilibrium generated by serpentinization can also promote other form of chemotrophy, such as sulfate oxidation, iron oxidation, and aerobic hydrogen oxidation.

Ultramafic exposures are common in slow-spreading areas of the mid-ocean ridges, comprising up to 25% of the seafloor in slow-spreading ridge environments (Fruh-Green et al, 2004). A prime example is the Lost City Hydrothermal Field (LCHF), located in the middle of the Atlantic Ocean (Kelley et al, 2001). Serpentinization at the LCHF releases warm (20 – 93°C), reduced, alkaline fluids at the seafloor through large carbonate “chimneys.” This slow-spreading ridge provides a unique habitat relatively independent of active magmatism. The elevated temperatures and chemistry of LCHF is influenced by exothermic reactions resulting from the interactions of ultramafic rock with the seawater, resulting in fluid temperatures up to 93°C (Kelley et al, 2001).

In addition to mid-ocean ridges, ophiolites also present a typical setting conducive for serpentinization. An ophiolite is a terrestrial exposure of peridotite rock, where younger, denser oceanic crust is superimposed upon lighter, granitic (also known as felsic) continental rocks (Schulte et al, 2006). This emplaces ocean-derived rocks onto the continents, where they can react with freshwater and undergo hydration and oxidation. Ophiolites offer a more accessible location for scientists to study serpentinization and collect samples due to the costs and other technical challenges associated with accessing the deep seafloor.

Specifically, the Tablelands Ophiolite is series of rock formations that is about 500 million years old (Cardace, 2009). It consists of exposed ultramafic rocks that originated from oceanic mantle and crust that were displaced onto the continental margin, located on present-day Newfoundland. These rocks are composed predominately of the minerals olivine and pyroxene, which become unstable upon exposure to water. This phenomenon results in active serpentinization at Tablelands. Previous field work has

shown that the reduced fluids of this ophiolite contain up to 500 ppm hydrogen and 6-8 ppm methane, with a pH's greater than of 11 (Cardace, 2009). The abundance of hydrogen is anticipated to fuel an array of microbial metabolisms that use hydrogen as a reducing agent, such as methanogenesis or hydrogen oxidation.

A critical attribute of serpentinization is the highly basic pH associated with these environments. Most enzymes function optimally at or near a neutral pH. This presents a major challenge for organisms trying to survive in alkaline conditions (Sorokin et al, 2005). At a high pH of about 12 there are far fewer dissociated hydrogen protons as compared to a more acidic setting. The hydrogen pumps used for ATP synthesis and other physiological functions are rendered unavailable. Microbes in these environments must adapt and develop alternative membrane pumps for transport involving sodium ions for instance (Skulachev, 1989), and/or use substrate level phosphorylation (SLP) to generate ATP. Organisms that have adapted to grow and survive in basic systems are known as alkaliphiles, and are expected to be prevalent at regions of serpentinization. Another challenge to life in serpentinization-influenced ecosystems is access to terminal electron acceptors to couple to the abundant electron donors. The highly reducing conditions which lead to hydrogen production and abiotic organic synthesis also minimize access to these critical compounds. One solution to both of these problems may be that serpentinite-adapted microorganisms rely upon the fermentation of organic compounds, which can serve as both electron donors and acceptors, and utilize SLP. Finally, dissolved inorganic carbon (*e.g.* the carbonate anion) is extremely low in these high pH systems due to its near spontaneous precipitation as calcium carbonate (such as the carbonate chimneys at LCHF) in the presence of calcium cations leached from the

ultramafic rocks. Therefore, serpentinite-hosted microorganisms must be carbon limited, or utilize other carbon sources such as methane, alkanes, and other organic acids created abiotically by serpentinization.

Relatively little empirical data exists about how serpentinization processes affect microbial life. Specifically, few studies have focused on the terrestrial settings of serpentinization (ophiolites) and it is largely unknown which microbial species are able to exist in these environments and how they obtain energy. Even less is known about whether life can and does exist in the serpentinite subsurface. The work describes the microbiological analysis of samples from the Tablelands Ophiolite in Newfoundland, Canada which provides a map of microbial communities are distributed within a serpentinite-hosted ecosystem, and how communities vary based on how close they are to sources of alkaline fluids.

Although there is a dearth of microbiological analyses of serpentinite-hosted ecosystems, previous research can be used as a template to predict diversity and abundance levels, and also to give an idea of what species may exist at the Tablelands site. A study of bacterial and archaeal diversity at the Lost City Hydrothermal Field revealed the presence of Bacteria that use metabolisms such as sulfur oxidation, sulfur reduction, and methane oxidation. Genetic analyses indicated there were archaeal species such as methanogens (species that use methanogenesis as their primary method of obtaining energy), as well as anaerobic methane oxidizers (Brazelton et al, 2006). From a physiological standpoint, methanogens are well-suited for these high pH, reduced systems because they are anaerobic and use the hydrogen released from the serpentinization reactions to fuel their metabolisms. Moreover, many of the bacterial

species found at LCHF belonged to a broad phylum known as the *Firmicutes*. This phylum constitutes predominately gram-positive bacteria that have the ability to survive in extreme conditions, such as the alkaline setting of serpentinization (Brazelton et al, 2006).

Sequences related to the *Firmicutes* were also recovered from alkaline groundwater in Cabeço de Vide, Portugal (Tiago et al, 2004). This site consists of water with a high calcium-hydroxide concentration (pH ~11.4), resulting from active serpentinization. Using sequence analysis of the 16S rRNA gene, it was determined that approximately 81% of the bacterial strains isolated from this site were closely related to one of three species of the Phylum *Actinobacteria*: *Dietzia natrolimnae*, *Clavibacter*, and *Microbacterium kitamiense*. Several strains were also identified as *Alpha-proteobacteria* and *Firmicutes* species (Tiago et al , 2004). The majority of the isolates from this site were found to be alkali tolerant, not necessarily alkaliphilic. Even though the pH of groundwater was 11.4, only two of the strains were able to grow on media of pH 11. Most isolated strains optimally grew on media with a pH between 7 and 9. This indicates that bacteria exposed to alkaline seepage at the Cabeço de Vide site have the ability to at least survive in highly basic water, even if they grow optimally at a more neutral pH. Moreover, cell counts estimated abundances in the range of $6.0 - 7.2 \times 10^2$ cells ml⁻¹, signifying a low standing stock of microbial biomass (Tiago et al ,2004).

The parallels between the LCHF site and the Cabeço de Vide alkaline groundwater site suggest that similar species would be expected to be recovered at the Tablelands Ophiolite, in particular members of *Firmicutes* and *Alpha-proteobacteria*, and that microbial diversity should be highly limited at Tablelands, or any other serpentinite-

hosted ecosystem. Similar to the Cabeço de Vide site, the Tablelands site should also host low cell abundances. It is also expected that Tablelands will host many alkali tolerant microorganisms that are able to adapt their metabolisms to the highly basic conditions, as well as alkaliphiles that grow optimally in hyperalkaline settings (pH 10-12). In general, biomass is expected to be low at sites of active serpentinization as a result of the highly reduced and highly basic fluids that limit resources for growth.

The unique conditions created by serpentinization (*i.e.*, redox interface, high pH, abundance of hydrogen, etc.) provide geochemical energy for a variety of different metabolic reactions, as listed in Table 1. As previously mentioned, slow-spreading ridges such as LCHF has been shown to harbor populations of methanogens, sulfate-reducing bacteria, and anaerobic methane oxidizers. Despite the low free energy yields for the reaction anaerobic methane oxidizers are presumed to rely on syntrophic relationships in which they are coupled to another microorganism, such as sulfate-reducing bacteria (Brazelton et al, 2006). Methane oxidation is essentially the reverse of methanogenesis, in which methane is consumed and converted to carbonate, using sulfate as the terminal electron acceptor. Hydrogen transfer between the two cells creates energetically favorable ($-\Delta G$) conditions for the methane oxidizer, allowing it to carry out its energy-yielding pathways (Nauhaus et al, 2002). While methane oxidation requires syntrophy to be energetically favorable, methanogenesis is a high energy-yielding pathway that does not require coupling to another reaction. Methanogens are able to take advantage of the abundance of hydrogen, reacting hydrogen (an electron donor) with carbon dioxide (a terminal electron acceptor) to produce methane.

Minimal research has been performed on what types metabolisms exist at terrestrial sites of serpentinization, like the Tablelands Ophiolite. Because sulfur concentrations are expected to be very low in these freshwater seeps in comparison to marine systems, sulfate reduction/ is unlikely to be a significant pathway at an ophiolite, where sulfate is a predominant anion. However, redox reactions such as methanogenesis and hydrogen oxidation would be highly favorable at an ophiolite due to the large quantities of hydrogen. Hydrogen acts as a reducing agent by reducing carbon dioxide in methanogenesis and reducing oxygen in hydrogen oxidation. The microbial communities at an ophiolite would likely differ substantially from those found at deep marine settings of serpentinization, like a slow-spreading ridge. Microorganisms in ophiolites will also have much more direct access to sunlight, making photoautotrophic metabolisms potentially possible, especially near the surface. Also, these surface freshwater springs have direct exposure to the atmosphere, and therefore have a more direct air-fluid interface. The presence of dissolved oxygen and sunlight would likely make Tablelands a very different environment than LCHF or any other deep ocean alkaline setting. The alkalinity of this site should greatly limit species diversity. However, there should still be a variety of chemolithotrophs (catabolize an inorganic carbon source), photoautotrophs (use light to yield energy) and heterotrophs (consume preexisting organic compounds). Finally, the occurrence of serpentinized ophiolites on continental margins throughout the world, widely separated in space and time provides a fascinating series of ecosystems to study microbial colonization and dispersal.

Figure 1: Serpentinitized ultramafic rock at Tablelands.



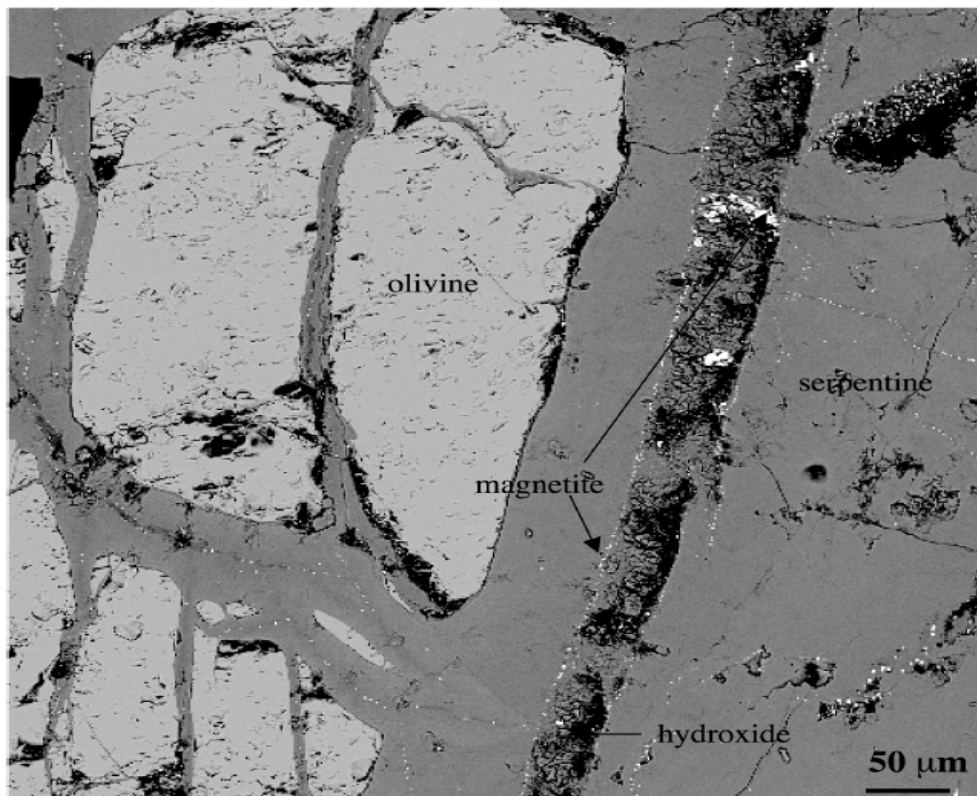
Vein pattern distributed throughout the rock's surface indicated where serpentinization has occurred, causing the olivine minerals to be altered, forming new serpentine minerals.

Figure 2: View of Gros Morne National Park, Newfoundland, Canada.



Tablelands Ophiolite can be seen in the background of this photo. The oxidized ultramafic rocks give the complex a distinct rust color that contrasts with the highly vegetated surrounding terrain. Ultramafic rock provides scarce opportunities for plant growth due to poor soil development, high pH, and arid conditions.

Figure 3: Electron micrograph of a section of olivine-containing peridotite.



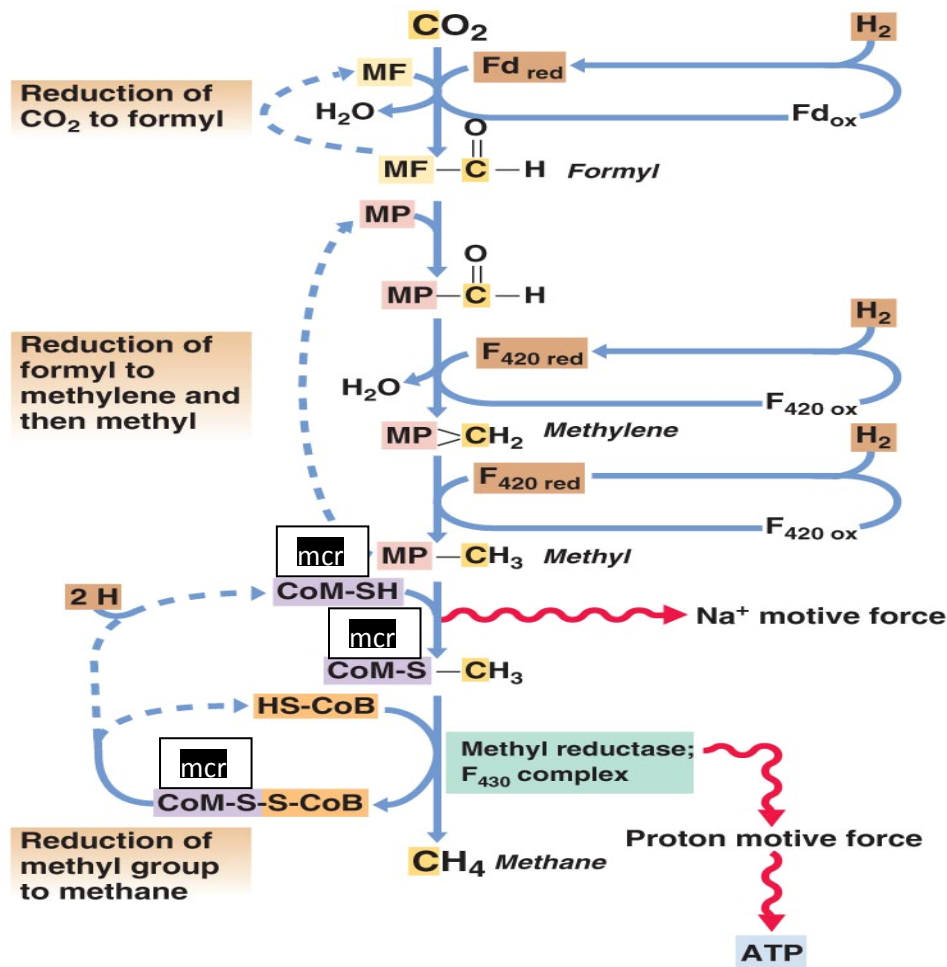
Sample recovered from the Coast Range Ophiolite in California. Image indicates where olivine has undergone alteration and has been transformed into serpentine. The appearance of the magnetite and hydroxide is indicative of serpentinization, as they are two chemical species produced by the reaction. From Schulte et al, 2006.

Table 1: Microbial metabolisms associated with sites of serpentinization.

Type of Metabolism	Reaction
Methanogenesis	$\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$
Hydrogen Oxidation	$2\text{H}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O}$
Sulfate Reduction	$2\text{CH}_2\text{O} + \text{SO}_4^{2-} \rightarrow 2\text{HCO}_3^- + \text{H}_2\text{S}$
Anaerobic Oxidation of Methane	$\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O}$
Acetogenic Fermentation	$\text{C}_6\text{H}_{12}\text{O}_6 \text{ (sugar)} \rightarrow 3\text{CH}_3\text{COO}^- + 3\text{H}^+$

The listed reactions represent a snapshot of some of the metabolic pathways that are utilized by microbes at sites of serpentinization. Sulfate reduction and anaerobic oxidation of methane are typically found in deep ocean settings, such as LCHF. Dissolved sulfate is found in abundance in marine environments, typically around 2,701 ppm (Anthoni, 2006). Chemical analysis of the nonsaline alkaline groundwater of Cabeço de Vide showed a minimal sulfate concentration of app. 0.03 mM (Tiago et al, 2004). However, the plethora of hydrogen and methane at ophiolites should promote pathways such as hydrogen oxidation and methanogenesis. Above reactions from *Brock Biology of Microorganisms*, 12th ed.

Figure 4: Molecular pathway of methanogenesis



The above schematic depicts the steps involved in methanogenesis. The critical enzyme, methyl-coenzyme M reductase (*mcr* or CoM), is shown to catalyze the reduction of the methyl group ($-\text{CH}_3$) to methane (CH_4). This process generates a proton motive force that yields energy in the form of ATP. Image courtesy of *Brock Biology of Microorganisms*, 12th ed.

Figure 5: Test strip indicating a pH ~11 for the WHC2 alkaline spring.



Chapter 2: Objectives

1. Site Description/Microbial Species Diversity

The primary focus of this project is to assess the relationships between the geochemistry and the microbiology of serpentinization-influenced ecosystems. Very little is known about the diversity of microorganisms, their physiology, or their associated metabolisms within serpentinite-hosted environments. This study is aimed at increasing the understanding of how serpentinization impacts the surrounding microbial ecosystem. The major objective is to assess the microbial taxonomic diversity of this site using the total genomic DNA extracted from seep fluids and rock samples from the Tablelands serpentinite in Newfoundland, Canada. A second goal is to determine differences in the biogeographic distribution of microbial communities at the Tablelands site, especially how these distributions vary near and away from the point sources of serpentinization-influenced fluids. Additionally, a survey of specific functional genes (MCR, hydrogen oxidation, etc.) likely to be utilized in the serpentinite ecosystem was conducted to determine what metabolisms may be operative in these habitats.

2. Cell Abundances

One of the first-order challenges at the Tablelands site was to determine the abundance of microbial cells at each location. Do these ecosystems support population sizes consistent with other habitats that have more moderate environmental conditions, or does its unique state put a limit on population growth? Collectively, microorganisms are able to thrive in a diverse range of habitats. However, the extreme conditions (i.e. high pH, lack of oxygen) should inhibit the survival and growth of most species. Cell

abundances measured from the fluids and rocks at the Tablelands provided a quantitative background to the analysis of extracted nucleic acids. Physical and chemical measurements from the same sites were also conducted to provide geographic and habitat data related to these samples.

3. Biogeography (Community Overlap)

An assessment of the spatial distribution of microorganisms was also conducted to determine community similarity between different sites. A goal of this study was to examine spatial patterns and see how species composition changed in based upon the sample's proximity to an active serpentinite-driven seep. Would differences in pH and red-ox values influence species distribution? Also, would sites with very similar conditions share many of the same species, or would they have their own, unique communities? Comparing the number of operational taxonomic units (or OTU's) shared between each site was used to determine species overlap between each site and to gain a sense of how serpentinization influences microbial communities.

4. Presence of Functional Genes (*mcrA*, Hydrogenase)

Little to no published research exists on the presence or absence of certain functional genes at terrestrial sites of serpentinization, such as the Tablelands Ophiolite (Blank et al, 2009). Sequence analysis using the 16S rRNA gene allows researchers to assess the diversity from a taxonomic standpoint. However, screening for specific functional genes is informative as to what reactions these microbes are utilizing to obtain energy. For instance, methanogenesis has been shown to occur at serpentinites such as the Lost City Hydrothermal Field (Schrenk et al, 2004). A crucial enzyme that is

required for this pathway is methyl-coenzyme M reductase (mcr). MCR catalyzes the terminal step of methanogenesis and is therefore a useful tool for determining if microbes use this pathway. MCR is composed of alpha, beta, and gamma subunits. Thus far, all sequenced genomes of methanogens have contained the mcrA subunit operon. Using primers specific for mcrA, it is possible to identify the potential for methanogenic activity (Hallam et al, 2003).

Another functional gene of interest is hydrogenase, an enzyme that catalyzes not only the oxidation of hydrogen, but also the oxidation of excess reducing equivalents of fermentative reactions, which produces hydrogen (Boyd et al, 2009). Screening for the gene that codes for hydrogenase, *hydA*, can be used to determine if a microorganism has the capability of performing hydrogen oxidation. To this point, evidence of *hydA* has been limited to anaerobic bacteria and a minimal amount of green algae (Boyd et al, 2009). The alkaline seeps at Tablelands provide a suitable environment for this metabolism, which may provide an interesting comparison to the acidic, terrestrial hot springs where this metabolism has been previously studied.

5. Presence of Archaea

Genomic DNA recovered from the environment was also screened for the presence of the 16S rRNA archaeal gene to determine if archaeal species were present at Tablelands. Gel electrophoresis was used to indicate if Archaea occurred at each of the 6 sites. Archaea are typically predominant in extreme environments, such as hydrothermal vents, hot springs, or other high-temperature environments. Because Archaea are usually much less abundant in lower temperatures and nonsaline conditions, this screening was

performed to determine if the serpentinite-hosted ecosystem could support archaeal communities in a low temperature terrestrial setting.

6. Culturable Microbial Diversity

This project also focused on isolating microbial strains in pure culture from the Tablelands site. Obtaining cultures of alkaliphiles and/or alkali-tolerant bacteria provided further information concerning how they obtain energy (heterotrophic, chemotrophic, etc.), their growth responses in the presence or absence of oxygen (aerobic/anaerobic), and the influence of pH upon growth. Phylogenetic relationships between the isolates and the environmentally-derived DNA were determined through sequence analysis of the 16S rRNA gene. Once strains were grown in culture, further experiments were conducted to assess how certain conditions (*i.e.* temperature, light, pH, and presence or absence of oxygen) affected microbial activity. Because the pH of the WHC2 and TLE1 sites were in the range of pH 10-11.5, pH 11 media consisting of yeast extract, peptone, and bicarbonate was used to grow these microbes on agar plates at a temperature of 15°C.

Chapter 3: Hypotheses

1. Species diversity will be lowest in the most extreme environments (*i.e.* high pH).

Microbial diversity at this site of active serpentinization is expected to be low. The extreme settings of these environments should significantly constrain which species are able to grow. Three major factors that limit life in serpentinite hosted ecosystems are high pH, a lack of electron acceptors, and low organic carbon. The high pH would create a lack of protons, which are necessary to drive the proton motive force across the cytoplasmic membrane. Also, serpentinite ecosystems are often low in electron acceptors, which are critical for oxidative catabolic pathways used to obtain energy. Furthermore, the availability and requirements for nutrients and trace elements are completely unconstrained. These factors necessitate specific adaptations for microbes to survive and to grow, likely limiting the number of different species that may be supported.

2. Species diversity will increase away from the alkaline seep source.

Substantial variation in the number of species present at each unique site is also expected. The sites of pH 11.6 (WHC2-A, WHC2-B, and WHC2-C) are predicted to harbor the lowest range of species due to its highly basic pH, as indicated in Table 4. The pH 10.19 (TLE1-B) and 10.37 (TLE1-D) sites should have a greater diversity of microorganisms than the WHC2 sites, due primarily to the lower pH, reported in Table 5. However, this site is also very basic and will likely have relatively low diversity as well. The control site (WHB), which had a pH of 7.19, is predicted to have a significantly

greater number of microbial species than either of the ultrabasic sites. The neutral pH should promote more growth and put less stress on microbial cells, while still benefitting from the fuels produced by nearby serpentinization. Therefore, the basic guideline being followed is that the higher the pH of the fluids, the lower the microbial diversity.

Diversity should be the lowest at the direct source points of serpentinization, where the fluids will be highly reduced (mean redox value of -457 mV for site WHC2 compared to -200 mV for TLE1-D, -67 mV for TLE1-B, and +208 mV for WHB, as shown in Tables 4 and 5) and have an alkaline pH. Higher reduction potentials promote higher concentrations of electron-donating chemical species such as hydrogen, which favors anaerobic metabolisms and limits opportunities for microorganisms that grow optimally in more oxidized, neutral pH environments. Moving away from the seep source, the pH should gradually decrease and promote higher diversity.

3. Community overlap between different sites will be low.

In addition to low overall microbial diversity, each site is anticipated to have its own unique community of microorganisms. The different pHs will select for different metabolic and physiological groups of microorganisms. Therefore, the WHC2, TLE1, and WHB sites should share little overlap and have only a small number of species in common. However, there should be substantial overlap between the WHC2-A, WHC2-B, and WHC2-C sites. These samples were obtained from different locations within the same alkaline pool, and the resident microorganisms would have been exposed to similar conditions (pH, temperature, red-ox values, etc.). The same concepts apply to the TLE1-B and TLE1-D sites, which should also have high overlap due to similar pH conditions. Diversity is also likely to be influenced by the availability of oxygen and the flow rates of

the reduced fluids. The WHB site is a creek with a higher flow rate and more dissolved oxygen compared to the WHC2 sites, which are from a stagnant, highly basic pool with little flow. Meanwhile, the TLE1 sites had the highest concentrations of dissolved oxygen (report the values here). The TLE1 sites were situated on a hillside where the water source was a shallow seep, which likely would cause the water to mix more vigorously with the air. These subtle differences are likely to have a great impact on not only what types of species are present, but the community diversity that each site is able to host. Overall, sites with similar pH values and flow conditions should share a relatively high number of species, while comparisons of sites with noticeably different pHs should reveal little overlap (*i.e.* unique communities).

4. Biomass will be lower at sites of active serpentinization.

Due to the distinctive nature of serpentinizing systems, cell abundance/biomass should be lower than what is expected in more moderate conditions. Only microbes that are specifically adapted to the environmental challenges of high pH, low solubility of dissolved carbonate, and low Eh should be able to take advantage of the available geochemical energy resources. The notion of low biomass is supported by a study of hyper-alkaline spring waters in Maqarin, Jordan. Cell concentrations in the high pH waters were estimated at 10^3 - 10^5 cells/mL, which is substantially lower than microbial concentrations of app. 10^8 cells/mL that are typically observed in neutral pH groundwater (Pedersen, 2004). It could be anticipated that the high pH Tablelands fluids support similar biomass levels. In contrast the WHB site, having a neutral pH, should have the highest cell abundances. In other words, diversity and biomass is expected to be vary proportionally when comparing the different sampling sites.

5. Functional genes for methanogenesis (*mcrA*) and hydrogen oxidation (*hydA*) will be present near sites of alkaline seepage.

Because of the reduced fluids high in volatiles, it is likely that methanogenesis and hydrogen oxidation both occur at the Tablelands serpentinite. Microenvironments in the alkaline seeps that are underneath rocks or in the subsurface where conditions are more anaerobic will likely promote these pathways. Methanogens would be able to use the abundant hydrogen to carry out their anaerobic metabolisms. Methanogenesis is energetically favorable in these highly reduced systems and should occur at each of the alkaline sites (WHC2-A, B, C and TLE1-B, D). The neutral pH brook, WHB, likely will not contain evidence of methanogens because of the more oxidized conditions that contain less hydrogen and likely promote aerobic and heterotrophic communities. The same concept applies for hydrogenase. Anaerobic bacteria should be able to catalyze the reaction of hydrogen with dissolved oxygen to produce ATP, releasing water as a by-product. However, hydrogen oxidation would not be a favorable pathway in a neutral pH, oxidized environment due to lower levels of molecular hydrogen and competition by other microorganisms such as heterotrophs or phototrophs.

6. Presence of Archaea will be confirmed.

The unique, reducing conditions should promote methanogenic activity carried out exclusively by a prokaryotic domain known as the Archaea. Therefore, coupled screens of the total genomic DNA for archaeal 16S rRNA genes as well as the *mcrA* gene, should indicate the presence of not only methanogenic microorganisms, but also confirm that Archaea occur at Tablelands ophiolite.

This Tablelands is located in a relatively cold, Northern climate and even in the summer when readings were taken, water temperatures were around 18-20°C in most locations. These temperatures were recorded in July; therefore, the water temperature of these alkaline seeps likely decreases significantly in the fall and winter months. This is significant because Archaea generally predominate in high temperature environments such as hydrothermal vents or hot springs. Archaea are also more likely to be abundant in the deep ocean seafloor, where conditions are highly anoxic and anaerobic metabolisms are commonplace. The proximity to the atmosphere and access to sunlight, combined with the moderate to low water temperatures, make it likely that overall Bacteria will be more prevalent than Archaea at the Tablelands ophiolite. However, archaeal species such as methanogens should still be present in low numbers.

7. Microbial isolates can be enriched on high pH media from the seep fluids and they will be alkaliphilic.

Culturing studies should reveal the presence of microorganisms physiologically and metabolically adapted to function at high pH. Because nutrient-rich, aerobic media was utilized for the enrichment cultures, it should promote the enrichment of microbes that are able to break down the organics. Because ophiolites are terrestrial sites they are exposed to the atmosphere, there are likely niches within the serpentinite habitat that provide opportunities for colonization by aerobic microorganisms. Rock and fluid samples recovered from the shallow, alkaline pools have access to both light and oxygen. Conversely, anaerobic metabolisms exist at Tablelands, they likely occur in deeper pools or in the subsurface, within rock layers that have close proximity to the active serpentinization seep.

Chapter 4: Methods

Field Work

The field work was completed in Newfoundland, Canada at Gros Morne National Park in July 2009. This park contains a series of ultramafic (peridotite) rock formations referred to as the Tablelands. This site contains abundant serpentinites, the alteration products of serpentinite rocks, and the resulting high pH springs. First, physical and chemical conditions were assessed. The YSI 85 Handheld Dissolved Oxygen/Conductivity Instrument was used to measure the temperature, pH, salinity, dissolved oxygen, and conductivity of the water. An ORP Redox Meter (Oakton) was used to quantify the red-ox values in terms of Eh, which is the reduction potential promoted between a platinum electrode and a reference hydrogen electrode (Vance, 1996). The water sources that were studied included stagnant pools of water generated by serpentinization, as well as a typical brook fed by snowmelt with an approximately neutral pH to serve as a control. Another site located on a mountain slope that exhibited calcium carbonate deposits and moderate water seepage was also studied.

A portion of the samples were placed in 50-mL Falcon tubes containing 3.7% formaldehyde fixative to preserve them for microscopy. A portion of the rocks from each of the field sites were put into 50-mL Falcon tubes, which were subsequently filled with a solution known as RNAlater (Ambion). This is an aqueous reagent that stabilizes and preserves nucleic acids. This alternative approach was used due to the unavailability of dry ice or a freezer. In addition to rocks, sterile 250 mL Nalgene bottles were used to collect fluid samples from each of the locations and refrigerated at 4°C until processing.

Distribution of Sample Collecting

Rock samples were analyzed from the following sites: WHC2-A, WHC2-B, WHC2-C, TLE1-B, TLE1-D, and WHB. WHC2-A, WHC2-B, and WHC2-C are three separate locations in the alkaline water pool containing a pH of approximately 11.6 and a mean reduction potential of -457 mV. TLE1-B is at the bottom of a precipitated calcium carbonate deposit with alkaline water seepage (pH=10.19 and a reduction potential of -67 mV), and TLE1-D is located at the top of this structure (pH=10.37 and reduction potential of -200 mV). WHB represents a flowing stream with a pH of 7.19 and a reduction potential of +208 mV. Water temperatures of each sampling site were anywhere from cool to moderate, giving an approximate range of 13-23°C. Tables 4 and 5 give a summary of the data from these sites, including pH, reduction potential, water temperature, conductivity, salinity, and dissolved oxygen readings.

DNA Isolation from Rocks

DNA extractions from rock samples were completed using a modified of the protocol of Schrenk, et al. 2004 (Schrenk et al, 2004). Rocks were transferred into sterile whirl pack bags, pulverized into smaller (few mm sized) grains using a rock hammer, and approximately one gram per extraction was placed into a 2-mL microcentrifuge tubes. Subsequently, they were mixed with 500 µL of Extraction Buffer A (200 mM NaCl, 200 mM Tris, 2 mM sodium citrate, 10mM CaCl₂, 50 mM EDTA; pH 8.0), 20 µL of 10% sodium pyrophosphate, and 30 µL of lysozyme (10 mg/mL), and then agitated for at 150 rpm for 1 hr. at 37° C. Following the addition of 10 µL of 20% SDS and 60 µL of Proteinase K (20 mg/mL), a second incubation was done at 50° C for 30 min. at 150 rpm.

A third incubation, also at 50° C for 30 min. at 150 rpm, required the addition of 80 µL of 5 M NaCl and 80 µL of 10% CTAB in 0.7 M NaCl. Three freeze-thaw cycles of -80°C and 65°C were completed after adding 200 µL of 20% SDS. The sample was mixed with 500 µL of phenol: chloroform: isoamyl alcohol (PCI (25: 24: 1)) and spun for 10 min. at $12000 \times g$ (4° C). The supernatant was transferred to a new microcentrifuge tube and mixed with an equal volume of PCI, and spun again for 10 min. at $12000 \times g$. The top phase was transferred to another tube and mixed with an equal volume of isopropanol and one-tenth the volume of 3 M sodium acetate to precipitate the DNA. The mixture was then spun for 20 min. at $13000 \times g$. The supernatant was decanted and for the pellet was washed with approximately 150 µL of cold 80% ethanol, then centrifuged for 10 min. at $13000 \times g$. Repeating the previous step, the supernatant was decanted and the pellet was air-dried for 10 min. The DNA was suspended in 150 µL of lysis buffer and purified using the Wizard® Genomic DNA Purification Kit (Promega). Finally, the DNA pellet was resuspended in 40 µL of sterile, molecular biology grade water.

Measuring the DNA Concentration

A Nanodrop 2000 Spectrophotometer (ThermoScientific) was used to give values of DNA concentrations isolated from the samples, reported in nanograms per microliter. A microliter of sterile water was used as the blank, after which 1 µL DNA samples were loaded onto the nanodrop to be measured. The nanodrop used absorbances of 260 nm and 280 nm.

PCR Assays

DNA was amplified using a polymerase chain reaction (PCR) assay. For each bacterial or archaeal 16S rRNA PCR reaction the following reagents were added: 5× green GoTaq buffer, 800 μ M of dNTP, 5 μ M of forward primer (27F for Bacteria, 21F for Archaea), 5 μ M of reverse primer (1492R for Bacteria, 958R for Archaea), 10.8 μ L of molecular biology grade water, 0.5 U of Taq, and 1 μ L of template. The tubes were mixed gently and briefly centrifuged. A BioRad C1000 thermal cycler was used to run the PCR. The primers used for each assay are reported in Table 3.

Gel Electrophoresis

Electrophoresis using a 0.8% agarose gel was used to confirm the presence of the correct sized amplicon in the PCR products. To make the gel, 0.4 g of agarose was added to 50 mL of 1× TBE (Tris-Borate-EDTA) buffer. Then, 2 μ L of GelRed (Biotium, Inc.) was mixed with the solution, which was microwaved for 1.5 min. Once the gel solidified, 2 μ L of 1-kb ladder (Invitrogen) was added to one well, with 5-10 μ L of PCR product in each of the other wells. After the gel had been run for 45-60 min. with a voltage between 95-110 mV, a BioRad gel imager (it would be good to report what this is, specifically) was used to visualize the DNA and measure the size and intensity of the amplicons.

Gel Extraction and DNA Purification

Distinct bands of PCR amplified DNA was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol. The purified PCR product was used for cloning reactions.

*DNA Cloning using Chemically Competent *E. coli* Cells*

Cloning was performed using the TOPO® TA cloning kit (Invitrogen). A ligation reaction at room temperature was prepared involving 4 µL of sample, 1 µL of salt solution, and 1 µL of TOPO® vector. The transformation procedure was completed in accordance with the One Shot® Chemical Transformation Protocol, using TOP10F' cells. The transformed cells were spread on LB plates containing 50 µg/mL ampicillin, 40 µL of 40 mg/mL X-gal, and 40 µL of 100 mM IPTG. After allowing the colonies to grow overnight in a 37°C incubator, the white colonies (which contain the plasmids of interest) were checked for the presence of the correct sized insert and subsequently used for sequencing.

Plasmid Preps and Sequence Analysis

Plasmid preps of the selected white colonies were completed using the QIAprep Miniprep Kit (Qiagen) according to the manufacturer's instructions. The clones libraries consisted of 70-90 partial sequences of bacterial 16S rDNA for each of the 6 sites, totaling 490 sequences for all 6 sites (DNA sequencing performed by Denise Mayer of the Genomics Core Facility for East Carolina University). The partial sequences were constructed using the 27F primer. Raw sequence data was cleaned using the software

Sequencher (Gene Codes Corporation). Each of the partial 27F sequences were 400-600 bases long going in one direction (forward).

The software Mothur was used to align the sequences, create distance matrices, calculate diversity statistics, and generate figures such as rarefaction curves and Venn diagrams (Schloss et al, 2009). The 494 partially sequenced clones yielded 176 operational taxonomic units (OTU's), with 37 of the OTU's containing 2 or more sequences. Microorganisms are considered to belong to the same operational taxonomic unit (i.e. "species") if they share at least 97% genetic similarity concerning the 16S rRNA genes that have been partially sequenced. Because of the highly variable functional diversity that is found between prokaryotes, it is difficult to define a species compared to the eukaryotic domain. Therefore, OTU's provide an alternative solution that allows microbiologists to group similar microorganisms together for classification and phylogenetic purposes.

One sequence from each of these 37 OTU's was chosen to be fully sequenced, using the primers 8F, 1492R, 926F, 907R, 515F, and 519R (sequences of primers listed in Table 3). Also, the four cultured isolates obtained from the Tablelands (WHC2-A Red, WHC2-A Yellow, TLE1-B, and TLE1-D) were sequenced using the same set of primers. The sequences from the cultures and environmental DNA were aligned and matched with their closest relatives using a program called Green Genes (DeSantis et al, 2006). The aligned sequences and the reference sequences were organized together using BioEdit, which was used to save all the sequences under the same Fasta file. The Fasta file of sequences was input into a tree-generating program called Geneious (Drummond et al, 2009). A phylogenetic tree were generated and used to study the relationship

between the cultures, environmental sequences, and the reference sequences that were identified as closely related species. The tree was a consensus of 100 replicate trees, using the neighbor-joining building method and the Tamura-Nei genetic distance model.

Functional Gene Screening

Functional gene assays were performed to confirm whether or not the functional genes *mcrA* and *hydA* were present in the recovered samples. Purified DNA from each site was used for PCR assays with primers specific for each functional gene. A gel was run to indicate the presence or absence of the targeted gene for each sample.

The PCR assay for *hydA* consisted of a 50- μ L reaction containing 1 $\times$ green GoTaq buffer, 800 μ M of dNTP, 0.5 μ M of FeFe27F primer, 0.5 μ M of FeFe27R primer, 0.25 U of Taq, 1.5 mM of $MgCl_2$, 0.4 μ g/ μ L molecular grade BSA (bovine serum albumin), 20 μ L of sterile water, and 2 μ L of template. The PCR assay used a 4 min. initial denaturation step at 94°C, 35 cycles of denaturation at 94°C for 1 min., annealing at 56.5°C for 1 min., primer extension at 72°C for 1.5 min., and a final extension at 72°C for 20 min (Boyd et al, 2009).

PCR for *mcrA* also used a 50- μ L reaction that consisted of 1 $\times$ GoTaq buffer, 800 μ M of dNTP, 0.25 μ M of *mcrA*-F primer, 0.25 μ M of *mcrA*-R primer, 27 μ L of sterile water, 0.5 U of Taq, and 2 μ L of template. This assay used an initial denaturation step at 94°C for 3 min., 34 cycles of denaturation at 94°C for 45 sec., annealing at 50°C for 45 sec., primer extension at 72°C for 1.5 min., and a final extension at 72°C for 5 min.

Microscopic Cell Counts

Cell abundances were determined using an Olympus BX61 Spinning Disk epifluorescence confocal microscope at 100× magnification. Fluid samples were passed through a filter using vacuum filtration, trapping the cells on a 0.22 µm black polycarbonate membrane filter (Millipore). The trapped cells were stained for 20 min using a 2.0 µg/ml solution of 4',6' diamidino-2- phenylindole (DAPI) prior to observation using blue excitation (Reference Porter and Feig, Limnology and Oceanography 1980). The area of the filter was 176.625 mm². The area of each microscopic field was 0.006710886 mm². The ratio of the area of the filter to the area of the microscopic field was calculated to be 26319.176, which represented the total number of microscopic fields contained on the filter. The number of cells in each field of view was counted to determine an approximate average of the cells present in a field. The mean cell count per field was multiplied by the total number of fields (26319.176) to attain the estimated number of cells on the entire filter. This number was divided by the amount of fluid in milliliters that passed through the filter. The final number indicated the number of cells estimated per milliliter of fluid (cells/mL).

The cell count estimates for the rock samples were measured in cells per gram of rock, or cells/g of mineral, following a brief sonication and extraction procedure. Rocks were crushed and transferred to Eppendorf tubes. A 2% paraformaldehyde/phosphate buffered saline (PFA:PBS) solution was added enough to submerge the crushed rocks in solution. The mixture was sonicated for 30 min., then briefly centrifuged at high speed. The supernatant was vortexed for 30-60 sec, and subsequently passed through a filter identical to the protocol for the fluid samples.

Culturing

Several strains of bacteria from Tablelands were enriched in cultures using high pH media. To make the pH 11 media, 2.5 g yeast extract, 2.5 g peptone, 450 mL water, 10 M NaOH, and 7.5 g bacto agar were added to a 1000-mL pyrex bottle. This mixture was autoclaved. After it was allowed to cool, 5 mL of 1 M Na₂CO₃ was added. The media was poured onto petri plates, where it solidified.

Fluid samples from selected sites (WHC2-A, TLE1-B, TLE1-D) were spread onto pH 11 agar plates using a series of dilutions. Growth of colonies was allowed to take place for up to two weeks at in situ temperature of approximately 15°C. Once different colonies had formed on each plate, each type of colony was streaked on a separate plate for isolation.

Additional analyses were performed on the cultures to assess their optimal growth conditions. Two isolates, WHC2-A Red and TLE1-D, were subjected to different conditions (either light and oxygen, light and no oxygen, oxygen and no light, no light and no oxygen). This was performed to determine if these strains relied on photosynthesis and to determine if they were strictly aerobic or anaerobic. To set up this experiment, dilutions of the colonies were prepared. A sterile loop was used to obtain a small amount of the streaked colonies. These colonies were mixed in 10 mL of the liquid pH 11 media. Once the dilution was made, 80 µL of the solution was spread on each plate. Four plates were used for each strain, using the four possible conditions previously described. A period of 5 days was allotted to allow for possible colony growth. The results were then quantified by counting the colonies that had formed.

Table 2: Tablelands Field Sites and Abbreviations

Site	Name	Abbreviation
Stream (pH=7.19)	WHB	W
Large Pool (pH=11.6)	WHC2-A, B, C	A, B, C
Top of CaCO ₃ deposits (pH=10.37)	TLE1-D	TD
Bottom of CaCO ₃ deposits (pH=10.19)	TLE1-B	TB

The above table indicates the 6 sites that were used for cell counts and genetic assays.

WHB stands for Winter House Brook, and is the control site. Three sites (A, B, and C) were used at WHC2, or Winter House Canyon Pool. Two sites (TLE1-B and TLE1-D) were picked from a hillside alkaline seepage. TLE1 stands for Tablelands East Facing Slope.

Figure 6: WHB (pH 7.19)

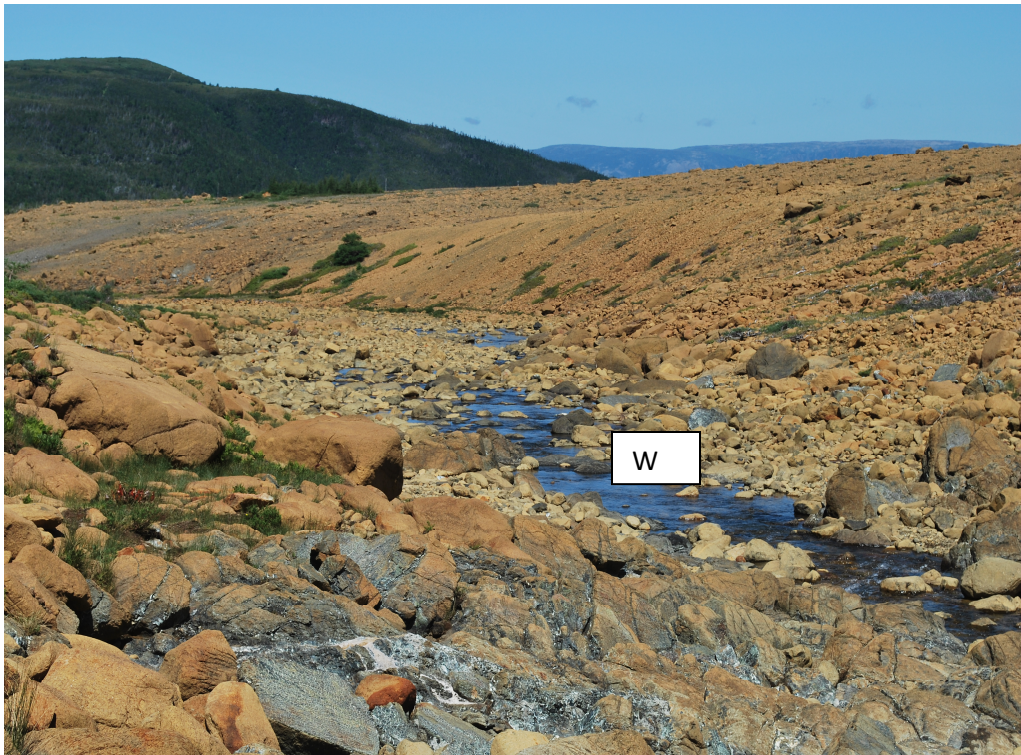


Figure 7: WHC2 (Large alkaline pool, pH ~11.6)

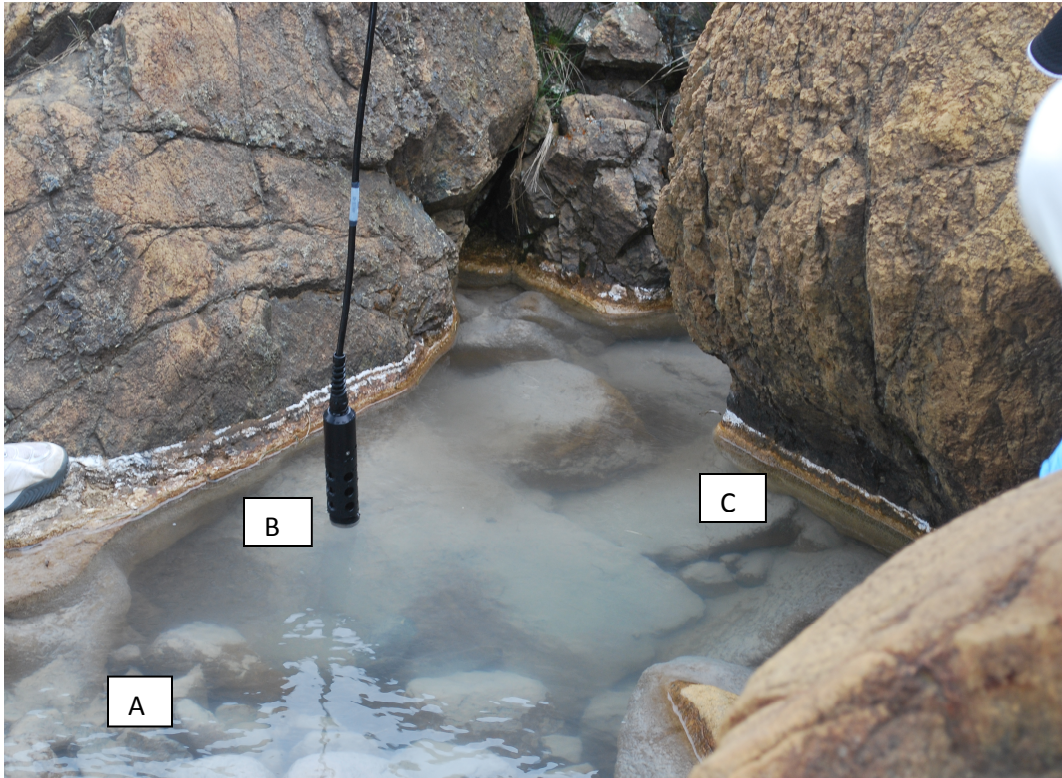


Figure 8: TLE1 (shallow alkaline seep, pH ~10.2)

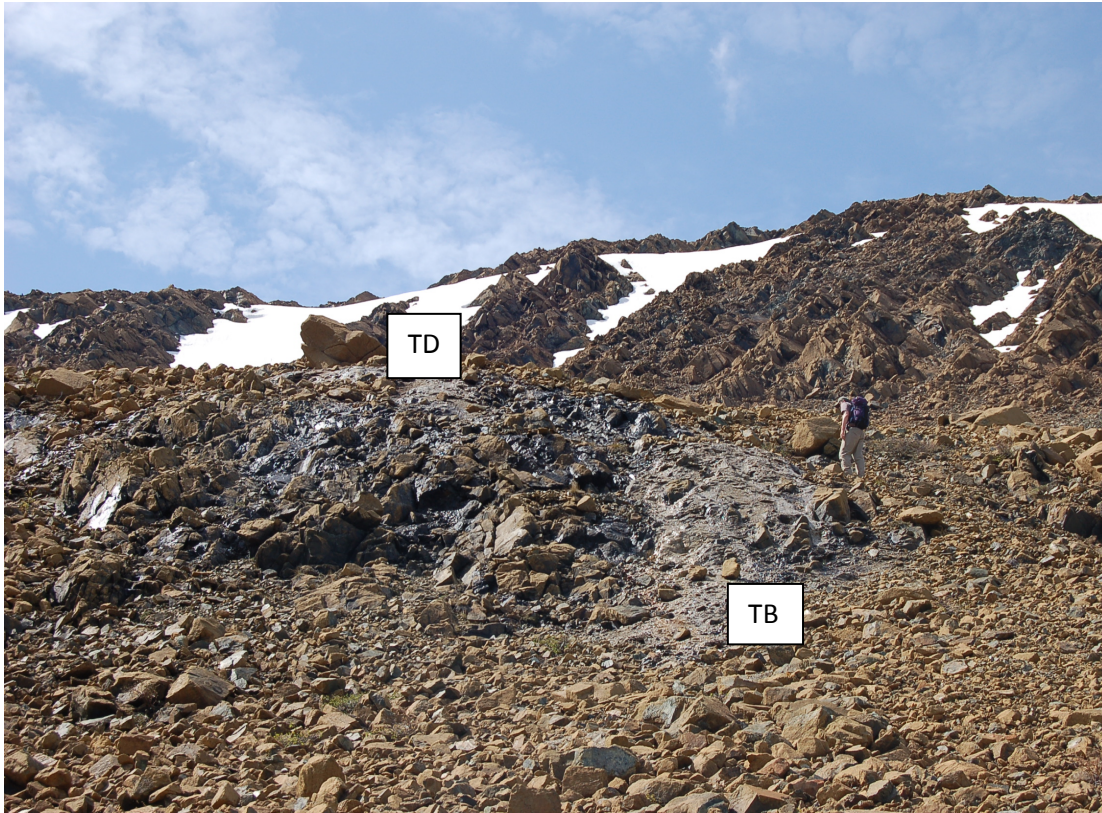
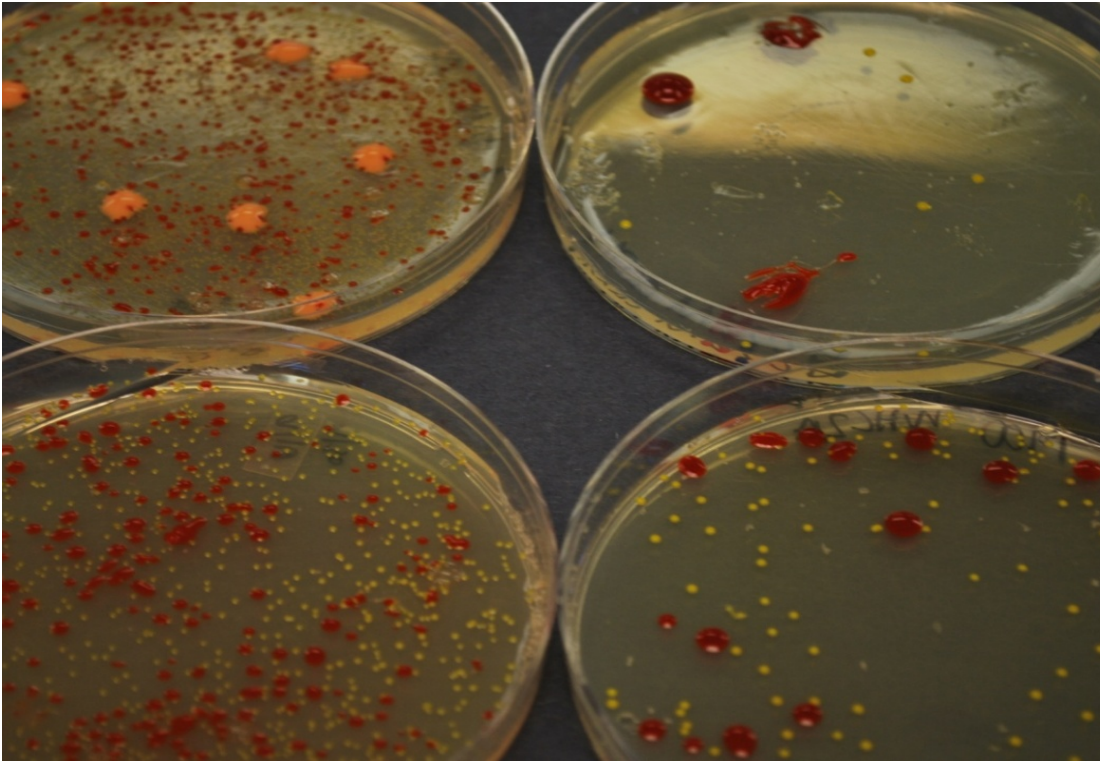


Table 3: Oligonucleotide primers used in the PCR Assays (Supplied by Invitrogen)

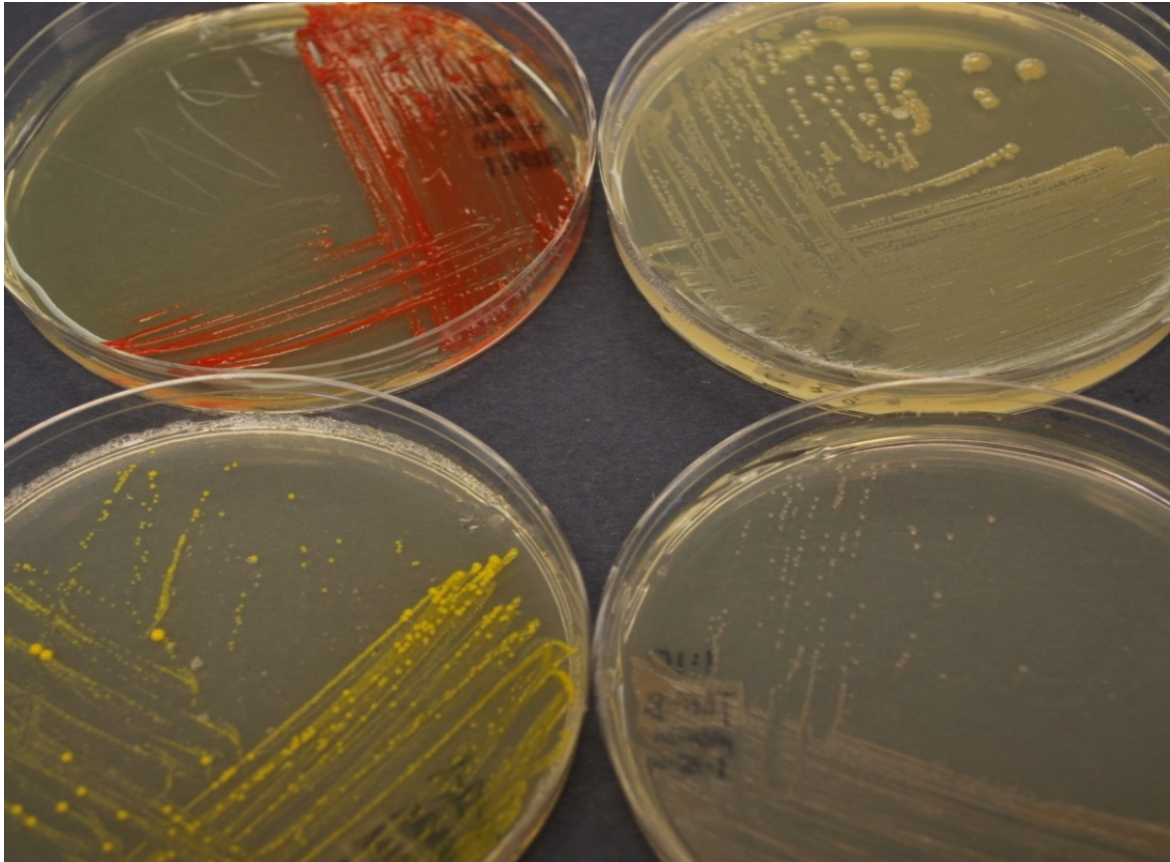
Target Gene	Primer Name	Primer Sequence
Bacterial 16S rRNA	27F	5'-AGAGTTTGATCCTGGCTCAG-3'
	1492R	5'-GGTTACCTTGTTACGACTT-3'
	515F	5'-GTGGCASC MGCCGCGGTAA-3'
	519R	5'-GWATTACCGCGGCKGCTG-3'
	926F	5'-GGTTAAACTYAAAKGAATTGACGG-3'
	907R	5'-CCGTCAATTCCTTTTRAGTTT-3'
Archaeal 16S rRNA	21F	5'-TTCCGGTTGATCCYGCCGGA-3'
	958R	5'-YCCGGCGTTGAMTCCAATT-3'
Hydrogenase	FeFe27F	5'-GCHGAYMTBACHATWATGGARGA-3'
	FeFe27R	5'-GCNGCYTCCATDACDCCDCCNGT-3'
MCR (Methyl-coenzyme M Reductase)	mcr-a F	5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'
	mcr-a R	5'-TTCATTGCRTAGTTWGGRTAGTT-3'

Figure 9: Image showing enrichment/growth of colonies from Tablelands samples.



These plates show colony growth after the addition of the serial dilutions onto the solid media. Each type of colony was streaked on a new plate for isolation. Image from Lauren Polli.

Figure 10: Photograph showing the process of obtaining isolated colonies from the Tablelands enrichment cultures.



The bacterial streaks in the above image show how each strain was isolated on a separate plate. The isolates are WHC2-A Red (top left), WHC2-A Yellow (bottom left), TLE1-D (top right), and TLE1-B (bottom right). Image from Lauren Polli.

Chapter 5: Results

Data obtained from field work, as well as laboratory analyses, are illustrated in the following figures and tables. Tables 4-6 give water data from the field sites and also comparisons to other serpentinite sites that have been studied, including temperature and pH readings. Table 7 shows DNA yields from rock samples. Table 8 reports the composition of bacterial species groups for the clone libraries. Table 9 lists several diversity statistics for each site. Tables 10-11 provides cell abundance data. Table 12 reports the results of the culturing experiment.

Figures 11-14 show gels for the bacterial and archaeal 16S genes. Figures 15-17 are epifluorescence micrographs, providing a microscopic view of the cell morphologies and abundances contained in the Tablelands fluid samples. Figure 18 is a rarefaction curve, which plots the number of 27F sequences versus the number of OTU's to give a representation of diversity. Figures 19-21 are Venn diagrams, which indicate the number of OTU's that are shared between the sites surveyed, providing a measure of community overlap. Figure 22 is a dendrogram, a phylogenetic representation of similarity between bacterial communities of different sites. Figure 23 is a phylogenetic tree of 37 fully sequenced bacterial OTU's of the Tablelands site and their closest phylogenetic neighbors.

Table 4: Water data from primary site (WHC2).

	WHC2 (2.4 m)	WHC2 (1.9 m)	WHC2 (1.4 m)	WHC2 (0.9 m)	WHC2 (0.4 m)	WHC2 (0 m)
pH	11.51	11.62	11.56	11.62	11.63	11.58
Dissolved Oxygen (mg/L)	10.58	10.39	9.62	7.70	8.08	7.45
Salinity	0.81	1.56	1.60	1.17	1.59	1.37
Conductivity (mS/cm)	1.60	2.99	2.86	3.37	3.09	2.65
Temp. (°C)	20.89	20.23	19.50	18.90	17.95	18.54
Redox (mV)	-2.5	-263	-906	-749	-683	-140

Readings taken at 0.5 m intervals across the pool. Red-ox values, or oxidation-reduction potentials measured in millivolts (mV). Mean pH of WHC2 ~11.6. Mean red-ox value of WHC2 is -457 mV.

Table 5: Water data from the control site (WHB), and the calcium carbonate site (TLE1-B and TLE1-D)

	WHB	TLE1-B	TLE1-D
pH	7.19	10.19	10.37
Dissolved Oxygen (mg/L)	13.15	14.40	23.84
Salinity	0.03	0.08	0.09
Conductivity (mS/cm)	0.069	0.174	0.199
Temp. (°C)	22.97	22.49	12.81
Redox (mV)	+208	-67	-200

The temperature and dissolved oxygen readings of TLE1-D likely deviated from the other sites because it was very shallow and had more direct exposure to the cool, oxidized atmosphere.

Table 6: Comparison of physical and chemical characteristics of Tablelands, Lost City Hydrothermal Field (LCHF), and Cabeço de Vide, Portugal.

Site	LCHF	Cabeço	WHC2	TLE1	WHB
Temp. (°C)	20-93	21	18-21	13-22	23
pH	9-11	11.4	11.6	10.19-10.37	7.19

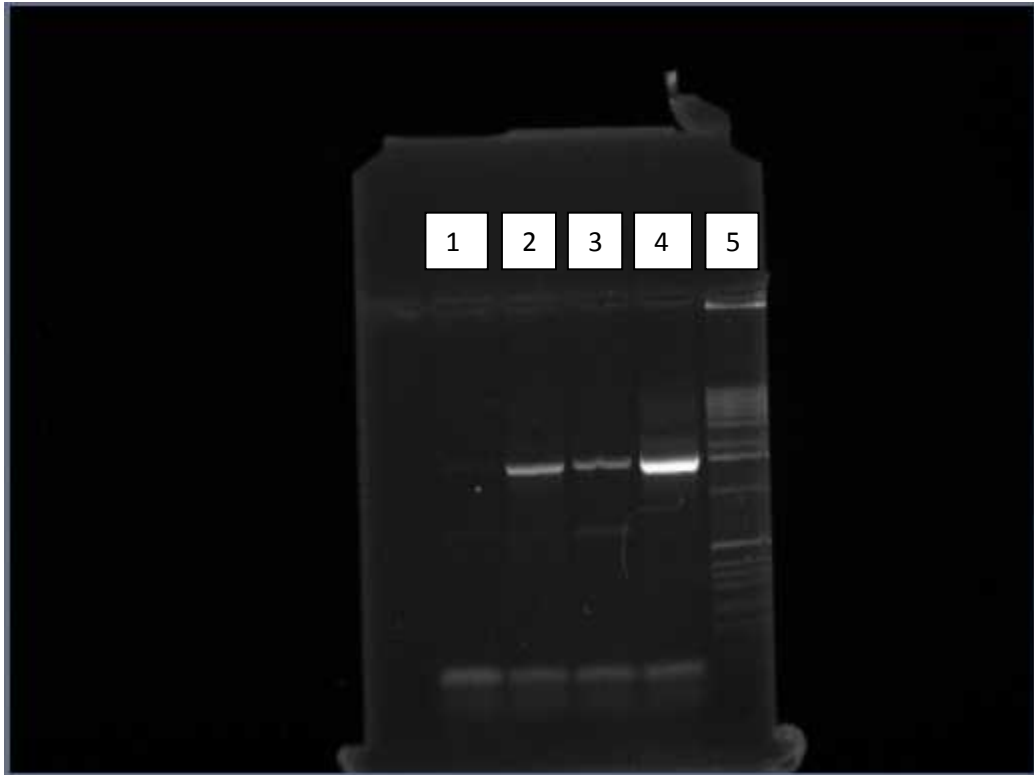
The above table compares pH and temperatures observed in Tablelands with two other serpentinite-influenced systems, LCHF and Cabeço de Vide. LCHF has a wide temperature range due to the interface created between the cold seawater and heated fluids released by the serpentinitization-driven hydrothermal vents (Kelley et al, 2005). The terrestrial serpentinite at Cabeço de Vide has very similar pH and temperature readings to Tablelands (Tiago et al, 2004).

Table 7: DNA yields from rock samples

Site	µg of DNA recovered per gram of soil
WHC2-A	0.408
WHC2-B	0.508
WHC2-C	0.356
TLE1-B	0.392
TLE1-D	0.260
WHB	0.600
Canadian forest soil (Roesch et al, 2007)	3.200

This table indicates how much DNA was recovered per gram of soil. Collected rock samples were crushed and used in the extraction protocol. DNA concentrations were relatively low from all recovered samples. The Canadian forest soil DNA recovered by Roesch and colleagues is used as a comparison to the low DNA recoveries of Tablelands. The extracted DNA was used for cloning and sequence analysis.

Figure 11: 1% agarose gel showing bacterial 16S rRNA bands for sites WHC2-B, WHB, TLE1-B, and TLE1-D. Band size of 1,465 bases.



Lane 1: TLE1-D Bacterial 16S rRNA gene

Lane 2: TLE1-B Bacterial 16S rRNA gene

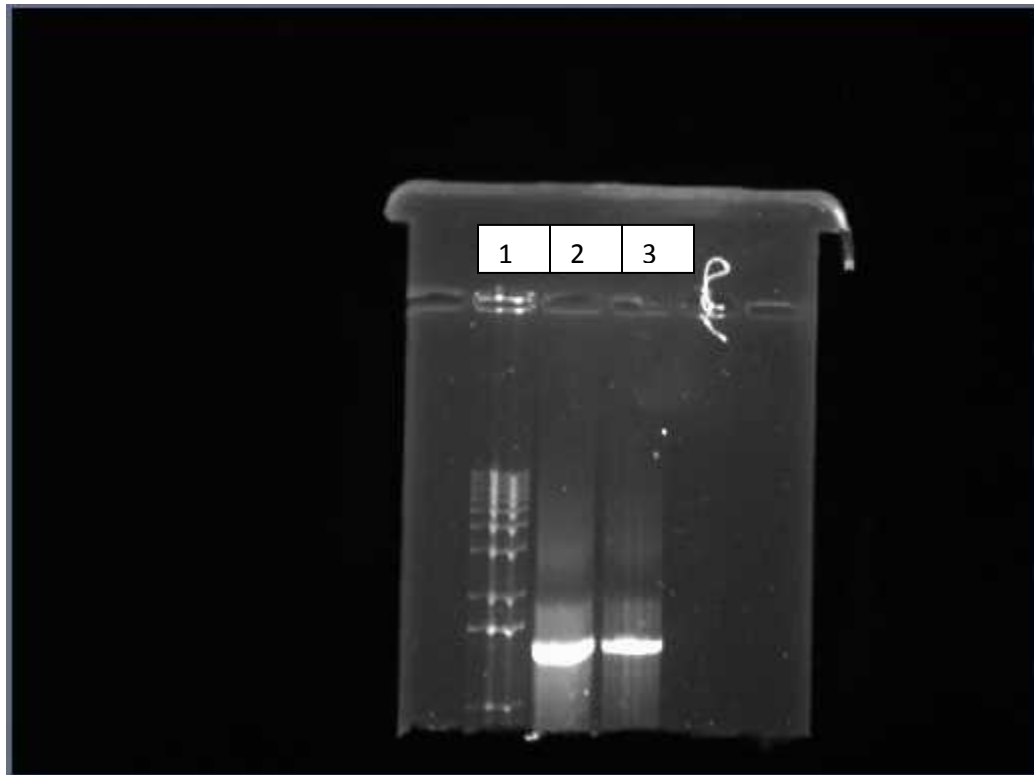
Lane 3: WHC2-B Bacterial 16S rRNA gene

Lane 4: WHB Bacterial 16S rRNA gene

Lane 5: 1-kb Ladder

Figure 12: 1% agarose gel showing bacterial 16S bands for WHC2-A and WHC2-C.

Band size is 1,465 bases.

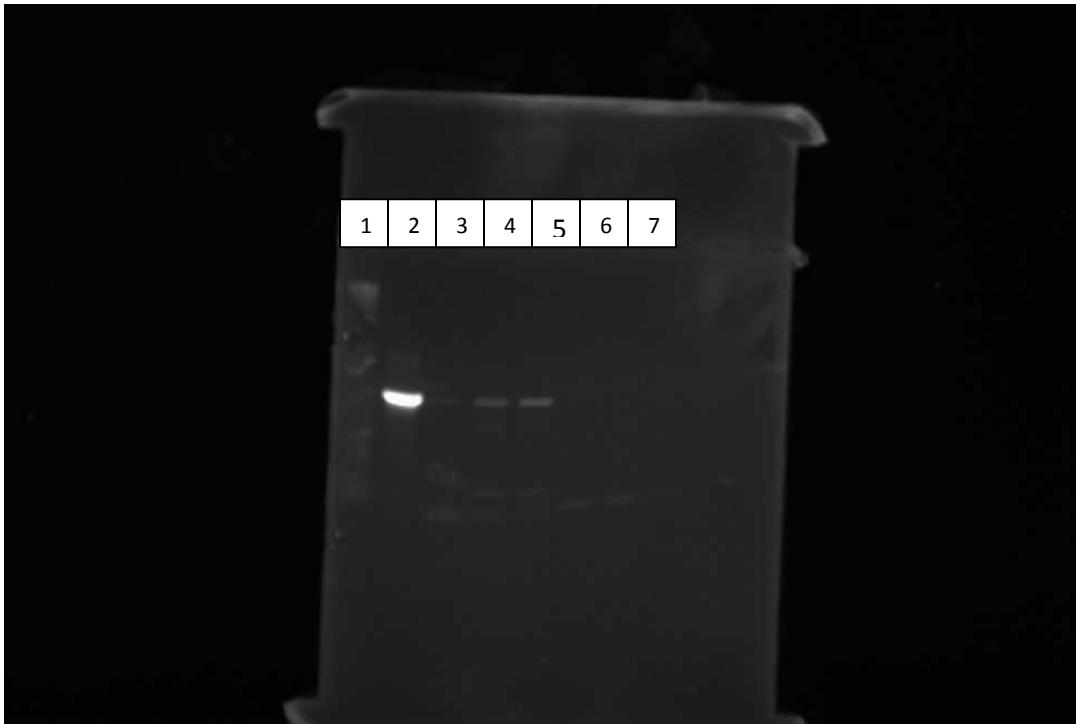


Lane 1: 1-kb Ladder

Lane 2: WHC2-A Bacterial 16S rRNA gene

Lane 3: WHC2-C Bacterial 16S rRNA gene

Figure 13: 1% agarose gel of archaeal 16S rRNA amplicons for an archaeal DNA positive control, WHC2-A, WHC2-B, WHC2-C, TLE1-B, and TLE1-D. Band size is 937 bases.



Lane 1: 1-kb Ladder

Lane 2: *Methanocaldococcus jannaschii* (an archaeal positive control)

Lane 3: WHC2-A

Lane 4: WHC2-B

Lane 5: WHC2-C

Lane 6: TLE1-B

Lane 7: TLE1-D

Figure 14: 1% agarose gel showing archaeal 16S rRNA amplicons for WHB. Band size is 937 bases.



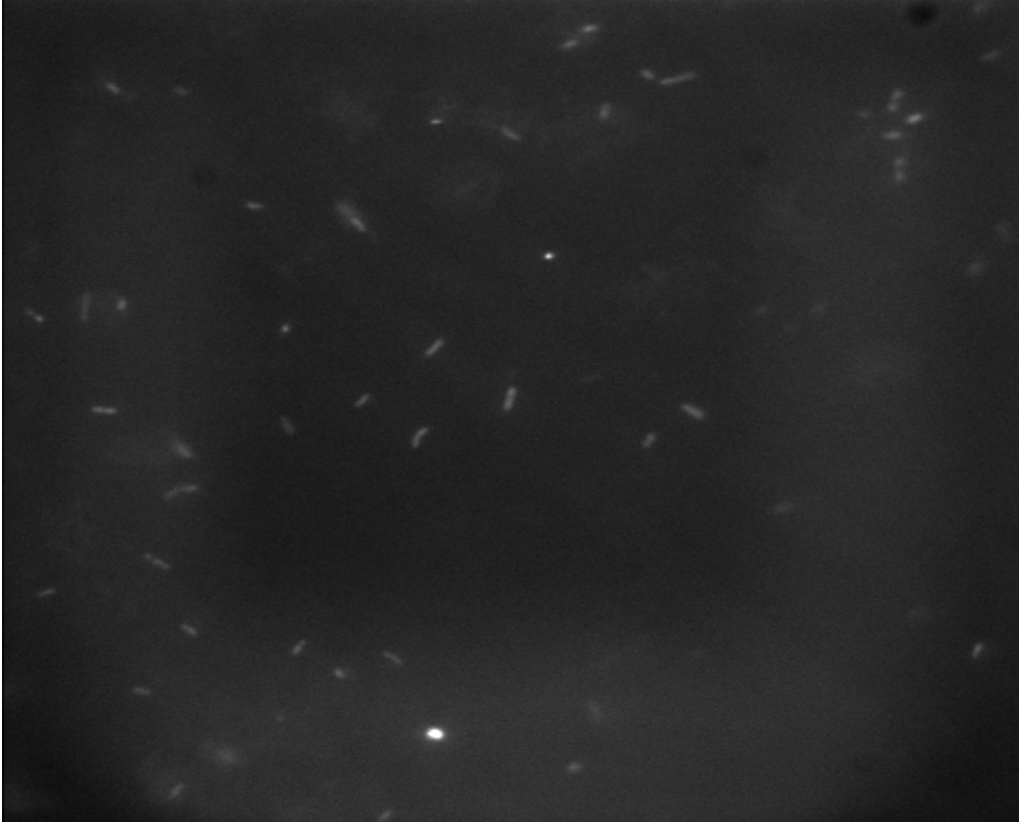
Lane 1: 100-bp Ladder

Lanes 2-5: WHB Archaeal 16S rRNA genes amplicons

Table 8: Percent compositions of bacterial groups in clone libraries.

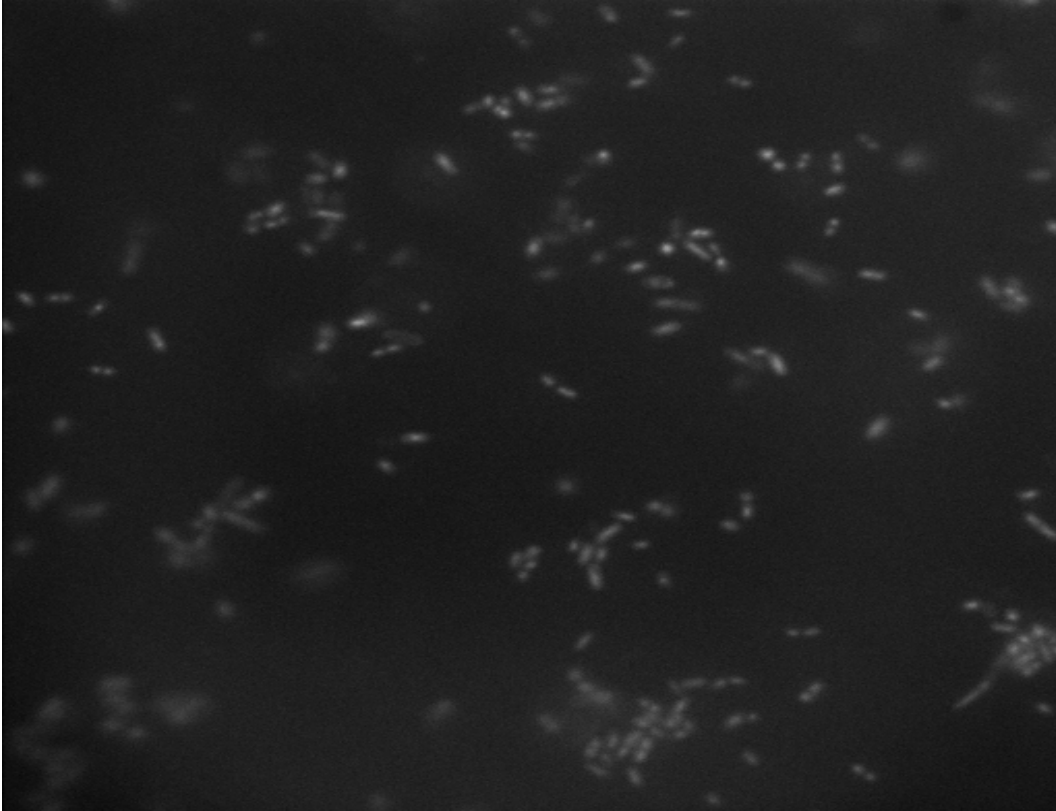
	WHC2-A	WHC2-B	WHC2-C	TLE1-B	TLE1-D	WHB	Total
Bacteria Group	% of library	% of library	% of library	% of library	% of library	% of library	% of library
Actinobacteria	2.8	18.2	1.1	1.2	27.9	8.8	9.9
Alpha-proteobacteria	1.4	0	1.1	1.2	32.5	17.6	9.4
Bacterioidetes	2.8	0	0	0	3.5	4.4	1.8
Beta-proteobacteria	23.5	3.9	58.2	72.1	16.2	23.1	33.8
Chloroflexi	0	0	5.5	0	1.2	6.5	2.4
Cyanobacteria	0	0	6.6	0	2.3	6.6	2.8
Deinococcus	0	0	3.3	0	1.2	0	0.8
Delta-proteobacteria	1.4	0	0	0	0	7.7	1.6
Firmicutes	66.7	77.9	5.5	0	8.1	11.0	25.8
Gamma-proteobacteria	0	0	16.5	25.6	4.7	2.2	8.5
Gemmatimonadetes	1.4	0	0	0	1.2	7.7	1.8
Nitrospira	0	0	2.2	0	1.2	4.4	1.4
# of bacterial clones	71	77	89	86	87	84	494

Figure 15: Epifluorescence micrograph of DAPI-stained microbial cells from fluid samples of WHC2. Magnification of 100×. Area of field $\sim 6720 \mu\text{m}^2$.



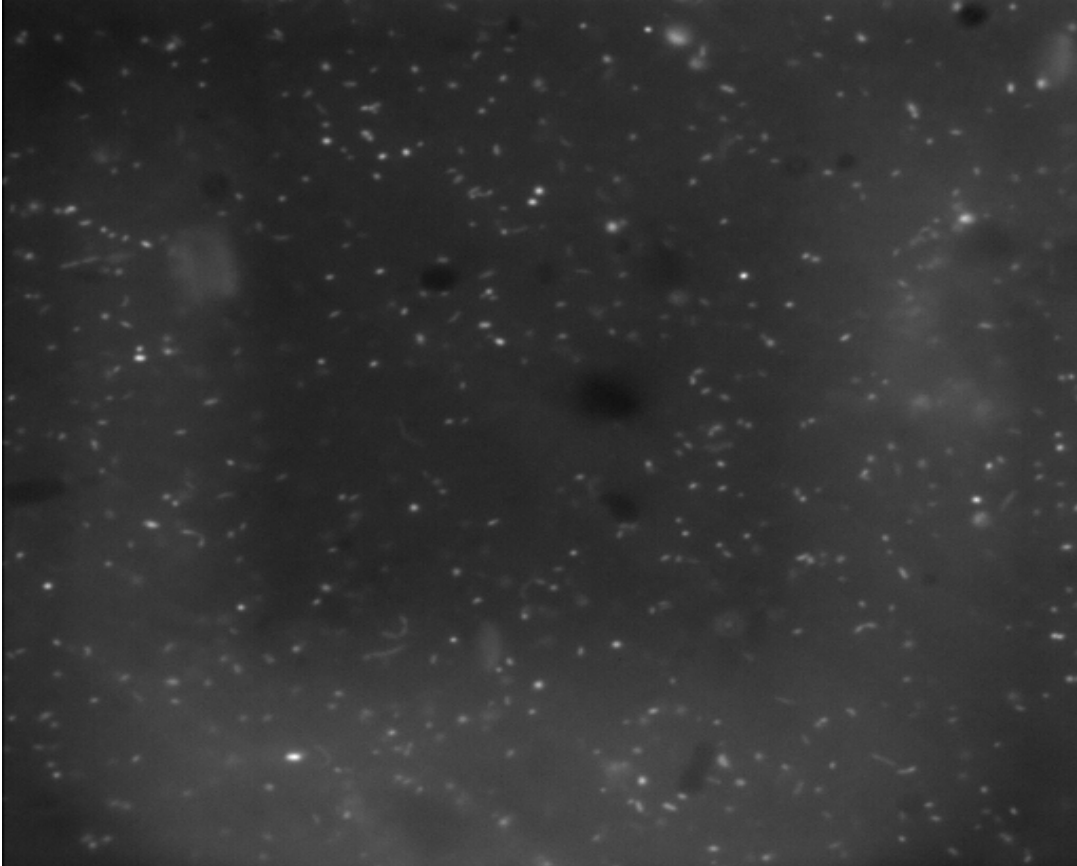
The micrograph of WHC2 shows that many of the cells in this samples have a rod, or bacilli shape.

Figure 16: Epifluorescence micrograph of DAPI-stained microbial cells from fluid samples of TLE1-B. Magnification of 100×. Area of field $\sim 6720 \mu\text{m}^2$.



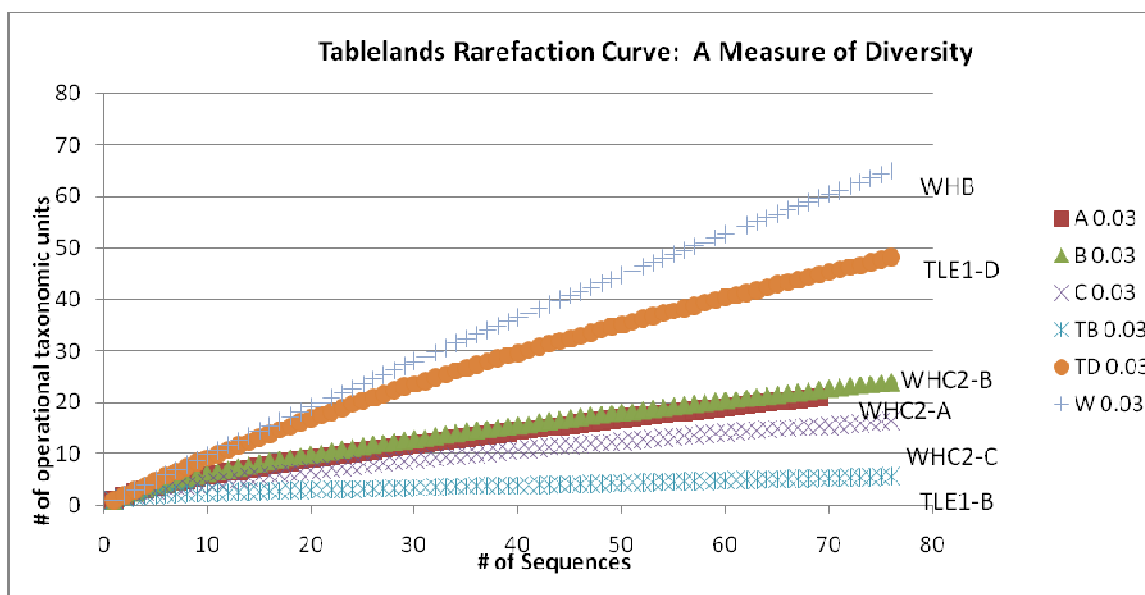
The microbial cells of site TLE1-B appear larger than the other two sites and have a rod, or bacilli shape.

Figure 17: Epifluorescence micrograph of DAPI-stained microbial cells from fluid samples of WHB. Magnification of 100×. Area of field $\sim 6720 \mu\text{m}^2$.



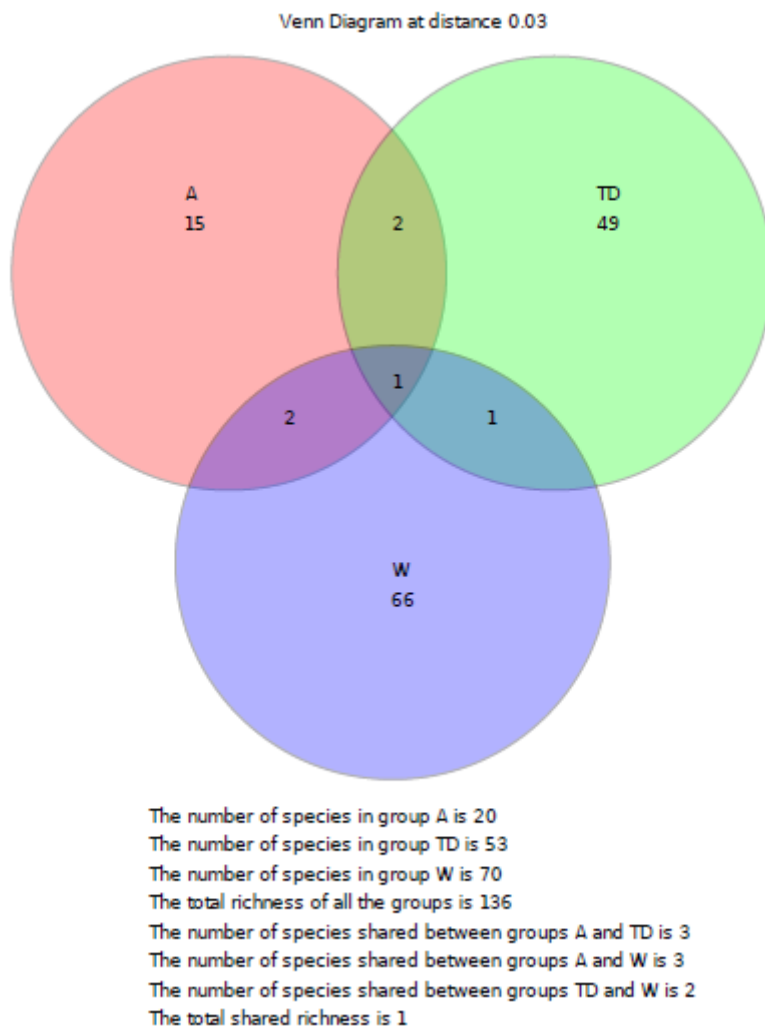
Cells of WHB appear to be smaller than those of WHC2 or TLE1-B. Most cells have a round, or cocci shape.

Figure 18: Rarefaction Curve (based on 97% similarity)



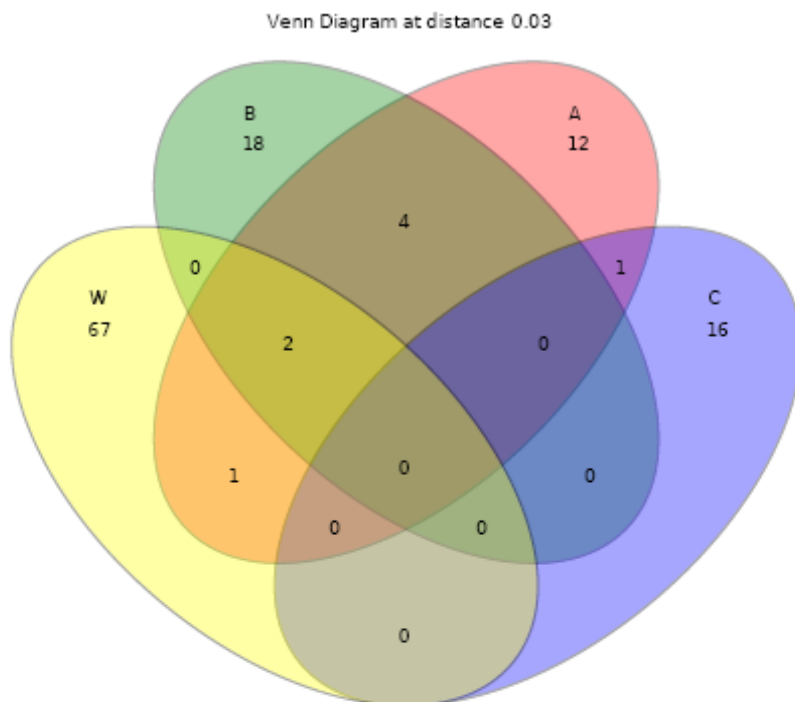
Rarefaction curves were generated measuring the number of partial sequences of bacterial 16S rDNA from each sample versus the number of operational taxonomic units present in all of the sequences using Mothur. The slope of each line is directly proportional to community diversity.

Figure 19: Venn diagram of WHC2-A, TLE1-D, and WHB.



Venn diagrams were also constructed using Mothur in order to look at community overlap and to determine how many species were shared between the sites. The Venn diagrams are based on a definition of OTU's as >97% sequence similarity. Below the Venn diagram is a list summarizing the number of species contained in each group and the number of species shared between each group.

Figure 20: Venn diagram of WHC2-A, WHC2-B, WHC2-C, and WHB.



The number of species in group A is 20

The number of species in group B is 24

The number of species in group C is 17

The number of species in group W is 70

The number of species shared between groups A and B is 6

The number of species shared between groups A and C is 1

The number of species shared between groups A and W is 3

The number of species shared between groups B and C is 0

The number of species shared between groups B and W is 2

The number of species shared between groups C and W is 0

The number of species shared between groups A, B and C is 0

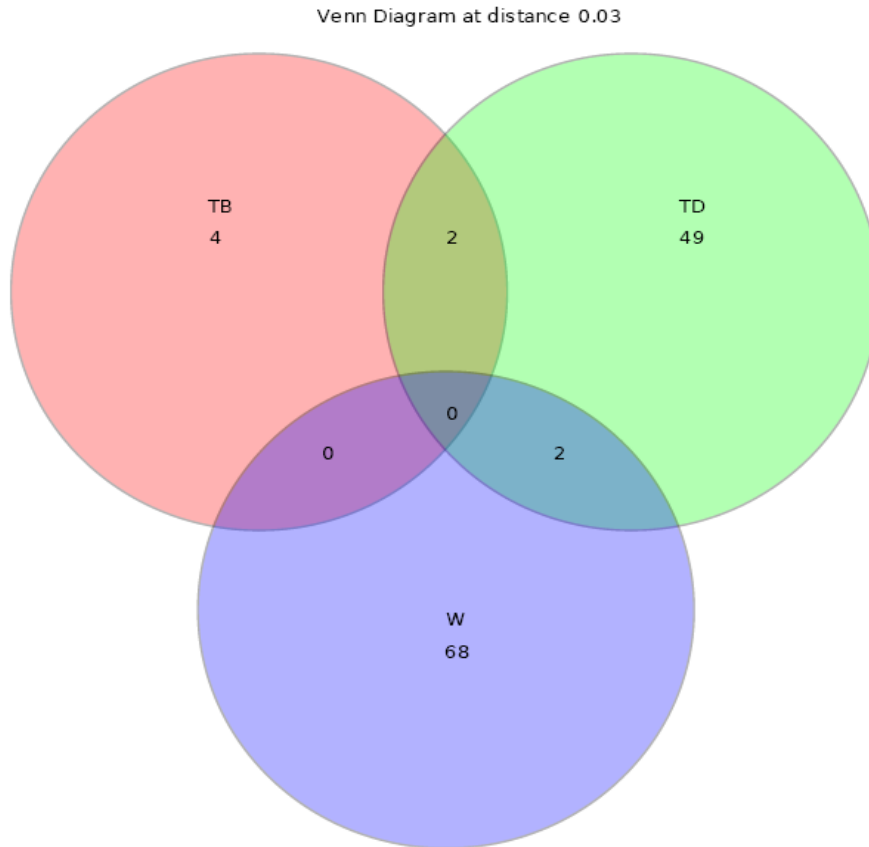
The number of species shared between groups A, B and W is 2

The number of species shared between groups A, C and W is 0

The number of species shared between groups B, C and W is 0

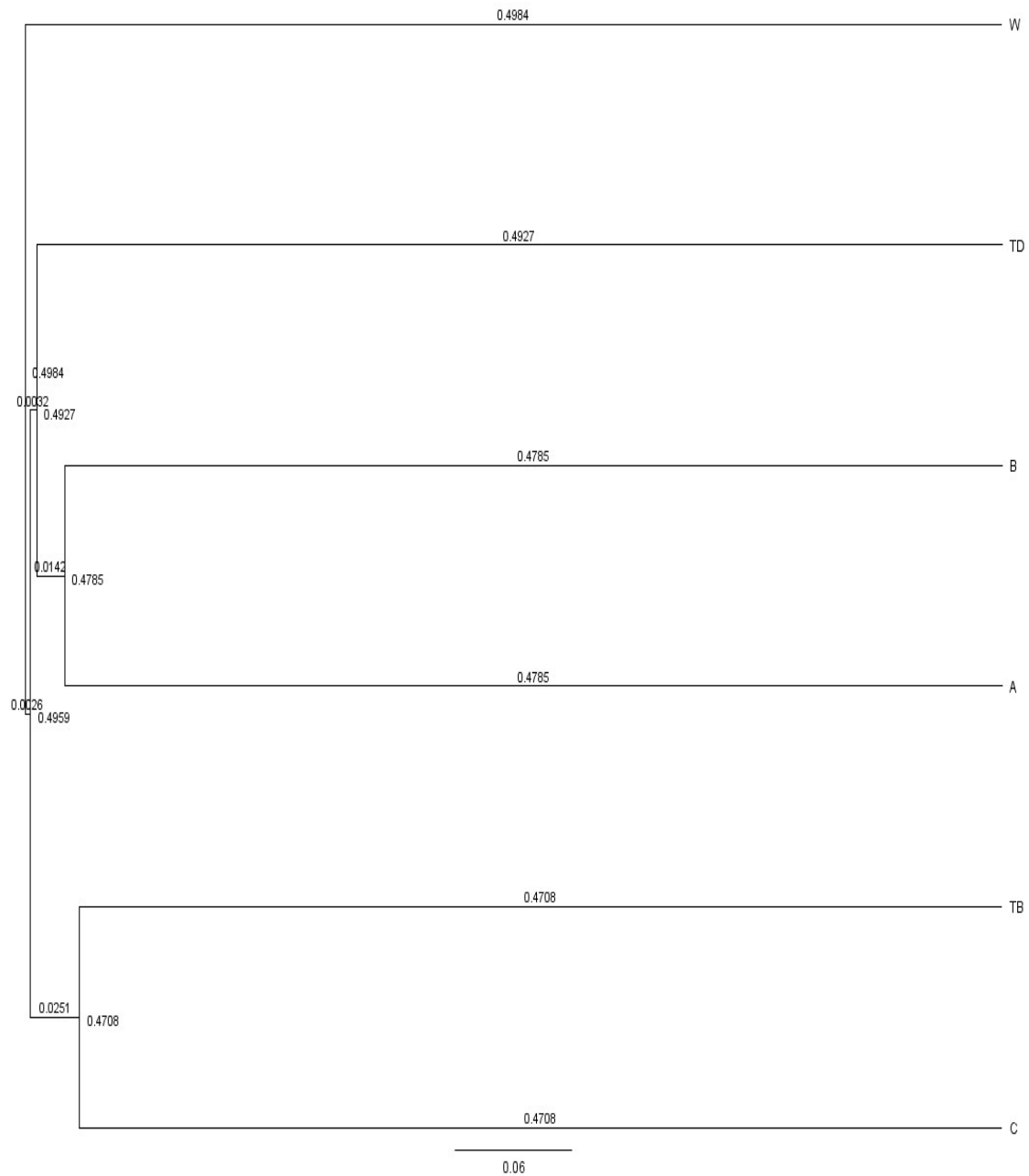
The total richness of all the groups is 121

Figure 21: Venn Diagram of TLE1-B, TLE1-D, and WHB.



The number of species in group TB is 6
 The number of species in group TD is 53
 The number of species in group W is 70
 The total richness of all the groups is 125
 The number of species shared between groups TB and TD is 2
 The number of species shared between groups TB and W is 0
 The number of species shared between groups TD and W is 2
 The total shared richness is 0

Figure 22: Dendrogram used to group closely related sites and express similarity between communities.



The dendrogram was generated using Mothur software. It is a representation of the similarity between the bacterial communities of each site. It groups genetic sequences into clusters based on degree of similarity, using the Jaccard coefficient (Schloss et al, 2009). The Jaccard coefficient is a statistical value that measures diversity and overlap between two groups by dividing the number of attributes the two groups share by the total number of possible attributes. The results are organized into a dendrogram, which resembles the shape of a phylogenetic tree. Groups with comparable diversity levels and strong overlap will be placed close to each other on the tree and paired into clusters, while farther distances on the tree indicate higher dissimilarity (Schloss et al, 2009).

Table 9: Diversity statistics for bacterial sequences obtained from the Tablelands site.

(distance=0.03, or 97% similarity).

	# of OTU's	ACE richness estimator	Chao1 richness estimator	Simpson's Reciprocal Index
WHC2-A	21	95.5-268.1	31-147	0.11-0.25
WHC2-B	24	108.0-249.2	43-264	0.10-0.24
WHC2-C	18	23.2-90.3	21-74	0.24-0.44
TLE1-B	6	0-0	7-43	0.46-0.65
TLE1-D	53	184.5-365.1	86-240	0.01-0.03
WHB	71	203.1-728.6	190-685	0.001-0.009

Value ranges reported as 95% confidence intervals. These statistics were generated using Mothur. The number of unique OTU's was calculated for each sample. Additionally, the ACE (abundance-based coverage estimator) and Chao1 richness estimators were used to give 95% confidence intervals for the species richness at each site. Simpson's Reciprocal Index is a numerical value that represents the microbiological diversity. Its values report the likelihood that two randomly selected organisms in a sampling are not members of the same species. Low Simpson's reciprocal index values indicate high diversity.

Figure 23: Phylogenetic Tree of Tablelands OTU's

The phylogenetic tree depicted above was generated as a neighbor-joining tree using the Tamura-Nei genetic distance model. The Geneious software generated 100 replicates of the tree, with the final result being a consensus tree of the replicates. The bootstrap values on the tree indicate how many times a particular branching pattern occurred out of the 100 replicates. For example, a value of 100 signified that the particular phylogenetic branch occurred in every tree that was generated. The lengths of the branches indicate the genetic distances between the sequences and represent the number of nucleotide changes per 100 base pairs. The sequences from the Tablelands clone libraries are indicated by rectangular boxes. The bacterial group representing each region of the tree is noted on the side.

Table 10: Cells per gram of mineral (wet weight)

Sample	Avg.	95% LCI	95% UCI
WHC2-A	1.78E+05	1.63E+05	1.92E+05
WHC2-B	5.45E+05	5.14E+05	5.77E+05
WHC2-C	8.03E+06	7.66E+06	8.40E+06
TLE1-B	2.36E+07	2.28E+07	2.43E+07
TLE1-D	5.02E+06	4.68E+06	5.36E+06
WHB	4.61E+06	4.41E+06	4.81E+06

Cell counts were completed to measure abundances for each of the sites. Counts were made for mineral and fluid samples. The values reported are the average cells counted per gram of mineral or milliliter of fluid. Also given is the 95% confidence interval for the average cell count data.

Table 11: Cells per mL of fluid

Sample	Avg.	95% LCI	95% UCI
WHC2-A	1.67E+05	1.49E+05	1.85E+05
WHC2-B	7.14E+04	6.32E+04	7.95E+04
WHC2-C	nd	nd	nd
TLE1-B	2.25E+05	2.05E+05	2.45E+05
TLE1-D	4.01E+05	3.53E+05	4.49E+05
WHB	nd	nd	nd

Nd= not determined

Table 12: Results of Culturing Experiment with isolates TLE1-D and WHC2-A Red.

Isolate	Oxygen	Light	# of Colonies	Description
TLE1-D	+	+	1000+	Spreading of many small colonies, some large colonies present.
TLE1-D	+	-	1000+	Spreading of many small colonies, very few large colonies.
TLE1-D	-	+	40	Almost all large colonies.
TLE1-D	-	-	112	Mostly large colonies, cluster of small colonies on middle of plate.
WHC2-A Red	+	+	0	No growth.
WHC2-A Red	+	-	0	No growth.
WHC2-A Red	-	+	4	Several small, red pigmented colonies.
WHC2-A Red	-	-	5	Several small, red pigmented colonies.

Chapter 6: Discussion

The overlying theme that the more extreme the environment (i.e. the higher the pH), the lower the microbial diversity was supported by the findings of this study. The WHB site (serving as the control with an approximately neutral pH) contained the highest number of different bacterial species, followed by TLE1-D, WHC2-B, WHC2-A, WHC2-C, and lastly TLE1-B. WHB samples yielded 71 unique OTU's, and had the highest predicted species richness (190-685) according to the Chao1 richness estimator. The Chao1 richness estimator represents a saturation point on the rarefaction curve. In other words, if more sequences were processed from this particular site, at a certain point the number of different OTU's that could possibly be contained within these sequences would level out. Therefore, any new sequence analyzed from this site would yield an OTU that has already been identified.

The three sites from the WHC2 pool of pH 11.6 (WHC2-A, WHC2-B, and WHC2-C), shared relatively similar diversity levels, although WHC2-C was noticeably lower in richness than the other two sites, based on the Chao1 and ACE richness estimators. The number of OTU's for WHC2-A, WHC2-B, and WHC2-C were 21, 24, and 18, respectively. Furthermore, samples from these three sites all generated very similar rarefaction curves. The Chao1 richness estimator predicted that none of these three sites could possibly contain more than 264 OTU's, while the neutral pH site WHB could have as many as 685 OTU's based on this estimator. Therefore, it can be reasonably postulated that the alkaline WHC2 pool has a relatively uniform distribution

of diversity throughout its sediments, and overall diversity at this site is much lower than the oxidized, neutral pH brook.

Opposite of the WHC2 findings, TLE1-B and TLE1-D differed remarkably in species diversity. Sequence analysis of the clone library revealed TLE1-B has only having 6 OTU's, while TLE1-D contained 53 OTU's. Even more, the Chao1 richness estimator predicts only 7-43 possible OTU's for TLE1-B, compared to 86-240 OTU's for TLE1-D. The likely explanation for this stark variation is the combination of many, small factors including the fact that TLE1-D had a more shallow water flow that would have been more directly exposed to the cooler, oxidized atmosphere. Even though the two sites had similar pH values and similar alkaline seep conditions, other factors less obvious could have influenced this outcome. One site could have had rocks recovered that were mostly underground or buried under layers of other rocks and consequently had less access to the sunlight and oxidized atmosphere. Also, chemical concentrations, such as hydrogen or methane levels, could have deviated greatly between the two locations. The rarefaction curve shows TLE1-D (pH 10.37) has more diversity than the pH 11.6 WHC2 sites as expected. Also, it is much less diverse than the neutral pH, control site of WHB. The diversity of TLE1-D is likely greater than the WHC2 sites because of less alkalinity (pH 10.37 vs. pH 11.6), less reducing conditions (-200 mV for TLE1-D vs. -457.25 mV for WHC2), and also because the TLE1 site is a shallow seep that will have more exposure to the oxidizing atmosphere and more access to sunlight. These findings support the hypothesis that WHB would have the most diversity, followed by the TLE1 sites and lastly by the WHC2 sites. The fact that TLE1-B is less diverse than all of the WHC2 sites is likely an anomaly and not the norm. This is because the TLE1 site is

much more oxidized and has a less basic pH than WHC2, which would make it more suitable to host a wider range of species diversity. More comprehensive analyses of 16S rRNA diversity between sites should help to verify this assumption. If more sites were selected from the TLE1 seeps, the majority of those sites should have more OTU's than the WHC2 pool.

Cell counts using fluorescent microscopy techniques served as a useful indicator for the general abundance levels expected to be seen at the Tablelands sites of serpentinitization. The results show that overall this environment has a relatively low biomass, similar to the abundance levels observed by Tiago and colleagues at the alkaline groundwater site in Portugal (2004). Abundances at the Tablelands site ranged from 4.54×10^3 to 6.03×10^5 in rock samples, while the cell count range of the fluid samples was 2.12×10^4 to 4.01×10^5 . Cell counts of the fluid and rock samples yielded no discernable differences, as both were relatively low. A neutral pH pool containing rock surfaces would be expected to host biomass levels of about 10^8 cells/mL (Hoehler et al, 2007). From a broad-scale view, it is evident that the low diversity of a serpentinite-hosted ecosystem correlates to low biomass. Harsh conditions (i.e. reduced fluids, high pH, lack of nutrients, etc.) should not only limit the different types of species this environment is able to host, but also curtail the population densities due to the amount of energy required to sustain habitability and cell integrity (Hoehler et al, 2007).

Community overlap (species shared between sites) was noticeably little, to nonexistent in cross-comparisons between the six sites. Venn diagrams, as well as a dendrogram, were used as representations for community overlap, indicating how many species, or OTU's, were shared between each of the sites. The highest degree of

similarity between sites was observed with WHC2-A and WHC2-B. The Venn diagram in Figure 7 reports that WHC2-A and WHC2-B contain 20 and 24 species or unique OTU's, respectively. Of these different species, 6 are shared between both groups. In other words, 30% of the species found at WHC2-A were also found at WHC2-B. The noticeable overlap is expected since both sites were from the stagnant, highly reduced, pH 11.6 pool. Both sample sites were near the rim of the pool (on opposite sides), at which the water was shallow and filled with ultramafic rock. In sharp contrast, although WHC2-C samples are from the same basic pool, it only shares one OTU with WHC2-A, and does not have any species overlap with WHC2-B. The WHC2-C site is subjected to roughly the same pH (11.6), however the rocks were from a deeper part of the pool, which could have influenced access to light, as well as the redox conditions. Redox conditions were found to be both highly heterogeneous and highly reduced at the WHC2 location. The various redox gradients of hydrogen, methane, hydroxide, and other chemical species distributed throughout the pool could have created numerous microenvironments, each of which were suitable for a specific range of microbes. This could explain that while all three sites had approximately equal pHs, there was little overlap. Even though WHC2-A and WHC2-B had a moderate correlation, each of these three locations within the pool appeared to host their own unique community of microorganisms. Also, efforts should be made in the future to better constrain these distinct niches and more accurately define the parameters of the site.

The TLE1 sites, TLE1-B and TLE1-D, were located on a hillside at the bottom and top of a carbonate precipitate, respectively. Both sites were subjected to similar pHs, and both had shallow, water flowing over the carbonate formations. Despite the parallels

between the sites, community overlap was at a minimum. Only two species were shared between the two sites. Each site hosted distinct microbial communities, indicating that microenvironments specific to each location likely had high degrees of variation in the concentrations of certain chemical species and had unique redox gradients. The rocks collected from each site could have also been exposed to different amounts of sunlight depending on how the rocks were positioned on the mountain slope.

The neutral pH brook, WHB, showed no discernable overlap with the other serpentinite-influenced sites. WHB only shared two OTU's with WHC2-B and TLE1-D, and had three OTU's in common with WHC2-A. TLE1-B and WHC2-C did not have any species identical to those identified in WHB. This is likely because these two sites had the lowest species diversity and therefore had a less likely chance of sharing similar species with other sites. Specific parameters unique to each location (*i.e.* pH, redox gradients, temperature, exposure to light) appear to create novel communities that have little relation to microbial communities of other sites.

The dendrogram supports the data presented by the rarefaction curve and the Venn diagrams. The structure of the dendrogram indicates which sites are similar and which sites have the most diversity. Groups that are clustered together have more similarity in terms of species present and diversity. Also, the tree roughly ranks the microbial species diversity from most diverse (at the top of the tree) to least diverse. The dendrogram supports the notion that WHC2-A and WHC2-B have more overlap and similar diversities than any other sites. WHB and TLE1-D, which had the first and second highest levels of species diversity respectively, were at the top of the tree and clustered together. TLE1-B and WHC2-C, which had the two lowest diversity levels and

had almost no overlap with the other samples, were clustered at the bottom of the tree. Essentially, the dendrogram provides further evidence that the control site of WHB is the most diverse, and that WHC2-A and WHC2-B shared the highest degree of similarity. WHC2-A (20 OTU's) and WHC2-B (24 OTU's) had 6 species in common, which shows substantially higher overlap than any other comparison between two sites. The fact that all three of the WHC2 sites (all highly reduced with pH ~11.6) promoted low diversity levels, especially compared to TLE1-D (less reduced, pH 10.37) and WHB (highly oxidized, pH 7.19), supports the hypothesis that the extremely basic, reducing conditions will limit diversity and promote unique microbial communities that have adapted to the parameters specific to their environment.

Table 8 lists the bacterial species composition of each individual site, and Tablelands as a whole. The bacterial clones contained a total of 12 different groups of Bacteria. Over half of the bacterial clones from Tablelands were either *Firmicutes* (25.8%) or *Beta-proteobacteria* (33.8%). Site WHB contains clones within 11 of the 12 bacterial groups identified at this location. WHB appears to have a much more uniform distribution of species than the other sites. This adds further evidence that WHB, with oxidizing conditions and a neutral pH, is the most diverse site. *Firmicutes* predominate the WHC2 site, comprising 47.1%, or nearly half, of the clones obtained from WHC2-A, B, and C. *Firmicutes* have been identified in a wide variety of environments and appear to be able to resist harsh conditions such as the alkalinity and high reducing values observed in WHC2. *Beta-proteobacteria* made up 30.4% of the bacterial clones from WHC2. These findings indicate that *Firmicutes* and *Beta-proteobacteria* represent the bacterial groups that are most suitable for adapting to the extreme conditions of the

alkaline seeps. The presence of *Firmicutes* correlates to the findings of Tiago et al, 2004 at the Cabeço de Vide alkaline spring, where *Actinobacteria* and *Firmicutes* were the most prevalent Bacteria cultured. However, while *Actinobacteria* made up 81% of the bacterial clones from the Cabeço de Vide site cultures, *Actinobacteria* only comprised 9.9% of the Tablelands library (Tiago et al, 2004). Distribution of bacterial species was noticeably different at the alkaline seep of TLE1 as compared to the stagnant WHC2 pool. The major bacterial groups identified at TLE1 were *Beta-proteobacteria* (44.2%) and *Alpha-proteobacteria* (16.9%). *Beta-proteobacteria* seem fairly ubiquitous at Tablelands, as they make up at least 30% of the clone library for each of WHC2, TLE1, and WHB. Because of the abundance of *Firmicutes* clones obtained from WHC2, the most basic and reducing site, it appears this bacterial group well-adapted to survive this extreme environment.

The phylogenetic tree shown in Figure 23 illustrates the relationships between the different bacterial species of Tablelands, as well as how they relate to species of other sites that have high sequence similarity. The three major groups of Bacteria present in the tree are *Firmicutes*, *Beta-proteobacteria*, and *Alpha-proteobacteria*. The Tablelands clones A10, A50, B42, B80, and B81, all *Firmicutes*, show high relatedness to 7 clones of *Firmicutes* of the Cabeço de Vide site (Tiago et al, 2004). The prevalence of *Firmicutes* at both sites indicates that this group of Bacteria comprises a significant proportion of the microorganisms present at terrestrial ophiolites such as these two sites. A reference clone obtained from an alkaline lake, Lake Van in Turkey, showed phylogenetic similarity to Tablelands clone TD93, both of which are *Alpha-proteobacteria*. Two other clones from the Cabeço de Vide nonsaline, alkaline spring

were incorporated into the tree. One showed very high similarity to clone TB27, a *Gammaproteobacteria*, while the other Cabeço de Vide clone was related to C54, a *Beta-proteobacteria*. The species distribution of Tablelands and Cabeço de Vide show significant parallels. Both sites consist primarily of bacterial groups such as *Firmicutes*, *Beta-proteobacteria*, *Actinobacteria*, and *Alphaproteobacteria*. A limited range of other groups, such as *Gamma-proteobacteria* and *Bacterioidetes*, are less prevalent at these systems. The similarities between these two sites in terms of diversity and species composition suggest that nonsaline, alkaline seeps throughout the world are likely to promote comparable microbial communities. Therefore, it can be reasonably hypothesized that serpentinite-hosted ecosystems promote certain patterns of species distribution, based on parameters such as pH, reduction potential, and temperature of the environment. Also of note, species compositions similar to those found in serpentinites are also observed in alkaline soda lakes. For instance, 16S rRNA analysis of recovered DNA from Mono Lake, California (a soda lake) revealed bacterial species belonging to groups such as *Firmicutes*, *Alpha-proteobacteria*, and *Actinobacteria* (Humayoun, 2002). All of these bacterial groups, particularly the *Firmicutes*, were found at the Tablelands site.

Four strains were isolated in pure culture: TLE1-B, TLE1-D, WHC2-A Red, and WHC2-A Yellow. The two WHC2-A isolates were named Red and Yellow respectively because they produced brightly pigmented colonies of those particular colors. Because they were able to consume organic materials and grow on the pH 11 media, each of the strains were determined to be alkaliphilic heterotrophs. Partial sequence analysis using the 27F primer indicated that the TLE1-B isolate belonged to the genus *Roseomonas*, a

member of the *Alpha-proteobacteria*. The TLE1-D isolate was identified as a *Paenibacillus*, which is a *Firmicute*. Both WHC2-A isolates belonged to the genus *Aquiflexum*, a member of the *Bacterioidetes*.

The culturing experiments in which two strains (TLE1-D, WHC2-A Red) were exposed to either oxygen and light, oxygen and no light, light and no oxygen, or neither indicated both strains were alkaliphilic heterotrophs. TLE1-D colonies were able to grow on the pH 11 media in all four conditions. The two TLE1-D plates exposed to oxygen had relatively uniform distribution of small colonies covering most of the surface area of the plate, each containing over 1,000 colonies. Both TLE1-D plates grown in anaerobic conditions showed much less growth, and the presence of fewer large colonies instead of many small colonies. The TLE1-D plate with no oxygen and no light revealed relatively higher growth (112 colonies) than the plate with light exposure but no oxygen (40 colonies). The TLE1-D isolate is likely a heterotrophic, facultative anaerobe. Because the aerobic plates had many more colonies than the anaerobic plates, TLE1-D appears to prefer oxygen, but is still able to grow in the in an anoxic environment. Also, because it had significant growth on pH 11 media, it is presumably an alkaliphile. Further experiments are required to definitively characterize these isolates.

The WHC2-A Red strain only showed colonies on the anaerobic plates following the 5-day incubation period. Neither of the plates exposed to oxygen, regardless of light exposure, showed any signs of bacterial growth. Both plates placed in the anaerobic chamber revealed roughly the same amount of growth (5 colonies in the absence of light, 4 colonies in the presence of light). Because the anaerobic chamber cannot 100% mimic an anoxic environment, there is likely a trace amount of oxygen in the chamber.

Therefore, WHC2-A Red is most likely aerotolerant, that does not utilize oxygen in its metabolism. Also, like TLE1-D, it is probably an alkaliphile since it was able to form colonies on pH 11 media. The two plates fully exposed to the oxygenated atmosphere were not able to grow because the oxygen concentrations were too high to support microaerophilic activity, or because they had slower growth rates.

The findings of the culturing experiments support what would be expected of microbial activity at the Tablelands ophiolite. The WHC2-A Red strain was grown from rock samples obtained from the bottom of the alkaline pool, where it was highly reduced and oxygen levels were low. Moreover, TLE1-D responded more favorably to aerobic conditions because it was isolated from rocks of a very shallow, flowing seep that was subjected to more oxidized conditions. The pHs of WHC2 and TLE1-D were 11.6 and 10.37, respectively. This correlates strongly to the culturing results that provide evidence that these species can grow in highly basic conditions, and can still grow when oxygen levels are low or even if the environment is completely anoxic. Overall, the findings of the culturing experiments support the hypothesis that microorganisms recovered from the alkaline seeps of Tablelands could be grown on high pH media.

The presence of functional genes for methanogenesis (*mcrA*) and hydrogen oxidation (*hydA*) was not confirmed in any of the sites. However it should not be assumed that neither of these metabolisms exist at the Tablelands serpentinite. One problem is that biomass is low at this site and DNA yields from recovered rocks were very low, ranging from 0.26-0.60 $\mu\text{g/g}$ of soil (Table 7). Also, the field site was relatively isolated within a national park and there was no access to liquid nitrogen or dry ice. Therefore, it was likely that a substantial quantity of DNA was degraded before the

samples were transported back to the laboratory and stored in a -80°C freezer. As a result, additional field work must be completed to make a more accurate assessment as to the presence or absence of certain functional genes, as well as their expression. The reducing and anoxic conditions produced by serpentinization create thermodynamically favorable conditions for methanogenesis and hydrogen oxidation. It would be premature to discount the existence of these anaerobic metabolisms at the Tablelands ophiolite.

Screening for the archaeal 16S rRNA gene indicated that each of the 6 sites contained Archaea in low abundances. Qualitative analysis of the 16S gel bands show WHB likely has the highest archaeal diversity due to its strong bands. The three WHC2 sites all gave very weak bands, while the TLE1 bands were miniscule and barely visible. Because the bacterial 16S bands were much stronger and brighter, Tablelands likely supports greater bacterial abundances than archaeal abundances. Alternatively, the DNA extraction and purification procedure could bias against the recovery of DNA from Archaea, although identical procedures have yielded Archaea DNA from the LCHF. The predominance of Bacteria is expected, as most archaeal-dominated habitats have been found in the deep ocean, subseafloor sediments, as well as other extreme environments (*i.e.* high salinity, high temperatures, low oxygen, etc.). Even though Tablelands presents a very unique environment, the non-saline water combined with close proximity to the oxidized atmosphere would favor heterotrophic Bacteria over Archaea, which typically utilize anaerobic metabolisms.

Overall, there are still many unknowns about how serpentinization reactions and life processes interact with each other, and exactly to what degree one influences the other. We know that life can exist at active serpentinization sites, but is there a unique

biosphere specific for this process? This is the first study to characterize the species distribution at the Tablelands site. The results point towards to low diversity and low biomass at serpentinite-hosted ecosystems. In general, the trend appears to be that the more alkaline and reducing an environment is, the lower the microbial species diversity will be at the site. Further research needs to be completed to fully assess which functional genes are present in such an environment. Knowing which functional genes are present will provide clues about metabolisms that exist at ophiolites. Also, to gain a better understanding of what species are currently present and active, RNA should be isolated and sequenced. While 16S rRNA analysis of DNA paints a picture of what species are at this site, it does not tell which species are currently active and what metabolisms they are using. DNA could be from an active cell or remnants of a dead cell. However, due to its high turnover rates, RNA indicates a species is active. RNA analysis will provide stronger evidence concerning what energy-yielding pathways are currently being utilized.

Biogeography is a field concerned with the spatial distribution and biodiversity over the course of time (Martiny et al, 2006). A long-standing issue has been whether or not microorganisms exhibit distinct patterns of biogeography as seen in animals and plants. Does the environment select for certain species and promote higher abundances for some microbes as compared to others? Sequence analysis of the Tablelands site provides evidence for biogeographical patterns, in which there is minimal community overlap between sampling locations. The parameters unique to each site (*i.e.* pH, reduction potential, temperature, etc.) appear to promote different species and different levels of diversity. It would be interesting to see how this biogeographical distribution of

microorganism changes over time by studying successional patterns. It would also be interesting to conduct consistent biogeographical comparisons between serpentinite ecosystems, not just at the Tablelands, but at other ophiolites such as Caeco de Vide, the Coast Range Ophiolite in California, etc. Is there a serpentinite-specific biosphere? How are communities transferred between sites? How long have they been isolated from one another?

One field that has a particular interest in serpentinization and its effects on life is Astrobiology. Astrobiology is the multidisciplinary study of the origin and evolution of life in the universe. It focuses on life in terms of the past, present, and future, as well as the prospect of extraterrestrial life. Understanding the interactions between ultramafic rocks and water and how it is able to sustain life on Earth could be used as an analogue for analyzing other planets and moons in our solar system, and even planets outside of our solar system (*e.g.* exoplanets).

The possibility of previous or current serpentinization-like reactions on Mars is of special interest. The plethora of peridotite rock on Mars makes it an ideal candidate to have possibly hosted serpentinization at some point in time (Schulte et al, 2006). If there was ever liquid water on Mars at any point in time, then the occurrence of this process would have been highly likely. The knowledge of how serpentinization impacts life on Earth could be used to predict whether or not this reaction could have hosted some form of life on Mars (Shock et al, 1997)). For instance, methane sources have been detected on Mars. Future missions will study these methane outputs to determine if they are abiotic or biotic in origin, and if serpentinization is involved (Krasnopolsky et al, 2004).

Also, because the early Earth had conditions highly conducive for serpentinization, it is hypothesized that this event could have played a role in the origin of life, or could have at least provided habitats for primitive life (Martin et al, 2008). High plate tectonic activity and the abundance of ultramafic rock/water interactions would have created scenarios where altered mineral compositions and electron-donating chemical species could have provided habitats for early life in the form of serpentinite-driven hydrothermal systems.

To understand how serpentinization may have occurred on the early Earth or possibly on Mars, it is critical to first understand its properties and how it influences environments on present-day Earth. The Tablelands study, along with other serpentinite studies such as the Cabeço de Vide alkaline spring and LCHF, can serve as a blueprint for predicting species diversity, metabolisms, and cell abundances at similar sites that have not been extensively studied. Parallels between different serpentinization sites will be informative about exactly how this process influences microbial colonization and growth, and how serpentinization may have fueled metabolisms of some of the earliest organisms.

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Appendix A: Glossary of Terms

1. ophiolite-a portion of the Earth's crust or upper mantle that has been displaced onto the continental margin as a result of tectonic activity.
2. serpentinization- the aqueous alteration of ultramafic rock, yielding altered minerals (serpentes), as well as reduced, high pH fluids containing chemical species such as hydroxide, hydrogen, methane, and other hydrocarbons.
3. alkaliphile- a microorganism that grows optimally at high, basic pHs. It is a type of extremophile, and is found in environments such as soda lakes and serpentes.
4. chemolithoautotrophy- using an inorganic carbon source as a substrate to obtain energy through chemical reactions.
5. operational taxonomic unit- a concept used to group organisms that have greater than 97% genetic similarity. It represents an alternative method to classify species in the realm of microbiology.
6. ultramafic- a type of igneous rock common in the mantle that is formed by the cooling and solidifying of magma.
7. methanogenesis- the process of reducing carbon dioxide to methane, with hydrogen being the reducing agent. The production of methane is an energetically favorable reaction that microorganisms can utilize to generate ATP.
8. hydrogen oxidation- the process of reacting molecular hydrogen with oxygen to produce water, catalyzed by the enzyme hydrogenase. It is energetically favorable.

9. substrate level phosphorylation- a reaction that yields ATP by the phosphorylation of ADP by an inorganic phosphate.

