Abstract

PHYSIOLOGICAL STUDIES OF ALKALIPHILIC ANAEROBIC ORGANOTROPHS IN A SERPENTINIZING SUBSURFACE HABITAT

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October 27, 2014

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Microbial habitats in serpentinizing ultramafic rocks represent one of the largest, yet least understood portions of the biosphere, with potentially major consequences for global biogeochemical cycles. Serpentinization is a process where ultramafic rock is uplifted to Earth's surface through tectonic activity, where it reacts with water to create a highly reducing environment rich in hydrogen, methane, and small abiogenic organic compounds. These compounds may serve as important sources of nutrients and energy to sustain microbial metabolism.

While serpentinites can provide the energy to sustain microbial communities, the conditions created by serpentinization can be harsh and challenge the limits of microbial physiology in terms of extreme pH (>11), and availability of terminal electron acceptors. Ongoing culture-independent studies by our research group have identified bacterial taxa related to the order Clostridiales as important components of the highest pH, most reducing subsurface habitats. The goal of this project was to systematically analyze the nutrients necessary to sustain life in a serpentinizing subsurface habitat located on McLaughlin Natural Reserve near Lower Lake, CA. The research consisted of creating microcosm enrichment cultures to evaluate the distribution and environmental controls on Clostridiales species within a subsurface microbial

observatory in serpentinizing rocks. Subsequently, the cellular abundance of a novel isolate from these experiments was measured in relation to organic carbon utilization, and utilization of iron and sulfur as terminal electron acceptors. We have also conducted a cursory analysis of annotated biochemical pathways in the genomes of two isolates in relation to respiration and metabolism of carbohydrates. From these experiments we have shown that Clostridiales may play important roles in the biogeochemistry of carbon, iron, and sulfur in serpentinite environments. The research also provides further insight into habitability in these extreme environments and sources of nutrients and energy. Knowledge on the subsurface metabolic capabilities gained through this research has implications for the development of future biotechnologies related to bioremediation and microbial fuel cells.

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A Thesis Presented To

The Faculty of The Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science Molecular Biology and Biotechnology

By Crystal R George

October 27, 2014

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Matthew Schrenk for me giving me the opportunity to conduct research in his lab and meet collaborators through travel to the Coast Range Ophiolite to further conduct hands on research for my project. Allowing me that opportunity gave me a once-in-a-lifetime experience of which I am thankful for. I would also like to thank Dr. Farwell, Dr. Overton and Dr. Heimann-Rios for providing their expertise and guidance with my writing and experimental design.

I also extend my gratitude to two of my senior labmates, Melitza Crespo-Medina, and Katrina Twing for providing moral support and guidance over the duration of my research, as well as providing environmental samples and showing me how to collect my own samples during our research trip to CROMO. Secondly, I would like to thank Denise Mayer, Manager of the Genomics Core Facility at East Carolina University for her help with sequencing. In addition, I would like to thank Alyssa Kloysuntia for her help with this project and contributions that she made to our lab. Additionally, I would like to thank Dr. William Brazelton who provided his expertise with several molecular biology methodologies along with structural guidance to several of my experimental designs. Lastly, I would like to thank my family and friends for their encouragement during this program.

Funding for this project was provided to Dr. Schrenk from the Division of Research and Graduate Studies at East Carolina University, the NASA Astrobiology Institute through the Carnegie Institution for Science, the Deep Carbon Observatory, and the Alfred P. Sloan Foundation

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Chapter 1: Review of Literature

Serpentinization is a widespread geochemical process that occurs when water interacts with ultramafic rocks, characteristic of the Earth's upper mantle. The process of serpentinization occurs on each of the world's continents, and over large areas of the seafloor, and has likely extended deep into Earth's history (Sleep et al. 2011). The upper mantle is mainly composed of peridotites. When these rocks interact with water, a geochemical reaction alters the peridotites into a less dense rock form, serpentinites. More specifically, it is olivine and pyroxene within the peridotites that undergo oxidation of ferrous to ferric iron to create new minerals and the resulting serpentinite. The water-rock reactions create an environment characterized by an alkaline pH (often >11) producing a highly reducing environment containing copious amounts of hydrogen and methane. The serpentinization process can generally be summarized by the following reaction (McCollom and Seewald 2013):

$$\text{Olivine} + \text{pyroxene} + H_2O \rightarrow \text{serpentine} \pm \text{brucite} \pm \text{magnetite} + H_2$$

Biogeochemistry of Serpentinization in Ophiolites

Although the geochemistry and petrology of serpentinites have been studied for a number of years, the microbiology of these systems has not received as much attention (Barnes and O'Neill 1967). The discovery of the Lost City Hydrothermal Field, near the Mid-Atlantic Ridge, in 2000, spurred intense interest in the process of serpentinization and its relationship to life (Kelley et al. 2001). These studies found that despite high temperatures (>90°C) and high pH (>11), hydrothermal chimneys at Lost City are

teeming with microbial communities that likely use the chemical products of serpentinization (Schrenk et al. 2004).

Serpentinization-influenced habitats are also found along continental margins, in features known as ophiolites, where ancient seafloor is uplifted through tectonic events. Recent work at the Tablelands Ophiolite in Canada (Brazelton et al. 2012; 2013), at Cabeço de Vide in Portugal (Tiago et al. 2013), and The Cedars in California (Morrill et al. 2013, Suzuki et al. 2013) has shown that microbial abundance and diversity may be related to the processes of serpentinization and microorganisms may be able to sustain growth by using by-products of serpentinization.

Through 16S rRNA and pyrosequencing analyses researchers at Cabeço de Vide were able to determine the microbial community composition associated with the serpentinite hosted environment. From their findings, a majority of the sequences obtained belonged to the class Clostridia with a smaller portion belonging to the Proteobacteria (Tiago et al. 2013). Similar to this study, through metagenomics, it was found that microbial populations at a serpentinite-hosted environment at the Tablelands, Canada, belonged largely to *Hydrogenophaga* spp., and anaerobic organisms believed to be related to Clostridia (Brazelton et al. 2012, 2013).

The question arises, what types of metabolisms allow for the bacterial compositions found in these studies? Do by-products of serpentinization provide the fuel necessary to sustain life of these bacterial microorganisms? Studies from The Cedars, CA, found that over a three-year period the differences in microbial communities were strongly correlated with the source of the serpentinizing groundwater (Suzuki et al. 2013). If water was from below the surface the majority of the taxa found belonged to

the class Clostridia and if it was a mix of deep and shallow groundwater organisms belonged to the Betaproteobacteria. This deems the biogeochemistry of serpentinization and microbe-mineral interactions highly important, as changes in the geochemistry can affect the microbial diversity, ultimately limiting or allowing certain metabolic pathways to become dominant. From this we can also deduce that the location within the ultramafic unit is important to microbial composition (Schrenk et al. 2013).

The mineral transformations associated with serpentinization can produce significant quantities of H_2. The H_2 in combination with CO and CO_2 under highly reducing conditions result in the formation of CH_4 and other hydrocarbons through Fischer-Tropsch Type (FTT) synthesis. These reactions provide energy and raw materials to support chemosynthetic microbial communities (Crespo-Medina et al. 2014).

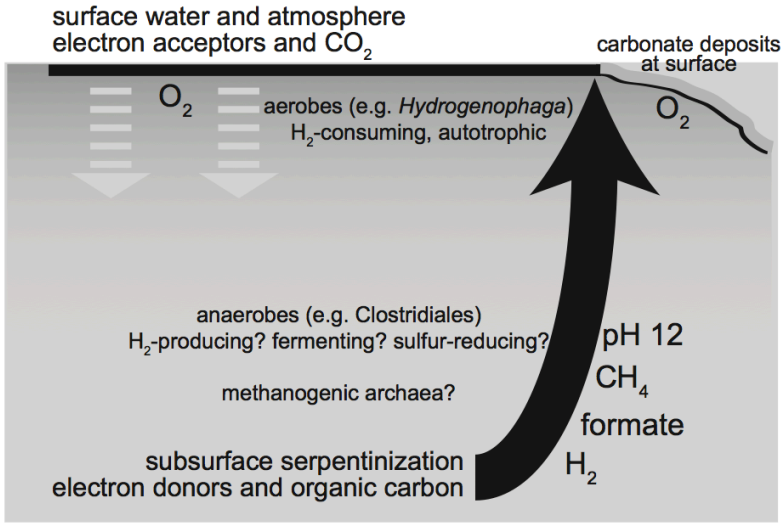

Figure 1: Biogeochemical processes that are occurring in continental serpentinization settings. Question marks indicate sources of uncertainty due to lack of data and should be considered speculative (Schrenk et al. 2013).

Study Site: Coast Range Ophiolite, Lower Lake California.

Ongoing work by our research team at the Coast Range Ophiolite Microbial Observatory (CROMO) in the McLaughlin Natural Reserve in northern California has enabled us to sample a range of different environments within the serpentinizing subsurface and begin to understand the relationships between geochemical and microbiological data (Figure 1). The Coast Range Ophiolites were emplaced in a series of orogenic events approximately 65 million years ago and contain extensive regions of serpentinized rocks that are intermingled with marine sedimentary deposits (Morrill et al. 2013). They were amongst the first sites in which alkaline seeps were identified and linked to the process of active serpentinization (Barnes and O'Neal 1967).

Serpentine soils occur throughout this region, with distinctive vegetation and ecology, all dependent on the sub-aerial weathering of ultramafic bedrock, specifically olivine and pyroxene minerals in peridotite (Cardace et al. 2013). At CROMO, a series of wells have been drilled in order to obtain subsurface water that is trapped in aquifers within the serpentinites. The subsurface water is used for many different studies, spreading across scientific disciplines (microbiology, geochemistry, hydrogeology). Of particular importance to this study is the objective of determining the diversity of microbial communities and to further study the geochemical environment and how it plays a role in influencing the diversity of microbial communities within the serpentinite subsurface.

Molecular Biological Analysis of Microbial Communities at CROMO

Recent work by our laboratory group has sampled the groundwater wells at CROMO to survey microbial populations using culturing and next generation sequencing

(NGS) approaches. Like with other sites, these studies have revealed the predominance of two major categories of bacteria, members of the β-proteobacteria class and members of the order Clostridiales (Schrenk et al. 2013). The 16S rRNA sequences indicated that the majority of these populations are closely related to *Dethiobacter alkaliphilus* (Figure 2).

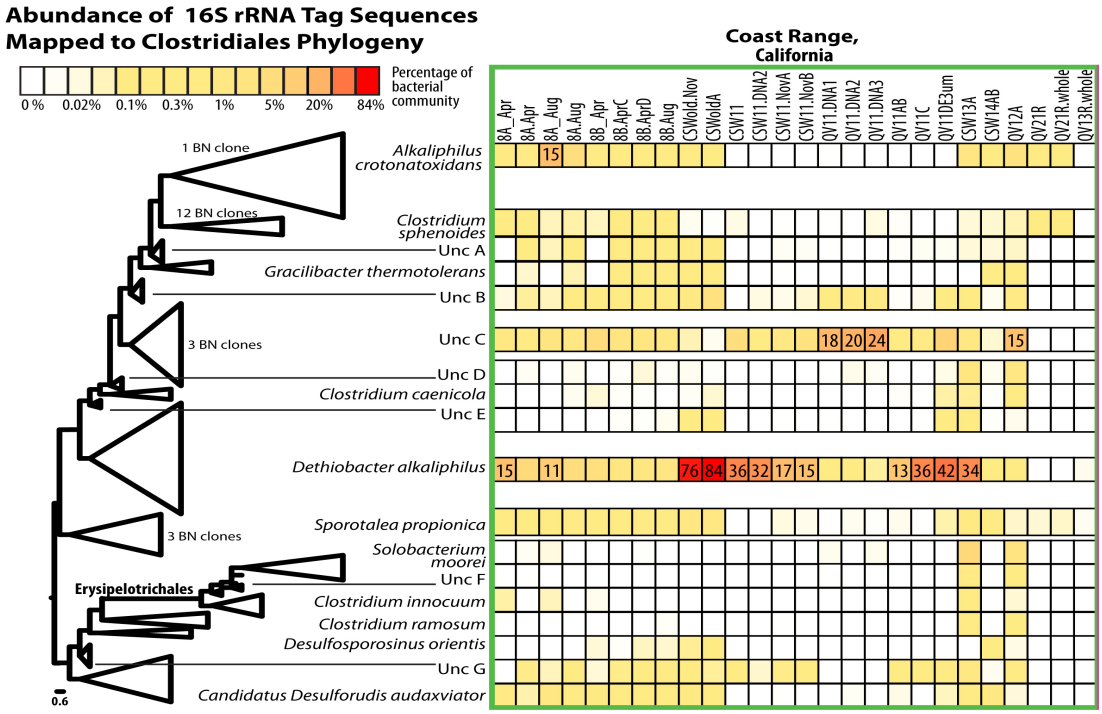

Figure 2: Abundance of 16s rRNA tag sequences, mapped to Clostridiales phylogeny. Focusing on samples from the Coast Range in California the abundance of 16S rRNA tag sequence is displayed in colors from yellow to red, with red being the highest percentage of the total sequences. *Dethiobacter alkaliphilus*, a member of the Clostridia family is found in high abundance at majority of the sampling sites at CROMO. (Brazelton unpublished)

Dethiobacter alkaliphilus appears in a majority of the wells and make up a large percentage of the bacterial populations within those wells. For example, *D. alkaliphilus* makes up 84% of the bacterial population of the "CSWold" well and makes up 42% of the bacterial population in "QV11" well. Figure 2 displays different wells at the CROMO site, which show the abundance of Clostridiales sequences based upon 16S rRNA analyses. Figure 3 also displays pyrotag sequences derived from bacterial communities from March 2013 CROMO well water. Of all the species present, a large portion of the

community is made up of Clostridiales (purple bars), thus proving that these microorganisms in particular must be well suited to this extreme environment.

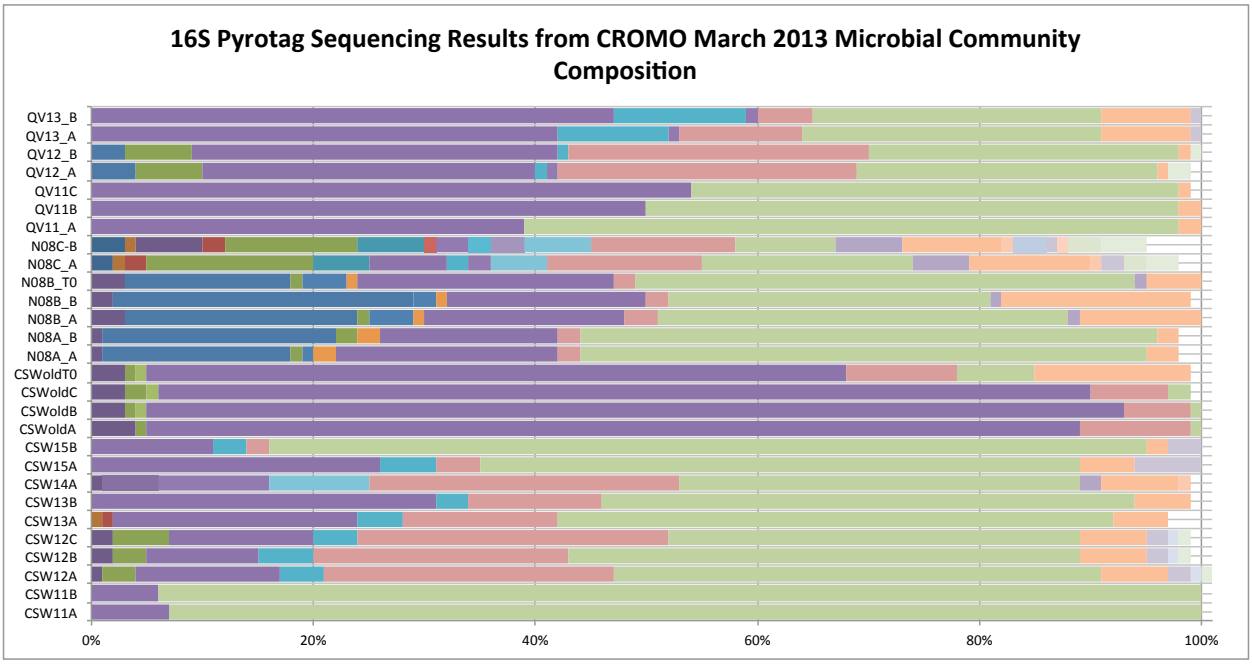

Figure 3 Pyrotag sequences mapping strains of bacteria from March 2013, in percentages of how they make up the community composition in each well. Light purple bars represent tag sequences mapped to Clostridiales. The highest percentages of Clostridia is found in the Core Shed Wells (CSW). (Schrenk unpublished).

More recently, a study of the microbial community and geochemistry of the subsurface seawater at CROMO was performed to confirm the results in Figure 2 (Crespo-Medina et al. 2014). Microcosm experiments were conducted using waters collected in March and August of 2013. From those experiments, molecular sequencing data revealed that *Dethiobacter alkaliphilus* was dominant in several wells in CROMO. A question that comes to mind is, what conditions allow for such domination of the bacterial population at these sites?

Over the past 3 years, samples from wells at the CROMO site within the McLaughlin Natural Reserve have been sampled and used to initiate anaerobic microcosm experiments. Total genomic DNA from these microcosms was extracted, PCR

amplified using 16S rRNA specific primers, and sequenced. In order to better understand the environmental conditions controlling the occurrence of *Dethiobacter*-like species, we designed a series of cultivation experiments modeled after the study that led to the isolation of *D. alkaliphilus* (Sorokin et al. 2008).

Importance of Dethiobacter alkaliphilus

Dethiobacter alkaliphilus is described and characterized in (Sorokin et al. 2008) as a sulfur-reducing microorganism, derived from soda lakes in north eastern Mongolia, that is able to grow chemolithoautotrophically with H_2 as the electron donor and can use thiosulfate, elemental sulfur and polysulfide as electron acceptors. The CROMO site offers an H_2-rich environment and when coupled to dissolved sulfur species most likely allows for the growth of this Dethiobacter or related taxa. One of the primary differences between the field sites studied by Sorokin and those of the current study is that serpentinization is a factor that serpentinization plays a role. Serpentinization may influence the availability of atmospheric carbon dioxide, the presence of the abundance and types of organic compounds, and the salinity of the fluids. Due to similarities between the microorganisms and sequences found at CROMO, and the habitat of *D. alkaliphilus*, it is likely that sulfur reduction also plays a role in the metabolism of Clostridiales in serpentinites. Ongoing studies focusing on the fluid chemistry of the alkaline springs in the serpentinite sites are being conducted in order to quantify and characterize the occurrence of sulfur compounds.

Similar work by Chivian and researchers in 2008 employed environmental genomic approaches in a hydrogen-enriched deep subsurface environment and found microorganisms similar to the sulfur reducers at CROMO (Chivian et al. 2008).

Candidatus *Desulforudis audaxviator*, a bacterium belonging to the *Firmicutes*, was the dominant member of the subsurface microbial community, comprising >99.9% of the microorganisms found in fluid samples from a South African gold mine. Although the South African gold mine is not a serpentinite environment, there are similarities that relate to the proposed research as well as CROMO, such as high hydrogen, moderately alkaline conditions, and the presence of sulfur. Sequences related to Candidatus *Desulforudis audaxviator* are also present at the CROMO site, although in low abundance (Fig. 2). One interesting note from the study of Chivian is that genomic analyses indicated that Candidatus *Desulforudis audaxviator* possess all the metabolic capabilities necessary for an independent lifestyle (Chivian et al. 2008). The same metagenomics approach can be used at the CROMO site to identify members of the microbial community. The use of environmental genomics can help in the confirmation that undiscovered bacteria at CROMO may be similar to Candidatus *Desulforudis audaxviator* and *Dethiobacter alkaliphilus*, and to determine whether the organisms possess the genes necessary for sulfur cycling and other adaptations to the subsurface environment.

Metabolic Capabilities of Alkaliphilic Anaerobes

Iron Reduction

Due to the challenges associated with the serpentinite environment, it is important to determine the microbe-mineral interactions that take place within the serpentinite

subsurface. With Clostridiales dominating this environment, it is important to understand how nutrients such as carbon, iron, and sulfur affect their metabolic capabilities.

Iron is the most abundant element on the Earth and the fourth most abundant element in the Earth's crust (Shelobolina et al. 2003). Ferric iron is available in the subsurface through water rock reactions that result in the oxidation of ferrous iron from olivine and pyroxene crystals resulting in the precipitation of ferric iron in magnetite and in the release of diatomic hydrogen (Schrenk et al. 2013). This process can be summarized by the expression:

$$2(Fe^{II}O)_{rock} + H_2O \rightarrow (Fe^{III}{}_2O_3)_{rock} + H_2$$

(McCollom and Seewald 2013)

One reason microbial populations may be able to inhabit such an extreme environment may be due to the adaptation to reduce ferric iron. Iron red-ox reactions have the potential to support substantial microbial populations in soil and sedimentary environments (Weber et al. 2006). The biotic mechanisms of Fe(III) reduction within soils and sediments are primarily described as either an indirect consequence of fermentation or microbial respiration, in which organisms couple the oxidation of carbon or molecular hydrogen to the reduction of Fe(III) (dissimilatory reduction) (Lentini et al. 2012).

Dissimilatory metal reducing bacteria (DMRB) are an important group of microorganisms that reduce metal oxides. Majority of the anaerobic bacterial species found at serpentinite-hosted environments fall within this category such as *D. alkaliphilus*

and *D. audaxviator*. Under anoxic conditions, these organisms can use Fe^{3+} as electron acceptors coupled to the oxidation of organic matter or H_2 and gain energy for maintenance and growth from such reactions (Zachara et al. 1998). Bacterial metal reduction has widened the realm of life supporting biological reactions. It has also been implicated as an important biochemical process on early Earth.

Previous work in our laboratory has shown that anaerobic microorganisms from these sites are able to reduce ferric iron, specifically ferric (III) citrate, although other types of Fe oxides are available to be used, and are more representative of the environment. Most oxidized Fe within soils and sediments exists as a variety of (oxy)hydroxides where the three most common are ferrihydrite, goethite, and hematite. Ferrihydrite the least crystalline and most soluble phase (at low pH), supports the greatest extent and highest rates of Fe(III)-reduction in laboratory incubations with common dissimilatory Fe(III)-reducing microorganisms. The current study addresses the issue of what processes and microbial groups are responsible for reduction of crystalline Fe (III) oxides within serpentinite environments.

The problem with using ferrihydrite is that it rapidly transforms into a more crystalline Fe(III) oxide with pH changes, which is poorly reduced by Fe(III)-reducing microorganisms. This fact leaves open the possibility that other subsurface microbes may be utilizing other metabolic pathways, such as fermentation. Various fermentative microorganisms have also been recognized to catalyze the enzymatic reduction of Fe(III). These fermentative microorganisms transfer only a minor fraction, approximately 5%, of the available reducing equivalents to Fe(III), and Fe(III) is not required for growth

(Weber et al. 2006). In this case, the iron serves as a sink for excessive reducing power generated through fermentative metabolism

High pH is a challenging condition for bacteria as it is difficult to maintain a proton motive force when the external pH exceeds that of the cytoplasm thus, highly alkaline conditions may favor fermentative metabolism (which rely upon substrate level phosphorylation) over respiration (Whittelston et al. 2013). The possibility that anaerobic microorganisms within the serpentinite subsurface may be utilizing other metabolic pathways is alluded to in several studies. In particular (Lovely et al. 1986) designed an experiment using organic glucose and hematite, which showed that iron reduction was a minor pathway, but when switching to amorphous ferric oxyhydroxide, iron reduction increased 50-fold because the fermentation products could also be metabolized with concomitant iron reduction. From these same studies, researchers concluded that a metabolism with Fe(III) as the electron acceptor is theoretically possible and more thermodynamically favorable than the mineralization of organic matter with sulfate reduction or methane production as the terminal step (Lovley et al. 1986). Still little is known about metal reduction under different extreme conditions and metal reduction under alkaliphilic growth conditions has not been demonstrated (Ye et al. 2004). Several studies refer to metal reducing microorganisms at acidic pH, though it should be stated that biologically induced iron reduction likely occurs in high pH environments as well (Whittleston et al. 2013).

Studies like those mentioned here could provide an explanation for microbial compositions in CROMO where alkaliphilic Clostridia is the most abundant species in a number of wells. With iron, sulfur, methane, and hydrogen being present within the

serpentinite subsurface at CROMO, this topic should be explored extensively to determine the metabolic capabilities of predominant microbial populations.

Reduction of Sulfur Species

Within the serpentinite environment, sulfur serves a biochemical and geochemical role similar to iron. Sulfur is among the most abundant elements on the Earth, the average crustal abundance of sulfur is 260 mg g^{-1}, and most sulfur on Earth is present as metal sulfides, gypsum, anhydrite and dissolved sulfate. As ophiolites originate on the seafloor and are at least partially altered by sulfate-rich seawater solutions, they likely contain a higher abundance of sulfur than rocks in typical freshwater continental environments. Biogeochemical cycling of sulfur is complex, because sulfur has a broad range of oxidation states, from -2 (completely reduced), to +6 (completely oxidized)- each of which can be influenced by microorganisms (Muyzer and Stams 2008). There are several intermediate valence forms of sulfur that can serve as both electron acceptors and donors for bacteria depending on environmental conditions; the most important ones are elemental sulfur and thiosulfate (Balk 2006).

Several strains of Clostridia are known to reduce sulfur in anoxic environment to gain energy. Sulfur-reducing Clostridia fall into a category known as sulfate reducing bacteria (SRBs). SRB's are anaerobic microorganisms that use sulfate as a terminal electron acceptor in the degradation of organic compounds. They are ubiquitous in anoxic habitats where they have an important role in both the sulfur and carbon cycles (Muyzer and Stams 2008). These microorganisms are able to form endospores depending upon the species, which allow the bacteria to survive in almost any habitat (Robles et al. 2000). The formation of endospores may play a role in sustaining life over long time

periods within the harsh serpentinite environment. For this specific reason, culture dependent and independent studies should be performed to test for growth in the presence of sulfur as well as the presence of functional genes that regulate sulfur cycles. Many species of Clostridia have been found to make up the bacterial composition within serpentinite sites and with a known utilization of sulfur, its possibility as an electron acceptor should be explored in conjunction with iron (Brazelton et al. 2006; Alt and Shanks 1998).

Assessment of Metabolic Capabilities of Clostridiales Isolates

From earlier culturing experiments with iron as the electron acceptor, members of the Schrenk lab were able to isolate two microorganisms within the Clostridiales. Recently these two microorganisms had their genomes sequenced and assembled and were used to conduct preliminary analyses of functional and physiological genes. Their 16S rRNA sequences represent microorganisms related to the genus *Clostridium sphenoides* and *Sporotalea propionica.* The two isolates are found to be a small yet idenitifable portion of the diversity at CROMO (Figure 2). There are few available publications related to *C. sphenoides,* however one early paper discusses its ability to ferment citrate to produce acetate, ethanol, carbon dioxide and hydrogen (Walther et al. 1977). *Sporotalea propionica* was originally isolated from the intestinal gut of the soil-feeding termite (also an alkaline environment) and is able to ferment lactate, fructose and pyruvate (Boga et al. 1997). These clues can be helpful in determining essential genes as well as answering questions about the role of Clostridiales in the subsurface.

Of particular importance to this study and to further understanding metabolisms that govern life in the serpentinite subsurface is the focus on functional genes that may be responsible for the reduction of metals. In conjunction with culture dependent studies, culture independent studies can be helpful to pinpoint specific genes that may serve importance to microbial sustainment within the subsurface. With two isolates cultured directly from CROMO well water, it is possible to analyze their genomes and discover clues to metabolic pathways that they may possess.

(Tiago et al. 2012) shows through 16S rRNA phylogenetic analyses genes encoding key enzymes for specific metabolic pathways including sulfur cycles such as APS reductase, alpha subunit encoding gene *aprA*, a gene that was found in several SRP bacteria including *D. alkaliphilus* and *D. audaxviator*. Other studies have noted genes that may be important to anaerobic metabolisms within the serpentinite environment. (Leang et al. 2003) discusses *OmcB* genes that are involved in Fe(III) reduction. *OmcB* genes encode outer membrane cytochromes that may be useful in the shuttling of electrons for energy. If isolates obtained from culture studies utilizing waters from CROMO contain genes that have been noted for fermentative iron reduction or other types of metal reduction, we may have the first solid clues on the metabolic properties of alkaliphilic clostridia strains from CROMO.

Relevance of Experimental Studies

The main problem when dealing with microorganisms in any environment is that we have only cultured a small percentage of the total microbial diversity that we know exists in nature. When the environment is challenging, the task of culturing microorganisms becomes even more complex. With the help of environmental genomics

and chemical analyses it is possible to decipher the elements that may be essential to growth. However, when replicating the environment *in vitro*, we may be unaware of critical elements that may be necessary to support growth. Systematically testing different compounds and analyzing the microorganisms will help in our quest of figuring out who is present in the serpentinite environment. This research will be helpful in understanding primitive metabolic pathways that may be the centerpiece of an independent anaerobic lifestyle and possibly provide clues to the early evolution of life on Earth.

Furthermore, anaerobic microorganisms from the serpentinite environment may aid in several biotechnological applications, including the ability to reduce metals that can be exploited for the bioreduction or immobilization of many toxic metals such as cobalt, chromium, uranium, and technetium (Ye et al. 2004, Whittleston et al. 2013). Microbially-mediated metal reduction has been proposed in deep geological disposal facilities (GDPs) (Williamson et al. 2013). Sulfate reducing prokaryotes (SRP) have an increasingly attracted interest due to their potential in various biotechnological applications such as biodesulfurization of flue gases (Kakosen et al. 2006) and SRPs can be beneficial by removing sulfate and heavy metals from waste streams (Muyzer and Stams 2008). Lastly, the harvest of electrical energy mediated by Fe-reducing microorganisms (FRM) in sediments and microbial fuel cells is a technological route to generate electricity. Previous studies have demonstrated that these FRMs can transform various organic compounds and heavy metal or radionuclide contaminants (Weber et al. 2006).

Chapter 2: Research Objectives

The overall objective of this study is to gain an understanding of the physiology of anaerobic, alkaliphilic bacteria present in the serpentinizing subsurface. This objective will be addressed by using cultivation experiments coupled to molecular biology approaches. These techniques are intended to enable future characterization of novel Clostridiales species and aid in understanding how their growth is influenced by different physical and chemical parameters within the serpentinite environment. Finally, the genetic content of these organisms has been investigated in reference to other closely related strains.

Systematically enrich for anaerobic microbial communities using different terminal electron acceptors

From previous work in our laboratory, it is known that bacteria within the order Clostridiales dominate subsurface environments in serpentinites. Previous experiments found that some of these groups can be enriched in anaerobic culture media at pH 11 using yeast extract as a carbon source and either Fe (III) citrate or Sodium Sulfide + Sodium Thiosulfate (i.e. polysulfides) as electron acceptors. Microcosm enrichment cultures were established using alkaline well water from a range of conditions at the CROMO sites. After 1-month of incubation, cell concentrations and taxonomic diversity within the microcosms were determined and compared to initial values. Results from the experiments allowed us to determine 1) if the same Clostridiales species are present at all sites, 2) whether certain metabolic conditions preferentially support their growth over others, and 3) potentially provide us with new microbial isolates that can be used in physiological characterizations.

Assess the diversity of microcosm experiments when different electron acceptors are employed

The genetic diversity of the microcosm enrichments can be examined through the use of terminal restriction length polymorphism to demonstrate the similarity of microbial communities based on where a restriction enzyme cleaves a specific sequence in bacterial 16S rRNA. Based on this approach we can determine the diversity of the communities within the microcosm experiments and how electron acceptors and environmental conditions influence the diversity.

To partially characterize an anaerobic novel Clostridia species derived from the serpentinite environment

A microbial isolates obtained from the microcosms was characterized in order to gain a better understanding of the physiology of Clostridiales strains within the serpentinite environment. Following isolation of the microorganism, the growth of the species was characterized, focusing on fermentation of simple sugars, and the presence or absence of iron and sulfur compounds.

Discover genes relevant to iron, sulfur and carbon metabolic pathways through genome analyses

The genomes of the new Clostridiales isolates were analyzed using the annotation pipeline RAST to determine possible metabolic functions, focusing specifically upon respiratory pathways and metabolism of carbohydrates.

Chapter 3: Hypotheses

The addition of ferric iron and sulfur compounds as terminal electron acceptors will increase cellular abundances in iron and sulfur microcosms relative to a yeast-extract only control

Little is known about terminal electron acceptor utilization patterns by alkaliphilic anaerobes in serpentinizing environments. However, preliminary evidence suggests that iron and sulfur may serve such a role in these ecosystems as they are present in the environment as byproducts of serpentinization. Preliminary evidence focusing on describing microbial community diversity also shows that microbial communities within the serpentinizing habitat are largely made up of Clostridia. Clostridia are known to actively reduce sulfur compounds and research suggests that they may have the capacity to reduce iron as well. The combination of important electron acceptors relative to a control containing only carbon sources, therefore should increase growth of microbes within the specific treatment. Ferric iron and sulfur are readily available in serpentinizing environments.

Using iron or sulfur as the electron acceptor in microcosm experiments will selectively grow microorganisms closely related to the Phylum Firmicutes

High pH, high concentrations of hydrogen gas, and limited availability of carbon sources are defining characteristics of the serpentinizing subsurface habitat. These same characteristics have been documented to selectively grow a very low diversity of Firmicutes, mainly Clostridia (Chivian, Sorokin.,, Due to the high abundance of Clostridiales species found through culture-independent studies of serpentinizing

habitats, I hypothesize that using iron or sulfur as the electron acceptor will promote the selective growth of Clostridiales-related species.

The anaerobic Clostridiales isolate WB is able to metabolize simple sugars, which is reflected in its physiology and gene content.

Several studies have demonstrated increased growth rates when coupling reduction of metals with carbon sources (Weber et al. 2006, Whittelston et al. 2013). With clues that similar microorganisms have a genome capable of an independent lifestyle, there is an assumption they may have multiple metabolic pathways to help generate ATP. Since the isolated bacteria are capable of life within the serpentinite environment, the isolates should be able to utilize a range of sugars and have increased growth in the presence of iron or sulfur compounds. These capabilities will also be reflected in the content of their genome.

Chapter 4: Methods

Sample Collection

Alkaline groundwater samples from CROMO and other locations were collected by our laboratory and used to initiate anaerobic microcosm experiments. In March 2013, our laboratory collected a comprehensive suite of samples and associated geochemical data that I used to generate results presented in this research. In August 2013, I traveled to California to collect a fresh suite of samples of groundwater. The CROMO site at the McLaughlin Natural Reserve consists of a series of wells whereby water is pumped from alkaline subsurface aquifers and collected (See Figure 4). With the assistance of colleagues in our lab, I was able to travel and collect samples following a method they have devised.

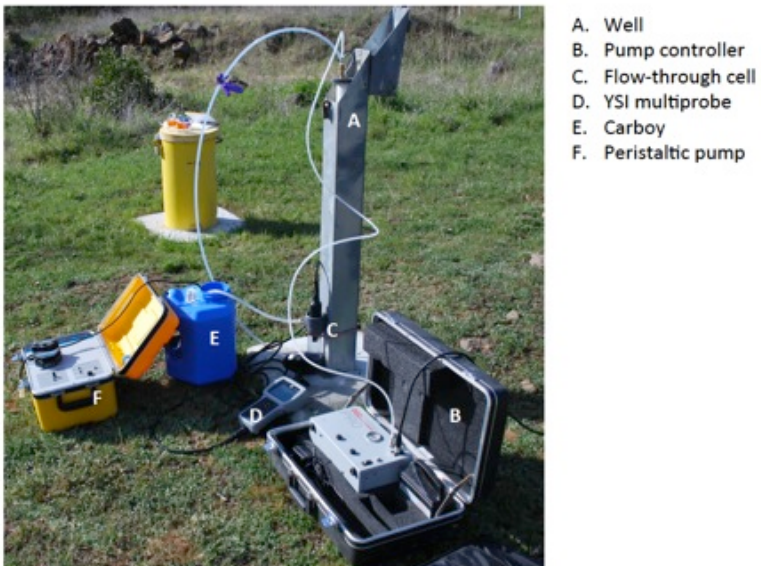

Figure 4. Collection of environmental samples from CROMO field site

In figure 4, the well (A) contains a low flow bladder pump sitting near the bottom of the well and is attached to two tubes – one for water and one for air. The bladder fills with water and the controller (B) sends a burst of air down through the air tube and into

the bladder, forcing the water up through the water tube. The water flows into the flow cell (C) where an YSI multi-probe is located. This allows for the measurement of pH, water temperature, dissolved oxygen, conductivity (salinity), and Eh (reducing nature) before it is exposed to any air. These values read out on the YSI handheld unit (D). The water then travels from (C) into a large holding container, or carboy (E), from where we can sample. The same method follows for different wells at the CROMO site.

For a complete dataset, I collected groundwater from all wells at CROMO in the summer of 2013. Table 1 displays all wells and their corresponding pH, eH (mV) and depth values. At each site, there is a series of wells. The CSW site has six wells (CSW 1-5 and CSWOld) and the QV site has six wells (QV 1-3 and N-08 A-C).

Well	pH	eH (mV)	Depth
CSW 1,1	12.23	-293	55 ft. (98-102ft.)
CSW 1,2	9.14	-140	63 ft.
CSW 1,3	10.08	-167	76 ft.
CSW 1,4	7.77	-126	29 ft.
CSW 1,5	9.95	-159	90 ft.
QV 1,1	11.41	-171	43-49 ft. (142 ft.)
QV 1,2	9.42	-112	55.5 ft.
QV 1,3	9.33	-153	78 ft.
N-08A	10.73	-186	130 ft.
N-08B	10.87	-127	86 ft.
N-08C	7.41	-128	45 ft.
CSWOld	9.84	-220	101 ft.

Table 1. Table displaying pH, reduction potential (Eh) in millivolts and well depths for all twelve wells at CROMO.

Culturing and Enrichments

In order to understand the abundance of Clostridiales at CROMO I cultured and enriched samples with different variations of electron acceptors, similar to a method described in (Pedersen et al. 2004). Through comparative analysis, I was able to determine which variations yield the most viable cells. From the fresh well water collected from each of the wells at CROMO, I ran three replicates each with varying electron acceptors to obtain enriched microbial populations (Figure 5).

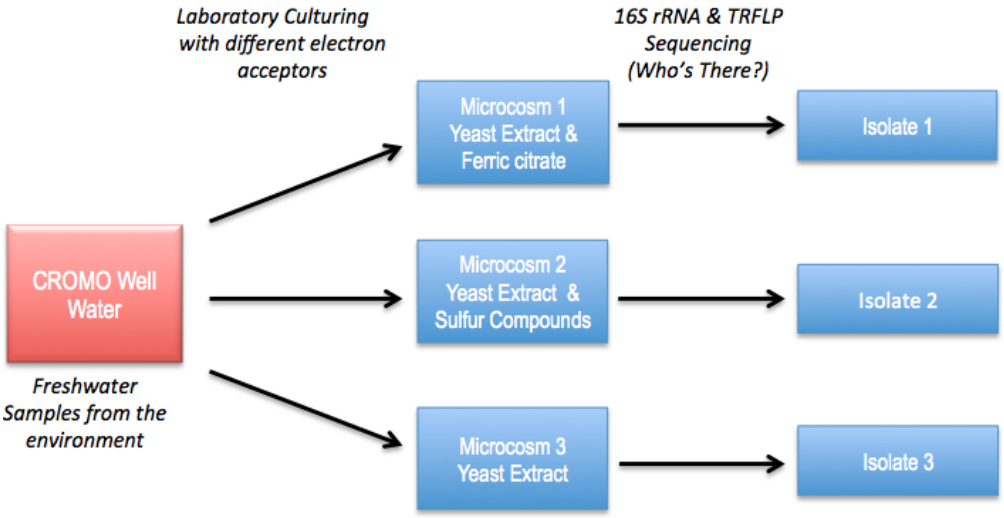

Figure 5. Flow chart of enrichment process for one well. Freshwater from one well will be taken to produce three microcosms in which the electron acceptor is the varying component.

To create an anaerobic environment, all manipulations took place in a Coy anaerobic Chamber filled with N_2. 20 ml Balch tubes were filled with 18 ml of the environmental sample and then enriched with the corresponding treatment compounds to give a total volume of 20 ml liquid. One milliliter of 4% (w/v) Yeast Extract was added to each of the three treatments as a source of carbon. 0.25g of Amorphous Fe oxyhydroxide (adapted from Geoglobus DSMZ medium 1210)was added to Fe treatment

microcosms and a mixture of sulfur compounds (20 mM Sodium Thiosulfate and 0.5 mM Sodium Sulfide) were added to the sulfur microcosms. The headspaces of the tubes were filled with a mixture of either H_2:CO_2 (80:20) or H_2:Ar (50:50). Tubes were capped with a butyl rubber stopper and incubated at 37°C for four weeks. From previous experimental trials, it was deduced that tubes would need to incubate a minimum of four weeks to allow sufficient growth for DNA extraction. The tubes were checked daily for visible iron reduction or for the production of a biomass within the tube. Maintaining pH is a critical factor for success of treatments. The pH of the treatment compounds was adjusted to a pH of 9.6-10. pH was checked weekly using pH test strips during incubation.

In earlier experiments microcosm treatments utilized filter sterilized 2% (w/v) pH 9.5 ferric citrate as an iron electron acceptor, but it should be noted that iron citrate is not readily available in the subsurface environment, and that the precipitation of iron solids may be the effect of citrate consumption and not associated with the iron itself. For this reason, before field sampling in August 2013, I compared growth of iron citrate to amorphous iron (oxy)hydroxides as the terminal electron acceptor. This experiment helped to determine if the microorganisms are utilizing the citrate or if they are utilizing the iron from the amorphous iron. From this point, we were able to determine that it was useful to carry out iron experiments using amorphous iron compounds rather than iron citrate.

Cellular Abundances

After the 4-week incubation period, a small portion of the microcosm fluid was fixed for determining the abundance of cells within different enrichment treatments. For the cell counts, 0.9 ml of the fluid was mixed with 0.1 ml of 37% Formaldehyde in a 2ml

microcentrifuge tube for preparation of slides. The fixed cells were then filtered onto 0.2 μm-pore-size black polycarbonate filters and stained with 4'6-diamidino-2-phenylindole (DAPI), a nucleic acid stain. Using epifluorescence microscopy, ten pictures were taken at random and all visible cells were counted and cell densities were calculated using appropriate conversion factors. The calculation for determining cells per milliliter of fluid takes into consideration the size of the black polycarbonate filter, filter area, as wells as the optical field the magnification of the lens, and the amount of sample that was loaded on the filter.

Dilution to Extinction

Dilution to extinction is an important laboratory method to isolate microorganisms for later use with DNA extractions and sequencing. In order to perform the Dilution to Extinction method, cell counts were used to mathematically calculate the number of cells that should be in a given test tube. The overall objective of this method was to isolate a single cell and allow it to grow under the assumption that is a pure culture.

Balch tubes were filled with 18 ml of a media used to replicate the alkaline environment. The enrichment media contains: 43 mM Sodium Chloride, 3 mM Potassium Chloride, and 3 mM Calcium Hydroxide. The media was buffered to a pH around 11 using Sodium Carbonate and finally autoclaved. In the attempt to isolate alkaline microorganisms, it must be noted that pH of this media is essential and should be checked often. The pH should always stay around 10.96-11.3. The Balch tubes were then injected using sterile 1 ml syringe and needles with attached dialysis filter, with 1 ml each of the

corresponding treatment components. Based on dilution to extinction calculations, I was able to manipulate the quantity of bacterial cells going into each tube to obtain an isolated bacterial species

DNA Extraction

After the incubation period of the microcosms or dilution samples, DNA was extracted in order to perform Terminal Restriction Length Fragment Polymorphism assays and 16S-rRNA sequencing. DNA was extracted following the protocol provided in the "UltraClean® Microbial DNA Isolation Kit" prepared by MO BIO Laboratories, Inc. (Carlsbad, CA). The extracted DNA was then quantified by using a NanoDrop 2000 spectrophotometer. The DNA extracted was quantified and had a purity range (260/280) of 1.7-2.0. The purity of the sample is a crucial quality that affects PCR amplification of the DNA templates and sequencing methods.

T-RFLP analyses

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is helpful when trying to view the diversity of a whole microbial community. DNA can be extracted from microcosm enrichments that serve as a small-scale replication of the environment. The extracted DNA is amplified via PCR with a fluorescently labeled primer. Primers used in PCR include a specific forward bacterial primer Bac518f [CCAGCAGCYGCGGTAAN] and a fluorescently labeled reverse primer Bac1046rFAM [CGACARCCATGCANCACCT]. The product is then purified and subjected to restriction enzyme digestion. The restriction digest reaction is allowed to incubate at

37°C for at least 20 hours to ensure that the amplified DNA is cut. Restriction enzymes used are *HaeIII*, *MspI*, and *RsaI*. Restriction enzymes cleave sequences within the 16S rRNA gene at specific sites that differ between the microorganisms. For this experiment, three restriction enzymes were used, *HaeIII*, *MspI* and *RsaI*. Cleavage sequences for these endonucleases are listed in Table 2. Cleavage of sequences within the gene in turn generates unique "fingerprints" to discover how similar organisms are to one another within the community based on their cleavage site.

Restriction Endonuclease	Cleavage Sequence
HaeIII	5' GG^CC 3'
MspI	5' C^CGG 3'
RsaI	5' GT^AC 3'

Table 2. Restriction Endonucleases used for TRFLP assay. ^character represents actual cut site within sequence.

This restriction reaction allowed for specific fragments of amplified DNA to be recognized as polymorphisms within the genome samples. Following the restriction

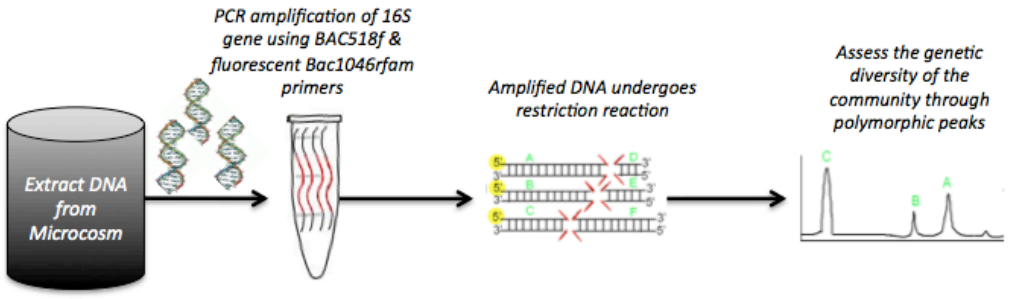

Figure 6. Flow chart diagram of TRFLP process. DNA is extracted from Microcosm, 16S rRNA gene is amplified through PCR. PCR product is purified and subjected to restriction enzymes, product is sequenced to create fluorescently labeled peaks.

digest reaction, the samples were washed with sodium acetate and ethanol to precipitate the DNA. The samples were sent to the Genomics Core Facility at East Carolina University to be sequenced. Analysis with GeneMapper software, distributed by Applied Biosystems, was used to determine which bacteria are present by presenting polymorphic peaks that create "fingerprints" for each sample (Figure 6)

Using T-RFLP was helpful in determining if a culture is pure or mixed bacterial populations. This molecular marker technology was extremely helpful with anaerobic Balch tube experiments, as they cannot be easily plated and isolated as a pure culture.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed to amplify the 16S rRNA region of the bacterial genome. Using a forward primer, Bac27f [AGAGTTTGATCCTGGCTCAG] and universal reverse primer, 1492r, [GGTTACCTTGTTACGACTT] allowed successful amplification of the 16S rRNA gene. After amplification, gel electrophoresis was performed to check for successful

amplification of the target region, which should produce a 1.6kb fragment in size. The PCR product can then be purified using a protocol and PCR purification kit provided by QIAGEN. The purified DNA was saved and used for sequencing and other molecular methods.

16S rRNA Sequencing

The 16S rRNA gene is a highly conserved gene that is used for phylogenetic studies. After extracting DNA from an isolate or microcosm environmental sample, I ran a PCR reaction to amplify the DNA and have it sequenced to determine if the bacteria isolated is in fact, a member from the order Clostridiales.

In order to prepare DNA samples for 16S rRNA sequencing, which is conducted by the ECU Genomics Core Facility, the amplified pure DNA should be at least at a 600ng/60µl sample (or 10ng/µl). To ensure the concentration, the nucleic acid content (NAC) was measured using the NanoDrop 2000 spectrophotometer. The NAC reading is displayed in ng/µl.

Characterization of an Isolate

Two strains of bacteria belonging to the phylum Firmicutes were isolated from Iron enriched microcosms. One of the two strains were characterized partially based on their ability to utilize different carbon sources (Fructose, Glucose, Lactose, and Ribose), and grow in the presence of either iron or sulfur as the terminal electron acceptor. Similar to microcosm treatments growth was analyzed through cellular abundance.

In 20 ml Balch tubes, 18 ml of media was supplemented with 1 ml 4% (w/v) Yeast Extract and either 0.25g Fe oxyhydroxide or Sulfur compounds as the terminal

electron acceptor. After four weeks, cellular abundance was assessed through epiflouresence microscopy.

The ability to ferment fructose, glucose, lactose, and ribose was also tested by using 18 ml of media supplemented with 0.25 g Iron then subsequently different sugars were added. 40 µl 2mM concentrations of Fructose, Glucose, Lactose, or Ribose were added to separate tubes and incubation occurred for four weeks. Growth was analyzed by cellular abundance through epiflouresence microscopy.

Genomics Analyses

The two isolates WB and WB1 genomes were sequenced using a HiSeq platform at Marine Biological Laboratory (Woods Hole, MA) and partially assembled into contigs. Partially assembled genomes were uploaded into RAST along with the genome of *Dethiobacter alkaliphilus* strain AHT1, obtained from the Joint Genome Institute IMG database. Automated annotation of genes was used to identify and quantify the presence of genes in different subsystems.

Chapter 5: Results

Electron Acceptor Microcosm Treatments

As mentioned briefly in the introduction, the process of serpentinization creates an extreme environment with high pH, low red-ox potential and limited availability of dissolved inorganic carbon. Organisms that succeed in such environments have evolved means to utilize carbon, iron, and sulfur compounds available in such ecosystems. In order to test how these compounds affect the growth of the microorganisms within the environment, we set up a series of experiments to test the growth of anaerobic microorganisms when coupled with either iron or sulfur as an electron acceptor. To best recreate the environment, the experimental microcosms were also provided with a hydrogen-argon headspace and supplemented with yeast extract as a carbon source.

During the duration of this study, two sampling trips were taken in March and August of 2013. All microcosm studies were performed twice using March and August samples respectively. Data collected from these experiments are displayed within this chapter. Grouped by sampling trip, March and August sample data were normalized and plotted against pH, Eh, and well depth to discover any possible correlations between (*n*) number of generations and environmental factors. Initial cell concentrations and initial pH readings were recorded, and compared to final cell concentrations and final pH (Tables 3 and 4).

Treatment	March Initial Cell Concentration	March End Cell Concentration		March Initial pH	March Ending pH
CSW 1.1	2.95E+05	Yeast Extract	1.92E+06	12.17	11
		Sulfur	1.26E+07		11
		Iron	2.14E+07		11
CSW 1.2	3.65E+06	Yeast Extract	5.01E+07	8.68	7
		Sulfur	5.88E+07		7
		Iron	4.23E+07		7
CSW 1.3	2.14E+06	Yeast Extract	4.42E+07	10.08	7
		Sulfur	3.71E+07		7
		Iron	1.12E+08		7
CSW 1.4	3.04E+05	Yeast Extract	3.54E+07	7.77	7
		Sulfur	8.38E+06		7
		Iron	4.55E+07		7
CSW 1.5	4.18E+06	Yeast Extract	5.82E+07	9.34	11
		Sulfur	4.04E+07		11
		Iron	1.45E+07		11
CSW Old	2.42E+05	Yeast Extract	7.43E+06	9.84	11
		Sulfur	1.29E+07		11
		Iron	1.38E+07		11
NO8-A	2.66E+06	Yeast Extract	1.52E+07	10.73	11
		Sulfur	2.98E+07		11
		Iron	9.26E+07		11
NO8-B	1.57E+06	Yeast Extract	1.19E+07	10.87	11
		Sulfur	8.35E+06		11
		Iron	6.13E+06		11
NO8-C	6.45E+06	Yeast Extract	5.92E+07	7.41	11
		Sulfur	8.77E+07		11
		Iron	9.75E+07		11
QV 1.1	8.97E+05	Yeast Extract	1.12E+07	11.41	11
		Sulfur	3.01E+07		11
		Iron	5.45E+07		11
QV 1.2	6.15E+05	Yeast Extract	8.66E+06	9.42	11
		Sulfur	1.00E+08		11
		Iron	1.81E+08		11
QV 1.3	5.57E+05	Yeast Extract	2.89E+07	9.38	11
		Sulfur	7.20E+07		11
		Iron	5.25E+07		11

Table 3. Initial and final cell concentrations for microcosm experiments started with samples from March 2013 CROMO sampling trip. Beginning pH readings from sampling and ending pH readings from microcosms are also displayed.

Treatment	August Initial Cell Concentration	August End Cell Concentration		August initial pH	August ending pH
CSW 1.1	1.49E+05	Yeast Extract	1.78E+07	12.39	9.5
		Sulfur	2.30E+07		9.5
		Iron	6.68E+06		9.5
CSW 1.2	4.33E+05	Yeast Extract	5.67E+07	9.15	8.5
		Sulfur	1.08E+08		9.5
		Iron	7.92E+07		8.5
CSW 1.3	3.68E+05	Yeast Extract	2.45E+07	10.2	9.5
		Sulfur	2.45E+08		9.5
		Iron	4.08E+07		9.5
CSW 1.4	2.54E+05	Yeast Extract	1.71E+07	8.04	9.5
		Sulfur	1.61E+07		9.5
		Iron	2.57E+07		9.5
CSW 1.5	2.85E+05	Yeast Extract	4.83E+07	9.95	9.5
		Sulfur	1.46E+07		9.5
		Iron	1.13E+08		9.5
CSW Old	9.22E+04	Yeast Extract	1.46E+07	9.82	8.5
		Sulfur	2.08E+07		8.5
		Iron	3.34E+07		10
NO8-A	9.05E+05	Yeast Extract	9.16E+06	10.42	7.5
		Sulfur	9.90E+06		7.5
		Iron	7.90E+07		7.5
NO8-B	3.81E+05	Yeast Extract	5.02E+07	10.98	9.5
		Sulfur	1.06E+07		9.5
		Iron	1.53E+07		9.5
NO8-C	1.49E+05	Yeast Extract	4.12E+07	7.55	7.5
		Sulfur	8.04E+06		7.5
		Iron	1.07E+08		8.5
QV 1.1	2.60E+05	Yeast Extract	3.02E+07	11.64	8.5
		Sulfur	6.06E+06		9
		Iron	1.41E+07		8.5
QV 1.2	2.29E+05	Yeast Extract	2.87E+07	9.07	7.5
		Sulfur	1.88E+07		7.5
		Iron	8.54E+07		7.5
QV 1.3	8.85E+05	Yeast Extract	5.27E+07	9.63	7
		Sulfur	6.09E+07		7
		Iron	7.62E+07		7

Table 4. Initial and final cell concentrations for microcosm experiments initiated with samples from August 2013 CROMO sampling trip. pH readings from sampling trip are displayed as well as ending pH of microcosm experiments.

To set up a baseline of comparison for experimental treatments, yeast extract was added to all microcosm treatments along with the respective electron acceptor.

March Sampling & Electron Acceptor Microcosm Experiment

Sampling Trip March 2013						
Sample	**Eh**	**pH**	**Well Depth (ft)**	**Fe (n)**	**S (n)**	**Ye (n)**
CSW 1.1	-293	12.17	55	6.33	5.57	2.87
CSW 1.2	-140	8.68	63	3.51	3.98	3.75
CSW 1.3	-167	10.08	76	5.67	4.09	4.34
CSW 1.4	-126	7.77	29	7.18	4.75	6.82
CSW 1.5	-171	9.34	90	1.78	3.25	3.77
CSW Old	-220	9.84	101	5.80	5.70	4.91
NO8-A	-186	10.73	120	6.65	5.02	4.06
NO8-B	-12	10.87	86	3.30	3.74	4.25
NO8-C	-128	7.41	45	7.40	7.25	6.69
QV 1.1	-171	11.41	46	4.33	3.48	2.06
QV 1.2	-112	9.42	55	6.80	5.95	6.92
QV 1.3	-153	9.38	78	3.00	3.46	2.15

Table 5 Microcosm sampling growth based on (*n*) number of generations using sample well water from CROMO March 3013. Resulting in majority of moderate growth (n= 3-6)

Table 5 displays (*n*) number of generations for microcosm experiments using sample well water from CROMO March 2013. The well sample name, Eh, pH, and well depths are displayed as well. The (*n*) values were taken directly from cellular abundance values and normalized using the equation: $n = 3.3 \log (T_1 / T_0)$, where T_0 represents bacterial the cellular abundance value at the beginning of microcosm experiments, and T_1 represents cellular abundance value at the termination of the microcosm experiments. (*n*) values less than 3 were recorded as "low growth" and assigned a red value in Table 5, (*n*) values between 3-6 were recorded as "moderate growth" and assigned a yellow value,

and (*n*) values greater than six were recorded as "strong growth" and assigned a green value.

From Table 5, the strongest growth occurred in wells CSW 1.1, CSW 1.4, NO8-A, NO8-C and QV1.2 for the iron enriched microcosms and only NO8-C resulted in strong growth through sulfur enrichments. The strongest growth occurred in microcosm enrichments that utilized iron or sulfur as the terminal electron acceptor. The highest (*n*) value of 7.40 was from well NO8-C iron enrichment, the second highest (*n*), 7.25 was from NO8-C sulfur enrichment. CSW 1.4 had the third highest (*n*) value of 7.18 in the iron enrichment.

The majority of microcosm experiments resulted in moderate growth, with (*n*) values ranging between three and six. Out of 4 (*n*) values resulting in low growth three of those occurred in the yeast extract control utilizing well water from CSW 1.1 ((*n*) = 2.87), QV 1.1 ((*n*) = 2.06) and QV 1.3 ((*n*) = 2.15), with the fourth occurring in an iron enrichment for well CSW 1.5 ((*n*) = 1.78).

After successful (*n*) calculations data was collected and input into SAS 9.2 statistical analysis software to determine if there were any possible correlations between environmental parameters and number of generations (*n*). Correlations were investigated between pH, Eh, and well depth with respect to number of generations. A summary of the statistical data from all correlations can be found in Table 6, with significant r^2 is listed in bold face print.

Treatment March 2013	Y-intercept	X-intercept	F-value	P-Value	R2
pH vs. (n) Iron	7.285	-0.2193	0.28	0.6097	0.027
pH vs. (n) Sulfur	7.125	-0.2488	0.88	0.37	0.081
pH vs. (n) Yeast Extract	**11.48**	**-0.749**	**8.99**	**0.015**	**0.4996**
Eh vs. (n) Iron	4.2072	-0.0005	0.47	0.5076	0.0451
Eh vs. (n) Sulfur	4.1392	-0.0003	0.37	0.5579	0.0354
Eh vs. (n) Yeast Extract	0.314	-0.01308	0.19	0.6715	0.0187
Well Depth vs. (n) Iron	6.387	-0.0174	0.73	0.4143	0.0676
Well Depth vs. (n) Sulfur	5.2647	-0.008	0.35	0.5671	0.0338
Well Depth vs. (n) Yeast Extract	5.0488	-0.012	0.5	0.4985	0.05236

Table 6. Statistical data based on enrichment microcosm treatment. Positive correlations in bold print based on a p-value less than 0.05.

There was one positive correlation found when using SAS 9.2. The relationship of pH and the number of generations in the yeast extract control (Table 6). The p-value (0.0150) and positive r^2 value (0.499) statistically validate that as pH increases the number of generations (*n*) decreases.

The extreme differences in n-values indicate that there may be a very small threshold for growth at an optimum pH, when outside of this small window logarithmic pH changes can have drastic effects on growth of bacteria. High pH is characteristic of the environment, however we see that a less basic pH still promotes growth of microorganisms, producing a higher number of generations; this can possibly be explained by the diversity within the well. There are more microorganisms that are able to survive at a more neutral pH hence, a spike in n-values once enriched with yeast extract could possibly facilitate growth of an array of bacteria within the water samples.

Tag sequencing was performed on CROMO March 2013 samples to analyze the microbial community composition. Through tag sequencing analyses, the wells with the highest (*n*) values correlate with wells that have the fewest members of Clostridia present (represented by purple bars in Figure 3). This indicates that different microorganisms proliferated throughout the duration of the experiment.

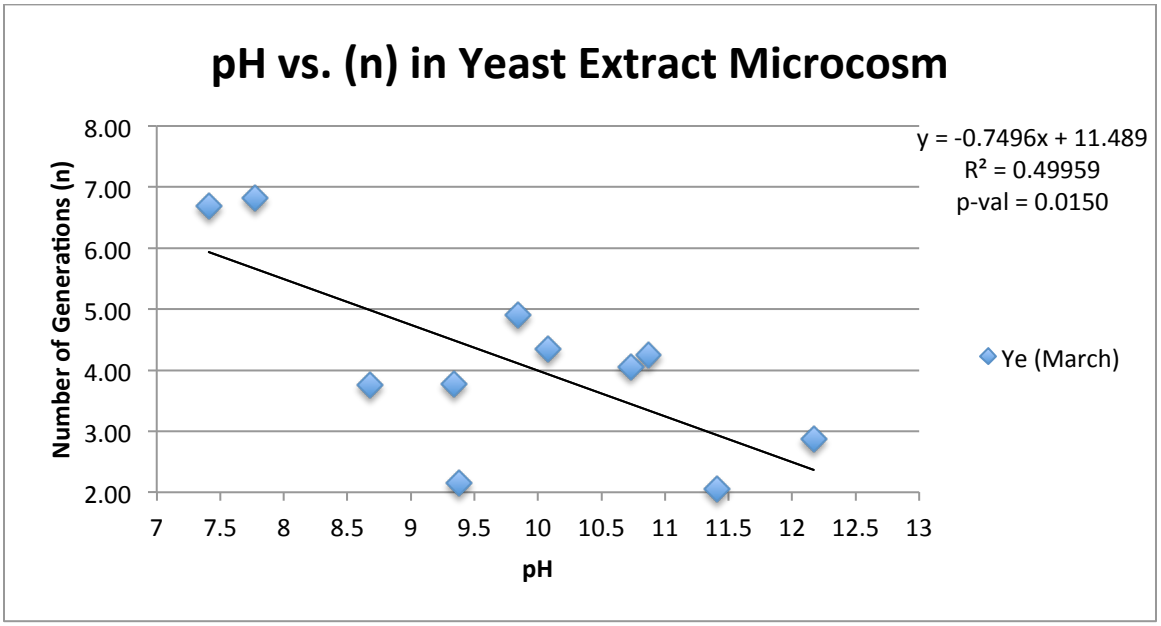

Figure 7. pH vs. (n) in March sampling set. Data here show a negative correlation between pH and (n) values. As pH increases to a more basic state the number of generations decreases

August Electron Acceptor Microcosm Experiment

Another round of well sampling took place at CROMO in August of 2013. Microcosm experiments were carried out on these samples using the same methodology as with March 2013 samples. After termination of microcosms, beginning and ending cell concentrations and pH were collected (Table 3), cell concentration data was normalized to calculate number of generations (*n*) to be compared against pH, Eh, and well depth. (Table 7). In Table 7 (*n*) values are grouped based on growth. Number of generations less than 3 were classified as "low growth" and shaded red, (*n*) = 3-7 was classified as "moderate growth" and shaded yellow and "strong growth" (*n*) = greater than seven was shaded green.

Sampling Trip August 2013						
Sample	Eh	pH	Well Depth (ft)	Fe (n)	S (n)	Ye (n)
CSW 1.1	-235	12.39	55	5.45	7.22	6.85
CSW 1.2	-130	9.15	63	7.47	7.91	6.99
CSW 1.3	-135	10.2	76	6.75	9.32	6.02
CSW 1.4	329	8.04	29	3.32	2.65	2.73
CSW 1.5	-224	9.95	90	8.57	5.64	7.36
CSW Old	-278	9.82	101	8.44	7.77	7.26
NO8-A	-139	10.42	130	6.41	6.73	3.31
NO8-B	-65	10.98	86	5.29	4.77	7.00
NO8-C	217	7.55	45	9.43	5.72	8.09
QV 1.1	-92	11.64	46	5.72	4.64	6.81
QV 1.2	-77	9.07	55.5	8.49	6.32	6.92
QV 1.3	-183	9.63	78	6.39	6.06	5.86

Table 7. Table displaying growth in number of generations (*n*) for each treatment microcosms for samples collected in August 2013, resulting in a majority of strong growth n= >7

The resulting (*n*) values from August 2013 have more moderate and strong growth than the March 2013 sample experiments. Of the 12 wells sampled, eight had strong growth with (*n*) greater than six in the iron enrichments (CSW1.2, CSW 1.3, CSW 1.5, CSW Old, NO8-A, NO8-C, QV1.2 and QV1.3). Sulfur treatments resulted in seven wells with strong growth (CSW 1.1, CSW 1.2, CSW 1.3, CSW 1.5, CSW Old, NO8-B, NO8-C, QV 1.1 and QV 1.2). CSW 1.4 had low growth with a long (*n*) of 2.65, in the same well low growth occurred in the yeast extract control resulting in a (*n*) of 2.73.

In the first round of microcosm experiments (March 2013), a hydrogen-carbon dioxide headspace (80:20) was used. The interaction of carbon dioxide with the unbuffered media was hypothesized to create a drop in pH, which could ultimately compromise growth of microorganisms. In August 2013 microcosm experiments, a hydrogen-argon headspace was utilized to avoid interference with the buffered media. The same representative electron acceptors were used to enrich samples and data collected is presented here. Based on the majority of enrichment microcosms from August samples resulting in strong or moderate growth, we can assume that changes in

headspace gas from hydrogen and carbon to hydrogen and argon, as well as changing from iron citrate to amorphous iron oxyhydroxides, between March and August 2013, samples may have been responsible for the increase of (*n*) values for August sampling.

The number of generations were then analyzed via SAS 9.2 for any correlations between (*n*) and environmental parameters, and these data are displayed in Table 8, with any statistically significant correlations listed in bold face print. Of all the correlations, there was a statistically significant positive correlation found between the reduction potential of wellwater and the number of generations.

Treatment August 2013	Y-intercept	X-intercept	F-value	P-value	R2
pH vs. (n) Iron	10.85	-0.4084	1.16	0.3063	0.1041
pH vs. (n) Sulfur	3.532	0.2721	0.48	0.5045	0.0457
pH vs. (n) Yeast Extract	4.91	0.1368	0.14	0.7185	0.0136
Eh vs. (n) Iron	6.58	-0.0026	0.8	0.391	0.0744
Eh vs. (n) Sulfur	**5.6912**	**-0.0006**	**7.15**	**0.02**	**0.4168**
Eh vs. (n) Yeast Extract	5.99	-0.0032	1.45	0.2567	0.1264
Well Depth vs. (n) Iron	5.749	0.014	0.6	0.4551	0.0569
Well Depth vs. (n) Sulfur	4.339	0.0265	2.17	0.1712	0.1785
Well Depth vs. (n) Yeast Extract	6.72	-0.006	0.12	0.733	0.0121

Table 8. Correlations made between number of generations at environmental factors such as Eh, pH and well depth for samples collected from CROMO in August of 2013.

The positive correlation is graphically displayed in Figure 7 and is denoted by a p-value lower than 0.05. Here the p-value was calculated to be 0.0234. As the reduction potential became more negative the number of generations (*n*) increased. Hence, signifying that sulfur may be the most suitable electron acceptor within the serpentinite environment, and its ability to be reduced rises as the reduction potential becomes more negative.

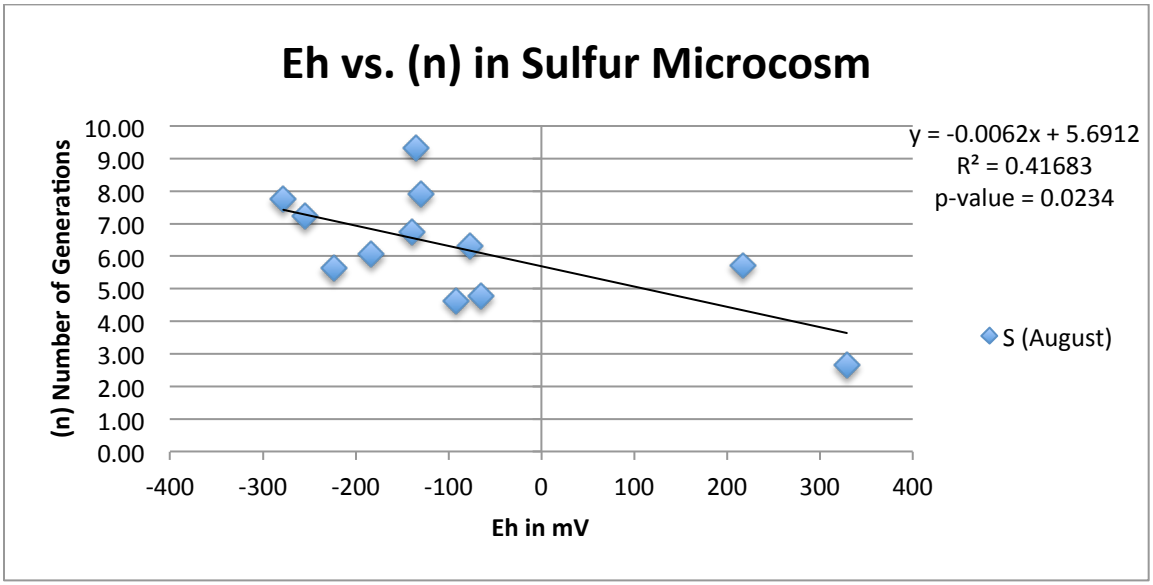

Figure 8. Scatter plot displaying the negative correlation between the reduction potential and number of generations in the Sulfur microcosm from August 2013 samples.

Assessment of Genetic Diversity at CROMO through TRFLP Utilization

By using terminal restriction fragment length polymorphisms (TRFLP) on 16S rRNA extracted from our community microcosms, we were able to determine the similarity of the species within our microcosm treatments. Microbial diversity in initial samples from CROMO was low with only a few taxa making up large portions of the community composition. Of those it is expected that Firmicutes and Proteobacteria compose the majority of the community (Figures 2 & 3). From these data, we were able to create cluster dendrograms based upon similarity of fragments through Primer6 software. Data generated from TRFLP peaks were normalized and transformed in Primer to generate a matrix of Bray-Curtis and Jaccard similarity values.

The Bray-Curtis (BC) similarity value is an estimation of the percent similarity between a pair of communities based on the composition and relative abundances of operational taxonomic unit (OTU's) while the Jaccard similarity value estimates the percent similarity between two communities based on the presence or absence of OTU's alone (Lie et al. 2013). The similarity values computed by PRIMER6 software were used to create cluster dendrograms to determine the similarity and absence/presence of the OTU's. For the objective of determining how diverse the microbial communities are within CROMO it was better to analyze samples through Jaccard similarity. Bray-Curtis cluster dendrograms can be found in Appendix A.

Grouped by sampling trip, Figure 9 displays a cluster dendrogram using the Jaccard interpretation of March 2013 CROMO microcosm samples when the restriction enzyme "*MspI*" is used. *MspI* was chosen to represent the environment data because it generated more peaks than restriction enzymes *HaeIII* and *RsaI*. Samples are grouped

based on similarity in restriction cut sites made in the 16S sequence by the restriction endonuclease. Depending on where the branching occurs we can determine how similar samples are to one another. If branching occurs, closer to the x-axis in the diagram the OTU's are similar indicating like species, whereas if the branching occurs further away from the x-axis the species are less similar. The similarity in the cluster dendrograms are displayed as a percentage.

Figure 10 displays Jaccard similarity cluster dendrogram for March 2013 CROMO samples when *MspI* is used and Figure 10 displays Jaccard similarity cluster dendrogram from August 2013 CROMO samples when *MspI* is used. In Figure 9, it is easy to recognize that all branching is high up further away from the x-axis, signifying that most samples are not very similar to each other. Of closest similarity at about 60% similar are the samples NO8-C from our Yeast Extract microcosm, and NO8-C from the sulfur microcosm, and QV 1.3 from the iron microcosm. This suggests that microorganisms in these three wells are 60% similar in their microbial composition.

In Figure 10, microcosm experiments seem to be more similar to one another with all sample branching occurring very close to the x-axis The extreme differences in similarity between March and August samples may be due to the chemical change in iron supplements from Fe-citrate to amorphous iron oxyhydroxide as well as a change in gas headspace from H_2:CO_2 (80:20) to H_2:Ar (50:50). Further culture dependent and independent studies should be run to conclude the correlations based on similarity of OTU's produced.

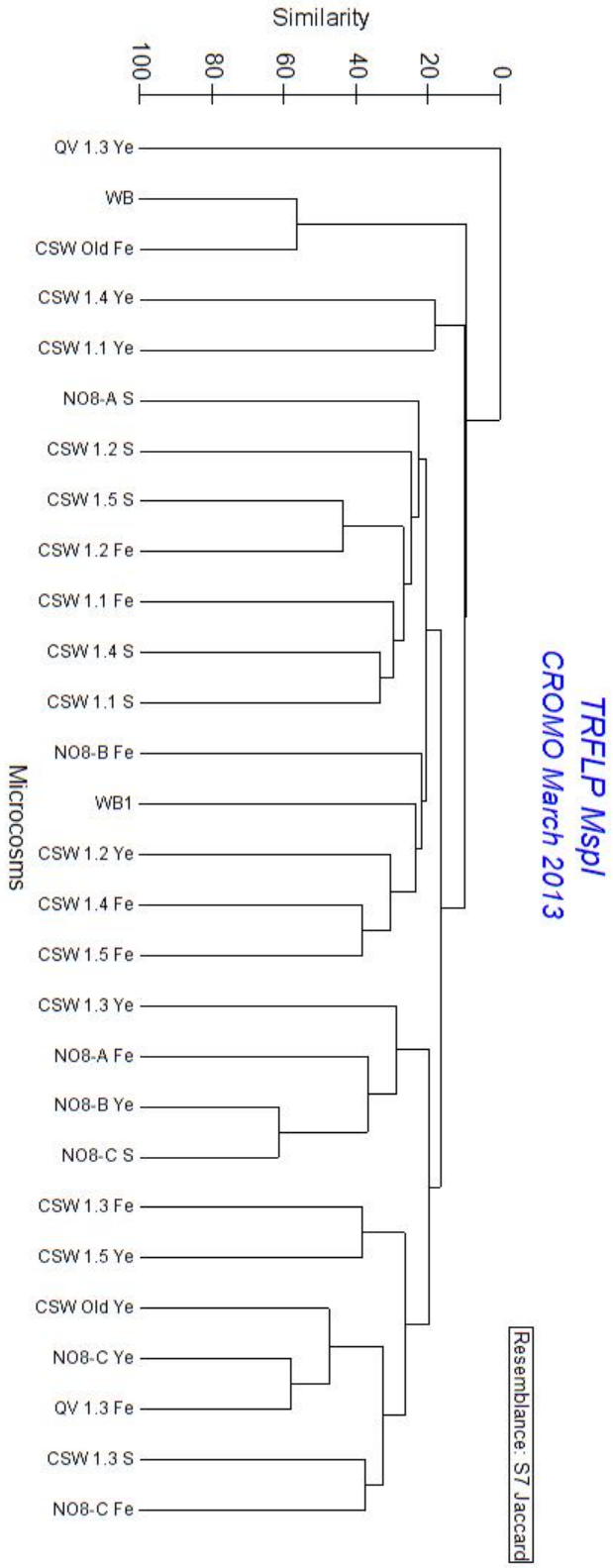

Figure 9. TRFLP cluster dendrogram created using Primer6. Displays the similarities between samples using a Jaccard analysis.

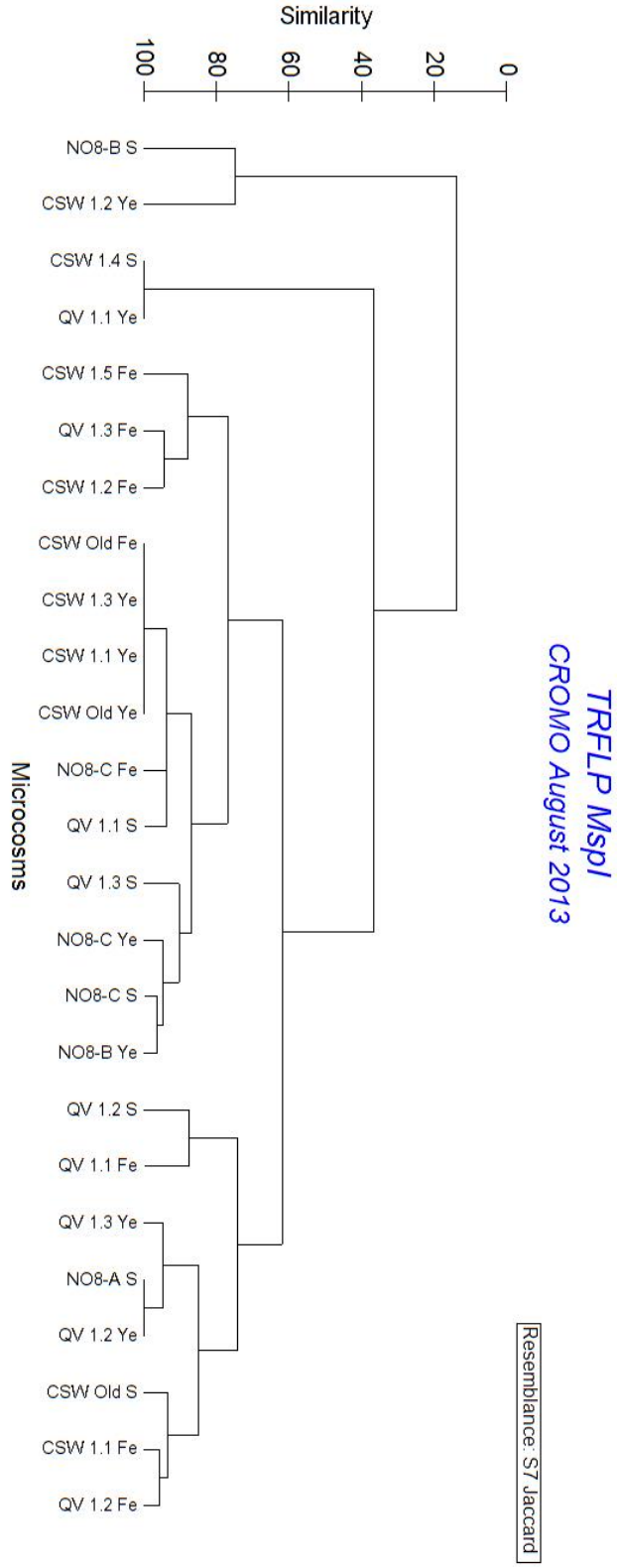

Figure 10. TRFLP cluster dendrogram displaying similarity based on Jaccard analysis using Msp1 restriction enzyme on August 2013 CROMO samples.

Sugar Utilization by Anaerobic Culture WB

From 16S rRNA sequencing and culturing methodologies, an isolate from CROMO was obtained, WB. Using Sequencher and NCBI Blast, sequences were closely matched to Clostridiales. Sample WB corresponded with *Clostridium sphenoides*. As discussed in previous chapters the order Clostridiales makes a large portion of the microbial populations within the subsurface environment (Figure 2). With this being a fact, it was important to further assess the scope of the metabolic capabilities on an isolate level. Doing so could help in future culturing experiments as well as serve as a key representative in the metabolic necessities of the majority of microbial populations within the subsurface.

Just as with our microcosm experiments, representative electron acceptors, iron, and sulfur were tested, to determine which of the two promoted high number of generations. Through the use of epiflourescence microscopy, cellular concentrations were calculated and (*n*)-values were generated. Table 9 displays ending and beginning cell concentrations for electron acceptor enrichment experiments.

Sample	Starting Cell Concentration	Ending Cell Concentration					
		Yeast Extract		Iron		Sulfur	
		Cellular Abundance	(*n*)	Cellular Abundance	(*n*)	Cellular Abundance	(*n*)
WB	8.97E+05	2.57E+07	4.81	1.11E+07	3.61	6.37E+06	2.81

Table 9. Number of generations produced when suitable electron acceptors were supplemented to isolate WB.

Another aspect tested on WB was the ability to ferment organic carbon sources. A characteristic trait of some anaerobic bacteria is to acquire energy in the form of ATP through fermentation. In order to test if the isolate obtained the ability to ferment sugars, four different sugars were supplemented to our purified cultures. Later growth in the presence of the sugars was analyzed by cellular abundance through epifluorescence microscopy. Beginning and ending cellular concentration is displayed in Table 10 below.

Sample	Starting Cell Concentration	Ending Cell Concentration							
		Glucose		Fructose		Lactose		Ribose	
		Cellular Abundance	(*n*)	Cellular Abundance	(*n*)	Cellular Abundance	(*n*)	Cellular Abundance	(*n*)
WB	1.79E+05	1.24E+06	2.77	1.23E+06	2.76	2.29E+05	0.35	3.03E+05	0.75

Table 10. Sugar utilization displayed by number of generations and cellular abundance for isolates WB and WB1

In our electron acceptor experiments, the yeast extract control resulted in the highest number of generations ((*n*)= 4.81) for WB. In the future these experiments should be run in triplicate or checked weekly for viability. Testing the ability of the isolates to ferment sugars concluded similar results, supplementing the experimental samples with glucose and fructose resulted in the highest number of generations for isolate WB with glucose having an (*n*) of 2.77 and Fructose 2.76.(Table 10).

Genomic Comparison of AHT1, WB, and WB1

Separate from testing growth in the presence of electron acceptors or carbon sources, WB and WB1 had their genomes partially sequenced and we compared their genomes to *Dethiobacter alkaliphilus* AHT1, a strain of bacteria found in high abundance in majority of the wells at CROMO (Figure 2; Sorokin et al. 2008). If isolates obtained from our CROMO microcosm experiments contained similar genes to AHT1 we could

have clues about the metabolic pathways that allow for life and growth within a serpentinizing habitat.

The first category of genes analyzed was genes regarding respiratory processes displayed in Table 11. Next, the genes associated with carbohydrate utilization were analyzed and are displayed in Table 12. WB and WB1 both have more genes that align more with fermentation as a means or respiration whereas AHT1. For example in the carbohydrate table, WB and WB1 both have more contigs present than AHT1 for oligosaccharide and monosaccharide utilization. Further tests of carbohydrates should be conducted in the future to determine if these isolates preferentially ferment carbohydrates that are more complex over reduction of metals.

	AHT1	WB1	WB
TOTAL	**86**	**225**	**236**
Biotin	16	78	74
ATP synthases			
V-type ATP synthase	8	9	14
F0F1-type ATP synthase		27	26
Electron accepting reactions			
Terminal cytochrome d ubiquinol oxidases		6	
Anaerobic respiratory reductases	12	24	15
Adenylylsulfate reductase	2	4	2
Terminal cytochrome oxidases		7	
Electron donating reactions			
Na(+)-translocating NADH-quinone oxidoreductase and rnf-like group of electron transport complexes		13	15
Respiratory Complex I	10	12	18
Respiratory dehydrogenaes I	1	16	20
NiFe hydrogenase maturation	5	13	16
Hydrogenases	13	25	23
Succinate dehydrogenase	4	5	6
NADH ubquinone oxidoreducatase			18
Respiration- no subcategory			
Carbon monoxide induced hydrogenase	8	3	10
Formate hydrogenase	5		15
Biogenesis of c-type cytochromes	7	10	6
Soluble cytochromes and functionally related electron carriers	11	15	26
Sodium Ion-Coupled energetics			
Na+- translocating decarboxylases and related biotin-dependent enzymes			6

Table 11. Table displaying contigs mapped to genes corresponding to respiration for isolates WB and WB1 compared strain AHT1

Pyruvate Alanine Serine Interconversions	4	19	28
Glycolysis and Gluconeogenesis, including Archaeal enzymes		34	
Dihydroxyacetone kinases		14	7
Glycolysis and Gluconeogenesis	15	49	48
Entner-Doudoroff Pathway		40	43
TCA cycle	11		22
Pentose phosphate pathway	5	31	43
Pyruvate metablism I: anaplerotic reactions, PEP	8	36	35
Gylcoolate, glyoxylate interconversions	1	9	11
Aminosugars			
Chitin and N-acetylglucosamine utilization		54	33
N-Acetyl-Galactosamine and Galactosamine Utilization		23	34
Di- and oligosaccharides			
Trehalose Biosynthesis		9	5
Sucrose utilization		7	
Fructooligosaccharides (FOS) and Raffinose Utilization		46	21
Trehalose Uptake and Utilization		33	28
Beta-Glucoside Metabolism		112	55
Lactose and Galactose Uptake and Utilization		49	43
Lactose utilization		17	6
Maltose and Maltodextrin Utiization		71	
Melibiose Utilization		6	
One-carbon metabolism			
Formaldehyde assimilation: Ribulose monopjosphate pathway			1
Serine-glyoxylate cycle	33	62	74
One-carbon metabolism by tetrahydropterines	7	13	21
Methanogenesis	3	3	2
Organic acids			
CitAB			1
Glycerate metabolism	2		15
lactate utilization		19	
Citrate Metabolism, Transport, and Regulation		19	27
Fermentation			
Butanol biosynthesis	11	51	49
Fermentations: Mixed acid		52	59
Acetolactate synthase subunits	2	9	5
Fermentations: Lactate		10	15

Table 12. Table displaying contigs mapped to genes corresponding with Carbohydrate utilization by WB and WB1 with a comparison to AHT1

Acetyl-CoA fermentation to Butyrate	18	57	52
Acetoin, butanediol metabolism		13	12
CO2 fixation			
Photorespiration (oxidative C2 cycle)			30
Sugar alcohols			
Glycerol and Glycerol-3-phosphate Uptake and Utilization		36	26
Mannitol Utilization		17	13
Inositol catabolism		16	15
Carbohydrates- no subcategory			
Carbon storage regulator	2	3	
Lacto-N-Biose I and Galacto-N-Biose Metabolic Pathway		12	
Polysaccharides			
Glycogen metabolism	4	21	16
Alpha-Amylase locus in Streptococcus		4	
Monosaccharides			
Mannose metabolism	3	47	40
D-Tagatose and Galactitol Utilization		21	20
Deoxyribose and Deoxynuceloside Catabolism		33	30
D-gluconate and ketogluconates metabolism		15	9
L-ascorbate utilization (and related gene clusters)		10	
Fructose utilization		53	23
D-galactarate, D-glucarate and D- glycerate catabolism- gjo		11	10
D-Galacturonate and D-Glucuronate Utilization		35	27
L-fucose utilization temp			9
L-rhamnose utilization		23	
D-ribose utilization		60	47
Xylose utilization		27	12
D-Sorbitol (D-Glucitol) and L-Sorbose Utilization		9	5
D-galactarate, D-glucarate and D-glycerate catabolism		11	10
L-Arabinose utilization		19	12

Table 13. Table displaying contigs mapped to genes corresponding with Carbohydrate utilization by WB and WB1 with a comparison to AHT1

Chapter 6: Discussion

Although serpentinite sites like CROMO have low microbial diversity, studies of anaerobic bacteria through genomic and molecular methodologies can present phylogenetic data that can in short map out "who's who" in the serpentinite environment. Characterization of novel species from the environment can determine optimum conditions for life in extreme alkaline environments. Exploring the physiological limits of growth of anaerobic bacteria is beneficial to the overall project by helping to understand the biochemistry of these organisms. However, microorganisms in the serpentinite habitat thus far prove to be a challenge to culture, particularly because they have low growth rates. The experiments performed over the duration of this study should serve as a basis for further exploration of anaerobic bacteria and the chemical processes that allow for habitability of serpentinite sites.

In particular, microbial growth and metabolism in serpentinites may mediate and control carbon and energy exchange between the deep Earth and the surface. Greater than 99% of Earth's carbon resides within its deep interior. Serpentinizing ultramafic rocks represent conduits between the deep Earth and the surface. Because Clostridiales and relatives appear to be common inhabitants of hydrogen-rich deep subsurface environments, physiological studies of these taxa may provide important insights into the roles and impacts upon elemental cycles.

In such an unstable and extreme environment, environmental parameters can have an affect on the ability for certain microorganisms to grow. As pH is a logarithmic function, minute changes in pH can cause rapid declines or increases in the number of generations. From these studies collectively, there seems to be an optimum pH range for

efficient growth of anaerobes, even with outliers, an alkaline pH of 9.5-11 seems to be optimal producing a "moderate growth" n-value in a range of 3-7. Outside of the above stated ranges, (*n*) values drastically increase or decrease, signifying that there may be error. More samples should be assessed to better pinpoint the optimal pH range for growth of yeast extract microcosms as well as the sulfur and iron enrichments.

While experimental error can account for some lack in statistical correlation, it should be stated that lack of correlation could also be contributed by several other factors. For example, differences in starting cell number and starting populations can cause fluctuations with growth stages for microbial populations. The fact that there is any correlation at all is surprising due to the simple fact that the enrichment experiments were performed on mixed rather than pure cultures. In addition, minor treatment differences between sampling sets, such as changes in gas headspace, can cause drastic changes in microbial populations leading to drastic statistical variations.

Future Direction

Similar to the study conducted within this paper, Williamson et al. 2013 utilized microcosm experiments with iron as the electron acceptor to determine the ability to grow in a similar environment. However researchers combined a series of vitamins such as riboflavin and humic substances which proved to increase growth within those microcosm experiments. Without significant changes in n-values in the study conducted, nutrients and vitamins should be added in future studies that are found within the environment to better pinpoint growth needs of microorganisms at an alkaline pH.

To better explore the diversity of the community experimentally, future studies should include replicated work by Pedersen et al. 2004, where pH can be adjusted to

assess the scope of bacteria within the sample or where selective growth can be controlled as in Pedersen et al. 2004, experimental cultures were grown aerobically and anaerobically. By systematically controlling pH and growth media, researchers would be able to fully assess the diversity within the microcosm samples. Taking into consideration the above mentioned studies, in the future, we can better pinpoint the microorganisms that grow within this environment by utilizing a collection of methods, including selectively choosing the range of electron acceptors, added nutrients and vitamins and pH.

From the current study, we can conclude that pH, Eh and well depth do not dictate the number of generations within a microcosm experiment, and iron and sulfur as the electron acceptors generally promote growth with higher (n) values when compared to yeast extract as the control. In the future, larger data sets and various electron acceptors and amendments could be used to optimize the growth of high pH anaerobes. This in turn will help decipher metabolic pathways utilized in extreme environments and moreover, yield the predominant taxa.

Studying the genetic diversity of microbial communities was another avenue of this project. By imploring Terminal Restriction Fragment Length Polymorphisms we were able to determine the scope of diversity within the serpentinizing subsurface habitat Studies of microbial growth rates were conducted and taxonomic diversity in microcosms experiments were initiated with high pH waters from the CROMO site. By combining this technology with culture dependent studies and cell counts, it was possible to determine which treatments were optimal for growth.

With recent advances in molecular biology technologies, sequence-based genomic approaches serve as a valuable tool to utilize when surveying the microbial functional and taxonomic diversity. Specifically, using molecular methodologies such as 16S rRNA sequencing along with polymorphic assays such as terminal restriction fragment length polymorphism (T-RFLP) we are able to study the diversity of enrichment cultures in high throughput and link these data to the deep sequencing we have in our NGS datasets. Along with information from collaborators, data on the chemical composition of serpentinite fluids provides further information on chemical factors, the availability of terminal electron acceptors, and nutrients that may influence microbial growth.

However, because TRFLP is such a low resoultion molecular assay, interpretation of results are often ambiguous and should be ran in tandem to eliminate errors in results The analysis of TRFLP data has developed considerably over the last decade but there remains a lack of consensus about which statistical analyses offer the best means for finding trends in these data (Culman et al. 2008).

Partial characterization based on carbon utilization and reduction of electron acceptors yielded results on the optimal conditions for growth, which has not been performed in the past. This portion of this research can be helpful in the future as it can be used to complement genomic data and functional gene surveys that inform which capabilities are necessary for survival in the serpentine environment.

Clues to The Past and Future Biotechnological Applications

It is important to study the microorganisms that inhabit these environments to gain a look into possible life on other planets that may have similar conditions. More specifically, it is important to study anaerobic bacteria that are present at CROMO

because these bacteria can give clues to life before the "great oxidation event" (Rampelotto 2010). Studying microbial life in these habitats can also help assess the possibility that other planets, having similar conditions to these extreme environments, can support life. For instance, there is evidence of serpentinization on Mars (Ehlmann et al. 2010). Several explorations of Mars have discovered trace amounts of methane, similar to extreme serpentinite environments on Earth. When we look at our own planet's most challenging environments, we are really looking for clues to what may be the normal conditions on other planets (Rampelotto 2010). Serpentinite associated life is likely associated with the boundary of the biosphere on modern-day Earth. Further, serpentinites likely played a role with the origins and early evolution of life.

From a biotechnological standpoint, alkaliphilic anaerobic bacteria may serve great importance in the future through use in biofuels and microbial fuel cells. Microbial fuel cells are an emerging technology which directly converts chemical energy stored in organic matter to electricity (Yang et al. 2010). The recent resurgence in Microbial Fuel Cells (MFCs) has launched several studies into optimizing the system for a thorough energy output that can be used for electricity in remote areas and in developing countries. The wide application of MFCs will significantly reduce the energy dependence on fossil fuel as well as the relative problems of climate and environmental pollution (Yang et al. 2010).

As metal reducers Firmicutes and Proteobacteria are shown to be dominant players in MFCs. These two groups of bacteria, mainly the Firmicutes, seem to be in high abundances at serpentinite sites, such as Coast Range, California. With further lab work some of the metal reducing Firmicutes from Coast Range sites will be isolated and

could possibly be tested for bioelectricity production. If this is the case, the isolates can possibly have a biotechnological significance and help jump-start bio-electricity production.

Alcohol production, specifically butanol and ethanol by *Clostridia* species, is also possible. As with the need for renewable electricity, the global energy crisis and limited supply of petroleum fuels have rekindled the worldwide focus towards development of a sustainable technology for alternative fuel production (Jang et al. 2012). Researchers have experimented with butanol despite of the somewhat new dawning of corn ethanol. Butanol is considered to be superior to ethanol due to its higher energy content and less hygroscopy (Jang et al. 2012). Different substrates used and fermentation strategies through anaerobic microorganisms within the phylum Firmicutes may be used to increase production and lessen the cost of daily necessities such as fuel and energy.

Fossil fuels cannot provide the increasing energy demand in the future. The evolution of sustainable and renewable energy resources and technologies is essential for sustainable development (Yang et al. 2010). Continuing studies on anaerobic bacteria for bioelectricity and biofuels will be important to lessening the reliance on current energy and fuel reserves. The biotechnological applications of microorganisms can serve as a catalyst to finding new fuels and energy sources that may serve as inexpensive alternative to costly and pollutant fuels of today.

References

1. Alt JC, Shanks WC (1998). Sulfur in serpentinized oceanic peridotites: Serpentinization processes and microbial sulfate reduction. *Journal of Geophysical Research* 103:9917-9929.

2. Balk, M. (2007). Life in the Absence of Oxygen: Alternative Electron Acceptors for Anaerobic Microorganisms in a Petroleum Environment. *Turk J Biol* 31:59-66

3. Barnes I, VC Jr, Himmelberg G. (1967). Geochemical evidence of present day serpentinization. *Science* 156:830-832

4. Blackwood CB, Marsh T, Kim SH, Paul EA. (2003). Terminal Restriction Fragment Length Polymorphism data analysis for quantitative comparison of microbial communities. *Appl. Environ. Microbiol.*(2) 926-932.

5. Boga H, Rong J, Ludwig W, Brune A (2007). *Sporotalea propionica* gen. nov. sp., a hydrogen-oxidizing, oxygen-reducing, propiogenic firmicute from the intestinal tract of a soil-feeding termite. *Arch Biol* 187:15-27

6. Brazelton, WJ, Morrill PL, Szponar N, Schrenk MO, (2013). Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. *Appl*. *Environ*. *Microbiol*. doi: 10.1128/AEM.00330-13

7. Brazelton WJ, Nelson B, Schrenk MO (2012) Metagenomic evidence for H_2 oxidation and H_2 production by serpentinite-hosted subsurface microbial communities. *Frontiers in Microbiology* 2: doi: 10.3389/fmicb.2011.00268

8. Brazelton WJ, Schrenk MO, Kelley DS, Baross JA,. (2006) Methane- and sulfur-metabolizing microbial communities dominate the lost city hydrothermal field ecosystem. *Appl. Environ. Microbiol.* 72(9):6257 doi: 10.1128/AEM.00574-06

9. Cardace D, Hoehler T, McCollom T, Schrenk M, Carnevale D, Kubo M, Twing K (2013). Establishment of the Coast Range ophiolite microbial observatory (CROMO): drilling objectives and preliminary outcomes *Sci. Dril.* 16:45-55.

10. Chivian D, et al. (2008) Environmental genomics reveals a single-species ecosystem deep within Earth. *Science* 322:275-278. doi: 10.1126/science.1155495

11. Crespo-Medina M, Twing K, Kubo MD, Hoehler TM, Cardace D, McCollom T, Schrenk MO (2014). Microbial metabolism of carbon compounds in continental serpentinite fluids. *Frontiers xx:xx-xx.in press*.

12. Culman SW, Gauch HG, Blackwood CB, Thies JE (2008). Analysis of TRFLP data using analysis of variance and ordination methods: a comparative study. *Journal of Microbiological Methods* (75) 55-63.

13. Ehlmann BL, Mustard JF, Murchie SL (2010) Geologic setting of serpentine deposits on Mars. *Geophysical Research Letters* 37:L06201, doi: 10.1029/2010GL042596

14. Jang Y, Malaviya, A, Cho C, Lee J, Lee SY, (2012) Butanol production from renewable biomass by clostridia. *Bioresource Technology,* 123, 653-663.

15. Kaksonen AH, Plumb JJ, Robertson WJ, Spring S, Schumann P, Franzmann PD, Puhakka JA (2005). Novel Thermophilic Sulfate-Reducing Bacteria from a Geothermically Active Underground Mine in Japan *Appli. Environ. Microbiol.* 72:3759-3762.

16. Kelley DS, Carson JA, Blackman DK, Fruh- Green GL, Butterfield DA, Lilley MD, Olson EJ, Schrenk MO, Roe KK, Lebon GT, Rivizzigno P, and the AT3-60 Shipboard Party. (2001). An off-axis hydrothermal vent field discovered near the Mid- Atlantic Ridge at 30°N, *Nature* 412:145–149.

17. Krulwich TA, Sachs G, Padan E (2011). Molecular aspects of pH sensing and homeostasis. *Nature* (9) 330-343.

18. Leang C, Coppi MV, Lovley DR. (2003). OmcB, a *c*-type Polyheme Cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*,*Journal of Bacteriology* 185: 2096-210

19. Lentini CJ, Wankel SD, Hansel CM, (2012) Enriched iron (III)-reducing bacterial communities are shaped by carbon substrate and iron oxide mineralogy. *Frontiers in Microbiology* 104: 1-19.

20. Lie AA, Kim DY, Schnetzer A, Caron DA. (2013). Small-scale temporal and spatial variations in protistan community composition at the San Pedro Ocean Time-series station off the coast of southern California *Aquatic Microbial Ecology* (70) 93-110.

21. Lovley DR, Phillips EJ, (1988) Novel Mode of Microbial Energy Metabolism: Organic Carbon Oxidation Coupled to Dissimilatory Reduction of Iron or Manganese. *Appli. Environ. Microbiol.* 54:1472-1480.

22. McCollom TM, Seewald JS, (2013) Serpentinites, Hydrogen, and Life. *Elements*. (9), pp. 129-134.

23. Miot J, Benzerara K, Morin G, Kappler A, Bernard S, Obst M, Ferard C, Guigner JM (2009). *Geochimica et Cosmochimica Acta*, 73, pp. 696-711.

24. Morrill PL, Kuenen JG, Johnson OJ, Suzuki S, Rietze A, Sessions AL, Fogel ML, Nealson KH, (2013). Geochemistry and Geobiology of a present-day serpentinization site in California: The Cedars. *Geochimica et Cosmochimica Acta*. 109 (2013) 222-240.

25. Muyzer G, Stams JA, (2008) The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews in Microbiology* 6:441-454.

26. Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: New insights. *Biochimica et Biophysica Acta (BBA)*, 1717:2 pp.67-88.

27. Pedersen K, Nilsson E, Arlinger J, Hallbeck L, O'Neill A (2004). *Extremophiles*, 8, 151-164.

28. Qi Y, Yul R, Caroll SL, Blair B, Zhou J, Zhang CL and Fields MW*Appl*. *Environ*. *Microbiol*. (2004), 70(9):5595. DOI: 10.1128/AEM.70.9.5595-5602.2004

29. Rampelotto PH, (2010) Resistance of Microorganisms to Extreme Environmental Conditions and its Contribution to Astrobiology. *Sustainability*, 2, 1602-1623; doi:10.3390/su2061602

30. Robles S, Rodriguez JM, Granados I, Guerrero MC (2000) Suflite-reducing clostridia in the sediment of a high mountain lake (Laguna Grande, Gredos, Spain) as indicators of fecal pollution. *Internatl Microbiol* 3:187-191.

31. Schrenk MO, Brazelton WJ, Lang SQ (2013). Serpentinization, carbon, and deep life. *Reviews in Mineralogy and Geochemistry*, *75 pp.575-606.*

32. Schrenk MO, Kelley DS, Bolton S, Baross JD, (2004). Low archaeal diversity linked to sub-seafloor geo- chemical processes at the Lost City Hydrothermal Field, Mid-Atlantic Ridge. *Environmental Microbiology* 6(10):1,086–1,095.

33. Shelobolina E, Vanpraagh CG, Lovely DR (2003) Use of ferric and ferrous iron containing minerals for respiration by *Desulfitobacterium frappieri Geomicrobiology Journal*, 20:143-156

34. Sleep NH, Bird DK, Pope EC, (2011) Serpentinite and the dawn of life. *Philos Trans R Soc Lond B Biol Sci* 366:2857-2869.

35. Sorokin DY, Tourova TP, Mußmann M, Muyzer G (2008) *Dethiobacter alkaliphilus* gen. nov. sp. nov., and *Desulfurivibrio alkaliphilus* gen. nov. sp. nov.:

two novel representatives of reductive sulfur cycle from soda lakes. *Extremophiles* 12:431-439

36. Suzuki S, Kuenen JG, Schipper K, van der Velde S, Ishii S, Wu A, Sorokin DY, Tenney A, Meng X, Morrill PL, Kamagata Y, Muyzer G, Nealson KH. (2013) Physiological and genomic features of highly alkaliphilic hydrogen-utilizing *Betaproteobacteria* from a continental serpentinizing site. *Nature Communications* doi: 10.1038/ncomms4900

37. Tiago I, and Veríssimo A (2012), Microbial and functional diversity of a subterrestrial high pH groundwater associated to serpentinization. *Environmental Microbiology*. doi: 10.1111/1462-2920.12034

38. Williamson AJ, Morris K, Shaw S, Byrne J, Boothman C, Lloyd JR (2013) Microbial reduction of Fe(III) under alkaline conditions relevant to geologic disposal *Appl. Environ. Microbiol.* doi:10.1128/AEM.03063-12

39. Weber KA, Abenbach LA, Coates JD, (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature*. (4) 752-764.

40. Whittleston RA, Stewart DI, Mortimer RJG, Burke IT, (2013). Enhancing microbial iron reduction in hyperalkaline, chromium contaminated sediments by pH amendment. *Applied Geochemistry* 28:135-144.

41. Yang Y, Sun G, Xu M (2011). Microbial fuel cells come of age. *J Chem Technol Biotechnol*, 86, 625-632.

42. Zachara JM, Fredrickson JK, Li S, Kennedy DW, Smith SC, Gassman PL, "Bacterial reduction of crystalline Fe3+ oxides in single phase suspensions and subsurface materials" (1998). US Department of Energy Publications. Paper 183. http://digitalcommons.unl.edu/usdoepub/183

43. Zhilina TN, Zavarzin GA (1994). Alkaliphilic anaerobic community at pH 10. *Current Microbiology*, 29, 109-112.

APPENDIX A

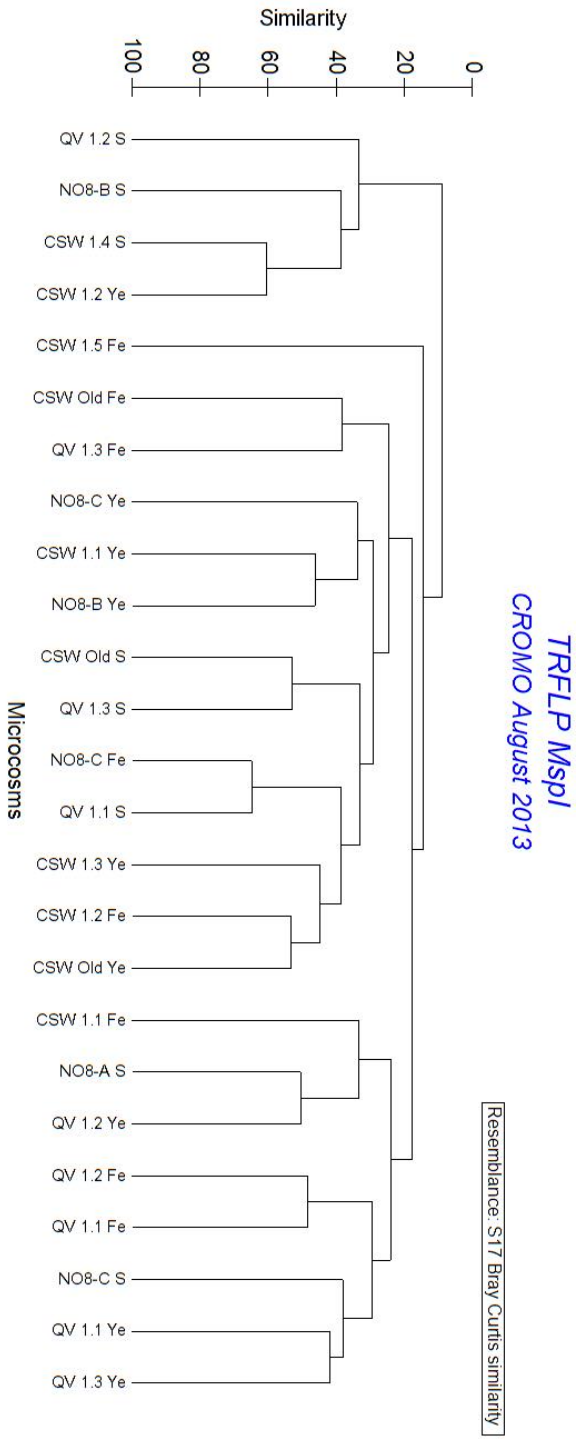

Figure 21. TRFLP cluster dendrogram displaying similarity based on presence of absence of OTU's when the restriction enzyme is used on CROMO August 2013 samples

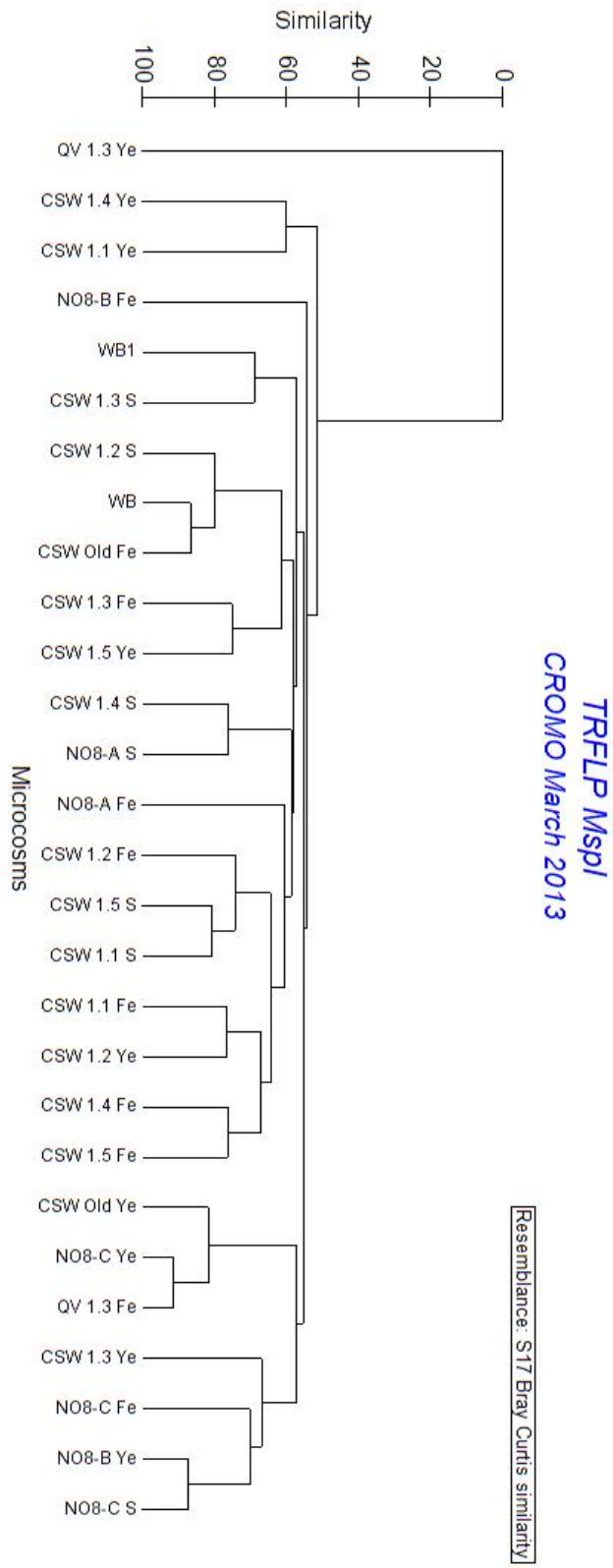

Figure 13. TRFLP cluster dendrogram displaying similarity based on presence or absence of OTU's when the restriction enzyme MspI is sued on CROMO March 2013 samples.