Biomineralization In Extreme Iron and Manganese Depositing Environments

By

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Dedication

This Ph.D dissertation is dedicated to my mother Donna Douglas whose unending support, encouragement and love has carried me through this journey.

To Shelby Jean Nicholson my beloved aunt who is always in my heart.

Nothing can fill your absence.

To my husband and children, Ken, Sterling, and Lauren

To my grandparents Alec and Ruth Douglas for unknowingly leading me to my passion for my tribal community and science.

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Abstract

An abstract of the dissertation of Wendy Francis Smythe for the Doctor of Philosophy: Institute of Environmental Health presented June 9, 2015.

Title: Biomineralization In Extreme Iron and Manganese Depositing Environments

Biomineral templating of Fe and Mn oxides is thought to be influenced by EPS, which determines the micro-environmental conditions for the microorganisms inhabiting the biofilm. The composition and morphology of EPS and its reactive side chains effect the porosity, density, water content, charge (i.e. reactive side chains), and mechanical stability of biofilm microbial diversity and biofilm morphology. Although it is known that microorganisms play a vital role in the biogeochemical cycling of metals, much is yet to be learned as to the mechanisms used in redox reactions. As we explore extreme environments we will gain more insight into the role microorganisms and their metabolic process play in the biogeochemical cycling of elements as well as identify novel microorganisms and unique metabolisms on Earth.

Research was conducted at two field sites selected as modern analogs to early Earth environments to the shallow seas from which ancient metalliferous deposits (i.e. banded iron and manganese formations) formed, and allows us to gain a better understanding of these novel ecosystems where unique biominerals, biosignatures and microfossils. At Soda Bay, Alaska cold-seeps represent a high Fe, low oxygen ecosystem along a salinity gradient, while Purple Pool, in Yellowstone National Park represents a Mn depositing, low oxygen ecosystem. Both sites are sourced with metalliferous oxygen deplete groundwaters and each site experiences extreme environmental conditions, which is reflected in the geochemistry and microbiology due to its influence on the composition of the microbial community inhabiting both ecosystems.

Our understanding of microbial evolution on early Earth will provide insight in how modern life will adapt to mineralizing environments. Gaining a better understating of the geochemistry, microbiology, microbial mineralization will allow us to address future important questions relating to whether these environments preserve traces of microfossil or chemofossils providing a glimpse into the history of early Earth.

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Wendy F. Smythe, her mother Donna Douglas and sister Bradeen Belknap

Chapter 1

Introduction To Mineral Templating of Biogenic Iron and Manganese Oxides and applications in K-12 STEM Education

Overview

This chapter introduces the general research topics that are the focus of this thesis: i) investigation of the role microorganisms play in the formation of iron (Fe) and manganese (Mn) oxide deposits; ii) how cellular surfaces and extracellular polymeric substances (EPS) influence mineral templating and microbial fossilization processes; iii) identification of novel Fe and Mn oxidizing microorganisms from different environments; iv) biogeochemical cycling of Fe and Mn; and v) evaluation of the effectiveness of coupling Western Science with Traditional Ecological Knowledge (TEK) to teach science, technology, engineering, and mathematics (STEM) disciplines to Alaska Native students.

Introduction

The role of microorganisms in the formation of banded Fe and Mn formations is a topic that is still passionately debated as is the identification of micro- (i.e., biominerals, fossilized microorganisms) and chemofossils (i.e., isotopic signatures, lipids) from these ancient deposits (Parenteau et al., 2010; Cady and Farmer, 1996). The identification of biominerals from morphology alone has proven difficult for some elements, even so, the morphology of biogenic Fe and silica minerals have been extensively characterized and are more easily identified as biogenic. Biominerals that have undergone diagenesis may be difficult to identify based on morphology alone, with identification relying on chemical signatures.

Microbial mineral templating of Fe and Mn oxides is thought to be influenced by EPS (Chan et al., 2004), which determines the micro-environmental conditions for the microorganisms inhabiting the biofilm. EPS has a profound effect on the porosity, density, water content, charge (i.e., reactive side chains), sorption properties, hydrophobicity, and mechanical stability of the biofilm (Flemming et al., 2007). While there is evidence for templating of biogenic Fe oxides on EPS (Chan et al., 2004), it has proven difficult to easily identify biogenic Mn oxides due to their amorphous

morphology. While it is known that microorganisms play an important role in the biogeochemical cycling of Fe and Mn, much is yet to be learned about the mechanisms of cycling and the extent to which the reactions are microbially mediated. As we explore these environments, we will gain more insight into the role microorganisms and their metabolic processes play in the biogeochemical cycling of elements as well as identify novel microorganisms and unique metabolisms on Earth.

Objectives

The specific objectives of this dissertation are broken down into four focus topics. Which are to i) characterize the morphology of biogenic Mn oxides from both a controlled laboratory and from complex environments in an effort to better identify biogenic Fe and Mn oxides from ancient geologic deposits, ii) characterize two complex Fe and Mn rich groundwater ecosystems to better understand the impacts environmental conditions have on biogenic Fe and Mn deposition, iii) observe the microbial diversity and identify microorganism from biofilms present in Fe and Mn rich groundwater ecosystems, and iv) develop an effective pedagogy for STEM education by coupling TEK with western geoscience education.

Study Sites

This research examined three field sites, which were selected as modern analogs to early Earth environments. The first site is Soda Bay, Alaska, which represents a high Fe, low oxygen ecosystem along a salinity gradient. The second field site, hot-spring LWCGNN05 (Yellowstone Coordination Network), referred to as Purple Pool, in Yellowstone National Park (YNP) was selected as a Mn depositing, low oxygen ecosystem. The third site located in YNP at Queens Laundry hot-spring was used to characterize mineral templating of silica minerals into biofilms. All three sites are supplied with metalliferous oxygen depleted groundwater, which is heavily influenced by the local geology. These sites experience unique environmental conditions, which is reflected in the groundwater geochemistry and influences the composition of the microbial community inhabiting both spring ecosystems.

These ecosystems may be viewed as modern analogs to the shallow seas from which ancient metal ore deposits formed, and allows us to gain a better understanding of these novel ecosystems and their potential to harbor unique biominerals, biosignatures and microfossils. These sites also allow us to expand our understanding of the metabolic diversity and the identity of the microorganisms that drive environmental redox reactions.

Knowledge about these modern ecosystems will allow us to better interpret biosignatures from the geologic record providing new insights into important geological questions such as the evolution of life on Earth and the role microorganisms played in shaping Earth as it evolved from an anoxic to oxic planet. Our ability to better understand modern microorganisms from mineralizing environments sheds light on the evolution of microorganisms present on early Earth and provides insight into how modern life adapted to mineralizing environments. Gaining a better understanding of the geochemistry, microbiology, and biomineralization will allow us to address future important questions relating to whether these environments preserve traces of microfossils (i.e., unique oxidation products or biogenic minerals) or chemofossils (i.e., lipids) that provide a glimpse into the history of early Earth.

Soda Bay is on tribal lands in Southeast Alaska, and is owned by an Alaska Native Corporation; both the location and ownership of this site ensures that it will remain protected for generations to come. The site is well known to inhabitants of Prince of Wales (POW) Island and as with other sites on the island has a written and oral history among the Alaska Native Haida. The TEK of this site is important for our understanding of the history, dating back 100 years. TEK about Soda Bay and the surrounding environment has existed for generations, providing details that are now becoming elucidated through analysis of the geochemistry and microbiology of the ecosystem. Interviews with elders from the Haida Nation describe a beautiful location with carbonated “soda water”, and a river where no fish or shellfish are present. Fishermen describe this site as having “bad water” (pers. comm. elder Claude “Miijuu” Morrison) where the “fish cannot breathe” (pers. comm. elder Robert Sanderson). Consequently, the river has historically been deemed of having no value, due to its lack of fishery resources. Even so, Soda Bay has been used for recreational activity rather than a fishery harvest site. For generations families have visited the site to picnic, swim, berry pick, or to

collect cedar bark for weaving. The name Soda Bay comes from recreational users who used the “soda water” to mix with powdered juice, making “soda” for their children to drink back when there were no stores and thus no “real” soda to drink (pers. comm. elder Alma Cook and Elsie Burton). Currently this location is rarely visited and current generations have forgotten about its value as a site for harvesting berries and cedar bark.

YNP, located in Wyoming, Montana and Idaho, spanning about 3,500 square miles, and managed by the National Parks Service, is located in the volcanically active Yellowstone Caldera centered over the largest supervolcano on the North American Continent. Yellowstone was known to and used by Native Americans 12,000 years before pilgrims arrived, with an estimated 26 tribes with ties to the park. Only one tribe, the Sheepeaters band of Shoshone, remained in the park year-round. The park was considered sacred and used for rituals such as vision quests. Most tribes were present in the park in the spring and summer for hunting, fishing and to acquire obsidian from Obsidian Cliff for tool making. The obsidian tools have been found across the continent and were used for trading with other tribes. Obsidian Cliff is now a national historic landmark as the first industrial area in North America. Hot-springs were used for cooking, bathing, and for preparing hides. There are many myths as to the Native Americans’ beliefs that the hot-springs were evil, possessing demons or evil spirts. These myths came about as the government wanted to encourage non-natives to move westward and visit the park and used these stories to reassure visitors that Native Americans, who were feared, were not in the Yellowstone region (Yellowstone Network).

The two study sites in YNP are located in the Lower Geyser Basin along the Firehole River. Queens Laundry is located in Sentinel Meadows and Purple Pool is located on Firehole Lake Drive.

Biofilms and EPS

Biofilms are complex consortia of single and mixed species microorganisms forming multicellular subpopulations, attached to surfaces and held together in an EPS matrix and cell-surface adhesion proteins. There are many benefits for microorganisms residing in biofilms such as protection from predation and resistance to antimicrobials. Microorganisms within biofilms experience a decrease in their growth rate due to the

confined space in which cells have to grow; some cells become metabolically inactive similar to stationary phase. In stationary phase, microorganisms have been found to increase production of secondary metabolites such as pigments and other small molecules (Flemming et al., 2007; Martin and Liras, 1989).

EPS are composed of hydrated biopolymers, proteins, glycoproteins, glycolipids and extracellular DNA (eDNA), produced by microorganisms and serve as the scaffolding for biofilms. However, the composition of the matrix varies greatly depending on environmental conditions in which the biofilm forms. The EPS matrix serves a variety of functions from attaching the biofilm to substrate surfaces to channeling nutrients sequestration of dissolved and particulate substances (Flemming et al., 2007). Studies examining biofilm architecture using *Pseudomonas* spp. mutants, which overproduce EPS found significant modification in the biofilm community with an increase in the rate of biofilm formation (Martínez-Gil et al., 2013).

Geology

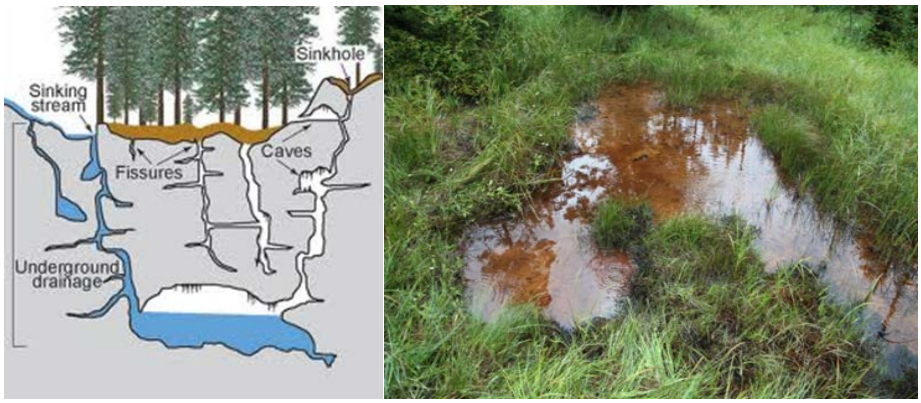
Soda Bay, Alaska: Iron Study Site

The Soda Bay watershed is a pristine low-temperature Fe-, Mn- and carbonate rich river that empties into the Soda Bay Estuary and is an important component of the nutrient transport into the estuary and in the near shore environment of Tlevak Strait. Dissolved minerals from the karst aquifer are transported through fissures to the surface of the limestone bedrock and along geologic contacts forming cold-seeps along the shore of Soda Bay River and in the hills above the river. In and around Soda Bay Fe- and Mn-carbonates occur as mounds up to three meters in height having formed due to the precipitation of dissolved minerals from waters supersaturated with dissolved carbon dioxide. Recent evidence has shown that microbial mats are necessary for the precipitation of carbonate minerals and the formation of tufa in lotic ecosystems, controlling both the conditions and the products of this biogeochemical process (Pedley, 2009).

The geologic history of POW Island determines the unique characteristics of Soda Bay. POW Island is on a landform referred to as the Alexander Terrane formed 570 million years ago (mya) at the equator on the seafloor of what is now the Pacific Ocean.

Tectonic drift caused the northward movement and uplifting of the landmass into shallow seas in the northern hemisphere, where extensive coral reefs formed during the Silurian (440 mya), resulting in the deposition of massive carbonate deposits (Rigby et al., 2008). During the Triassic (245 mya), the land mass continued on its northward trek, undergoing metamorphic processes due to rifting, eventually colliding with the North American Continent and being uplifted above sea level while it continued to move northward during the Cretaceous (144 mya). The rifting and continental collision resulted in the formation of Fe rich metamorphic green schist that now comprises the bedrock of POW Island. These metamorphic events resulted in the emplacement of swarms of magmatic Mn rich dikes. The presence of schist indicates that the region experienced low temperature and low pressure metamorphism, however, the intrusion of Fe and Mn rich dikes indicate the region experienced significant tectonic activity (Snelling, 1991).

Extensive tracts of Silurian limestone, metamorphic green schist and Mn rich magmatic dike complexes characterize this region, which experiences large amounts of annual precipitation (~2.5 meters). Studies of other karst systems have found that the dissolution of limestone due to precipitation, water temperature (>7.2^{o}C), and highly acidic water chemistry is accelerated along faults and shear zones (Allred, 2004). The development of a karst landscape is the result of underlying limestone where erosion and dissolution by ground water and chemical weathering produces fissures, sinkholes, and underground streams and caverns, creating a network of interconnected fissures in the limestone bedrock through which mineral saturated fluids flow (Fig. 1).



www.esi.utexas.edu/outreach/caves/karst.php

Figure 1. Top (Left) Cartoon of shallow soil and underlying karst conduit system (credit: www.esi.utexas.edu/outreach/caves/karst.php). (Right) Field photo of water logged muskeg at Soda Bay. Bottom (Left) Sink hole that is filled in with water and overlain by a thick microbial mat. (Right) Fe depositing (orange) cold-seep coming out the side of a cliff.

The region is well known for its shallow acidic soil referred to as muskegs. Muskegs are (pH 3.0 - 6.3) common in Arctic and boreal regions, consisting of dead plants in various states of decomposition (as peat), and ranging from fairly intact sphagnum moss to highly decomposed humus. Muskegs tend to have a water table near the surface, with the moss holding 15 to 30 times its own weight in water, allowing the spongy wet muskeg to form on sloping ground. Muskegs form where permafrost, clay or bedrock prevents water in rain and snowmelt from draining, forming permanently waterlogged vegetation and stagnant pools. Muskegs are prevalent on POW Island and in the Soda Bay River drainage and are a likely source of nutrient input into the spring effluents via surface runoff and percolation into the groundwater.

These shallow acidic bodies of water accelerate the formation of karsts on POW Island. Karsts are governed primarily by dissolution processes driven by water and carbonate rock interactions, referred to as karstification. Karst spring distributaries form branched conduits that rapidly discharge groundwater to multiple springs that are distributed along the bank of a short reach of the receiving stream, much like those seen at Soda Bay River. A karst aquifer is comparable to a roofed-over creek with similar branching drainage patterns and has characteristic geomorphic features, such as caves, subterranean conduit drainage systems, a disrupted topography caused by sinkholes and disappearing streams. Surface features indicative of karst terrains are often difficult to identify (Bennett and Engle, 2005). Aquifer conduits and streams fill from pore spaces between limestone grains and fractures formed by joints, bedding planes, and faults, where the openings forming the karst aquifer may be partly or completely filled with water. Conduits transporting water converge forming successively larger passages and a continual increase in groundwater flow, eventually discharging from springs at the surface. Groundwater circulation in karsts is highly variable; rapid flow occurs in locations with dissolution-enlarged fractures and conduits, while slow flow occurs through fine fractures and pore spaces (Bonacci et al., 1997).

Sinkholes and sinking streams draining into karst aquifers may be several miles away from the spring effluents as groundwater rises to the surface from water-filled conduits (Fig. 1). A majority of the water stored in karsts is underground in the epikarst, the area above the main part of the aquifer where water is stored in enlarged joints and bedding planes, in spaces around pieces of float, and in smaller conduits in the bedrock. Sinkholes are indicative of the formation of subsurface epikarst development and are sites of active transport of insoluble sediment and dissolved rock into the subsurface. Such surface features are evident from maps of the Soda Bay region. As groundwater from conduits approaches a permanent surface-flowing stream, the water seeks the lowest available exit and is continually creating new springs downstream. Along low-gradient streams, several openings may develop almost simultaneously, resulting in the formation of several springs draining a single groundwater basin. Karsts typically have significant amounts of heavy metals that precipitate within the karst ecosystem; however,

groundwater has very low concentrations of dissolved heavy metals, favoring transport of colloids or particulates.

Groundwater comprises the largest reservoir of freshwater in the world, accounting for over 97% of freshwater available on Earth, not considering glaciers and ice caps, and is an important component of the global hydrological cycle (Gibert, 2001). Prior studies have found that the microbial biomass of groundwater dwelling prokaryotes in the unconsolidated subsurface accounts for a significant portion of the Earth's total microbial biomass (Whitman et al., 1998). Subsurface groundwater environments that have not been impacted by eutrophication or high inputs of organic matter are often dominated by chemoautotrophic microorganisms. These microbes are critically important to global geochemical cycling due to their ability to oxidize inorganic compounds and fix inorganic carbon They serve as catalysts for reactions that would either not occur or would proceed exceedingly slowly over geological time scales (White, 2009). Traditionally karst formation has been thought of as an abiotic, chemical process occurring near the water table, through dissolution of carbonate rocks (Li et al., 2005). The dissolution of limestone results in the enrichment of groundwater fluids with carbon dioxide, which serves as an inorganic carbon source for chemotrophic microorganisms. The low temperature of the groundwater allows for carbon dioxide saturation; once dissolved into the groundwater CO_2 forms carbonic acid further acidifying the groundwater. As groundwater, fluids emerge from cold-seeps they are rapidly mixed with oxygenic surface waters resulting in the precipitation of dissolved metals (Fe- and Mn) and carbonate resulting in the formation of numerous carbonate mounds, or tufas.

Tufas form from alkaline waters that are supersaturated with dissolved carbonate. As groundwater fluids emerge from spings/seeps fluids degas CO_2 due to the lower atmospheric pCO2 and the resulting increase in pH which decreases carbonate solubility. Recent studies by Pedley et al (2009) demonstrated that microbially induced carbonate precipitation may be more predominant than abiotic precipitation.

At Soda Bay groundwater interacts with the green schist parent rock and Mn rich magmatic dikes supplying reduced Fe and Mn to the anoxic groundwater fluids. Concentrations of Fe and Mn vary seasonally due to the seasonal influx of oxygenated water from the surface during periods of high precipitation (Fig. 2), resulted in low

dissolved metal concentrations during these recharge events. Once the influx of oxygen has been consumed, Fe and Mn concentrations in the groundwater will again increase.

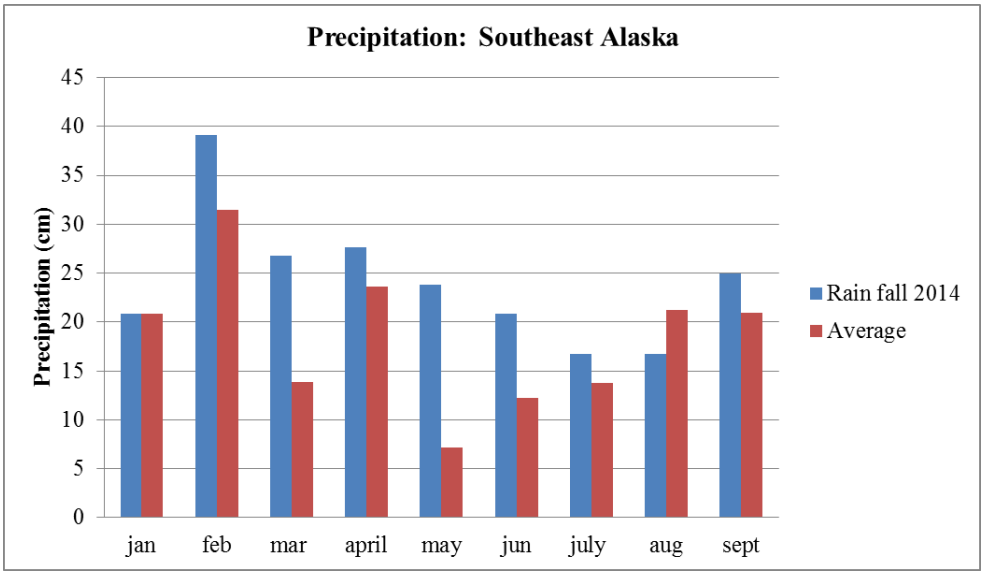


Figure 2. Graphical illustration of average annual precipitation and the increased precipitation for 2014 (NOAA, National Weather Service, http://www.arh.noaa.gov/public).

Purple Pool YNP: Manganese Study Site

YNP is located on a high volcanic plateau in a region of active major crustal extension. The region has experienced significant seismic activity over the last 2.2 million years. Eruptions of lava and tuff have been predominantly rhyoltic and subordinately basaltic due to its location on the North American Continent. The caldera sits atop a shallow magma pool at depths from 3 to 10 km (Fig. 3). The composition of the crustal rock has significant impact on the geochemistry and subsequently the type of volcanic activity. Crustal rock is composed of weathered and eroded material thereby enriching the rock in silica, which is seen all over the park as the white deposits around YNP hot-springs (Inskeep and McDermott, 2003; Braunstein et al., 1996).

YNP receives on average 218.11 cm of precipitation annually. This precipitation along with meteoric water that is derived from the mountains to the north and northwest percolates downward through fissures and faults recharging the five geyser basins and becoming super-heated (Fig. 4). This super-heated groundwater interacts with the silica rich, rhyoltic crustal rock enriching hydrothermal fluids in silica ions. High temperature

water convects upwards resurfacing as boiling hot-springs and fumaroles in regions where faults transect topographically low basins. Hot-spring waters have been measured between 180 – 270°C at depth beneath their source vents that range from 100 to 550 meters (Inskeep and McDermott, 2003). Geochemical studies have shown that although the behavior of hot-springs has changed in the park due to seismic activity, the composition of the waters has not changed much over the last century (Fournier, 2005).

Geochemical measurements conducted across YNP by the park service in collaboration with (Chaffee et al.) allow us to observe distributions of Fe and Mn (Figs. 5 and 6). Mn concentrations across the park average 0.311 µM, while Mn concentrations at Purple Pool average 1.14 µM, showing enrichment in Mn at this field site.

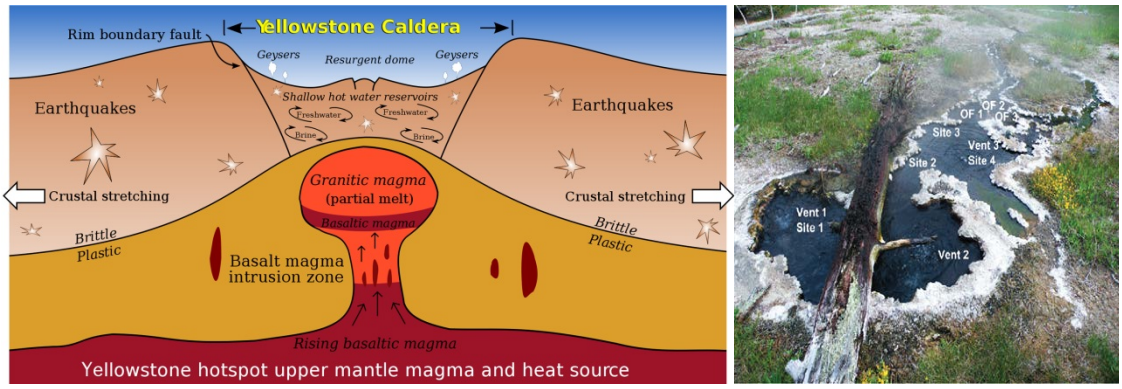


Figure 3. (Left) Cartoon illustrating the Yellowstone caldera and underlying magma chamber (whyfiles.org). (Right) Field photo of Purple Pool.

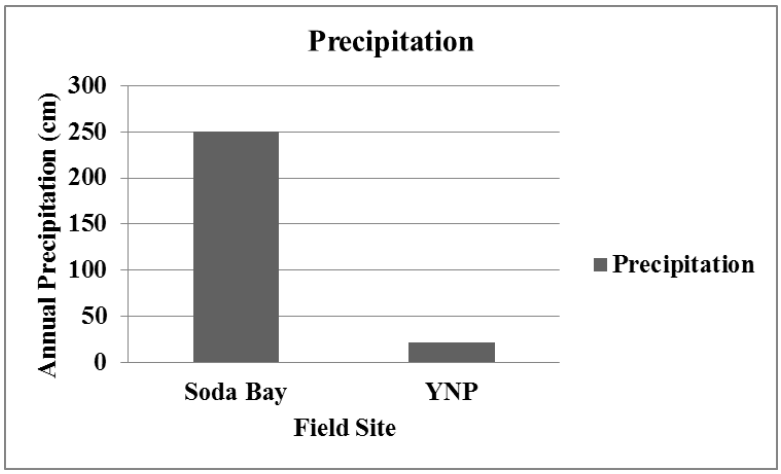


Figure 4. Graphical of annual precipitation from both Soda Bay, Alaska, and YNP, Wyoming.

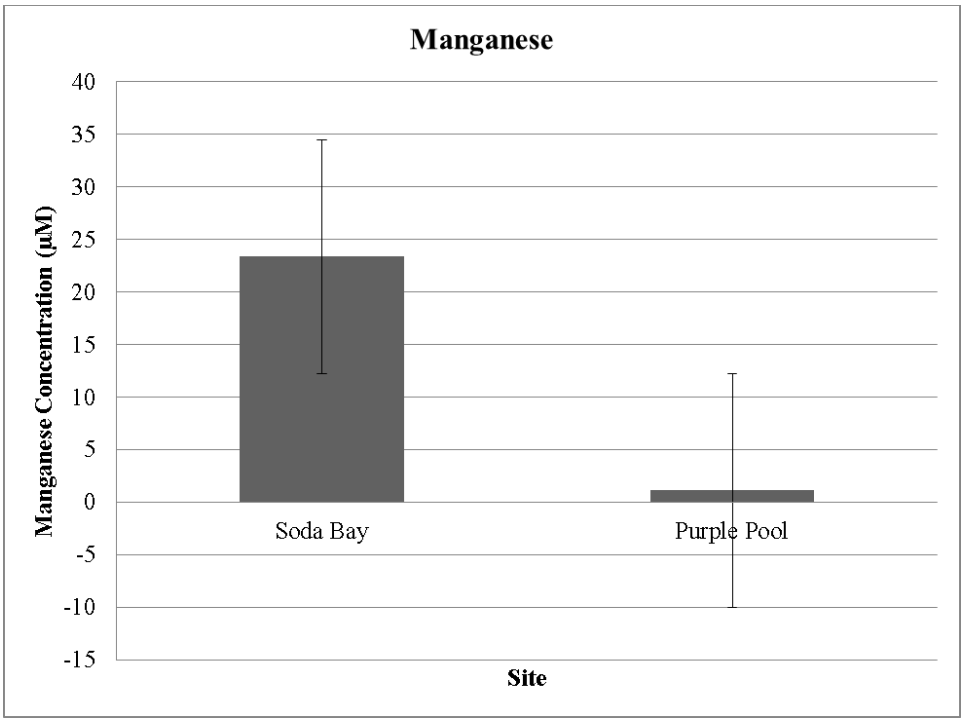


Figure 5. Distribution of Mn across YNP (data reported by USGS: Chaffee et al.).

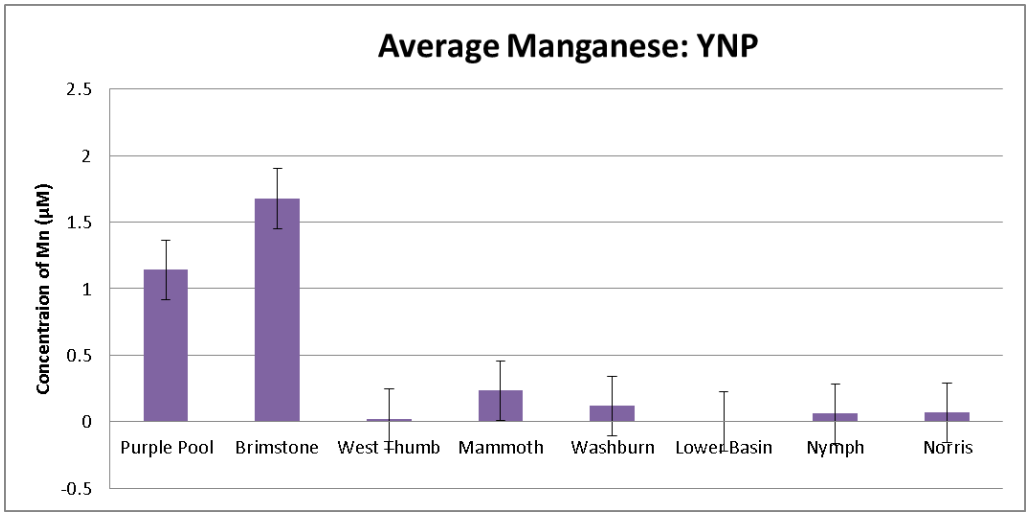


Figure 6. Graphical representation of Mn concentrations across YNP and at Purple Pool (data reported by USGS: Chaffee et al.).

Queen's Laundry Hot-spring: Mineral Templating of Silica

Queen's Laundry is a circumneutral silica-depositing hot-spring that is a member of the Sentinel Meadows Group located in the Lower Geyser Basin of YNP, USA at 44°33'48" N, 110°52'13" W (Fig. 4). The perimeter of the outflow apron is dominated by

the sheathed filamentous cyanobacterium *Calothrix*, which typically populates neutral to alkaline waters between 25 - 50°C. This gently boiling and surging hot-spring is comprised of a large deep thermal pool with two source vents discharging approximately 10 L/min of silica-saturated hydrothermal (92˚C) fluids into a narrow stream that meanders around the outflow apron creating a thermal and pH gradient (Hugo et al., 2011; Braunstein and Lowe, 1996). The outflow channel broadens into a large braided channeled apron that gently slopes, draining into marshy grasslands.

Biogeochemistry

Climatic Forcing

Climate forcing and thus climate change have a direct effect on microbial communities that return greenhouse gases to the atmosphere. For example, temperature and precipitation affect greenhouse gas production, photosynthetic productivity, microbial diversity, the supply of carbon to soils, and the structure and metabolic activity of microbial communities involved in decomposition processes and carbon release from soil (Bardgett et al., 2008). Groundwater recharge is the hydrologic process where surface water (i.e., precipitation, lotic, or stream sources) travel from the surface into groundwater aquifers. Recharge events occur both naturally (through the hydrologic cycle) and as a result of anthropogenic processes (i.e., "artificial groundwater recharge"), where rainwater and or reclaimed water is routed to the subsurface. Recharge of a karst aquifer results in an input of dissolved nutrients into the groundwater conduit system; as the excess groundwater is flushed from the aquifer the system returns to its steady state.

Iron

Iron is the most abundant transition metal in the Earth’s crust (Rudnick and Gao, 2003). In the deep crust and mantle iron exists primarily as zero valent iron, resulting in a large change in redox potential when exposed to surface oxidizing conditions. Soluble Fe(II) exists under acidic conditions and at circumneutral pHs under reducing conditions, such as in anoxic groundwater, hot-springs, stratified lakes or on early Earth when atmospheric oxygen concentrations were significantly lower than today (Parenteau et al.,

2010; Stumm and Morgan, 1996). In contrast Fe(III) is generally insoluble in oxic environments at neutral pH.

Biogeochemical cycling of Fe exploits both common oxidation states. Fe(II) may serve as an electron donor for chemotrophic growth of neutrophilic Fe oxidizing bacteria such as *Zeta-proteobacteria* and *Gallionella* (Emerson and Moyer, 1997; Hallbeck and Pedersen, 1991) and supports photosynthetic growth of purple and green phototrophs in a form of anoxygenic photosynthesis known as photoferrotrophy (Heising et al., 1999; Straub et al., 1996; Ehrenreich and Widdel, 1994a). Fe is an essential constituent of cytochromes and iron-sulfur proteins, which have a critical role in the electron transport chain for energy generation (Vuori, 1995). In contrast to Fe(II) oxidizing bacteria there are reducing counterparts, referred to as dissimilatory Fe-reducing bacteria, such as *Shewanella* spp. and *Geobacter* spp, which utilize Fe(III) as the terminal electron acceptor for respiration.

Manganese

Manganese is the second most abundant redox active transition metal in the Earths' crust, forming over 30 known Mn oxide/hydroxide minerals. Manganese exists in several oxidation states with Mn(II, III, and IV) most commonly found in nature. Due to their high reactivity Mn oxides are an important driver of environmental redox reactions strongly influencing the precipitation and dissolution of solid phase Mn(III/IV) oxides. Soluble Mn(II) is thermodynamically stable in the absence of oxygen and at low pH and may form a solid phase mineral when bound to phosphates or carbonates or as a minor constituent of other minerals (Tebo et al, 2007). The Mn(III) intermediate oxidation state is most stable in acidic solutions; however, in environmental conditions it is thermodynamically unstable, and does not accumulate unless bound to complexing ligands. Mn(IV) is thermodynamically favored in the presence of oxygen and at a high pH, forming insoluble brown/black oxides. Mn(II/III/IV) ions all form a 6-coordinate octahedron with six ligands coordinating to a central Mn ion (Tebo et al., 2004; Tebo et al, 2005). Mn oxides are commonly found in geologic formations, sediments, and in fresh water and marine systems. Mn oxides are also present in extreme environments such as

hydrothermal systems, cold seeps, and in the deep ocean at volcanically active sites (Emerson and Revsbech, 1994; Mita et al., 1994).

Mn(III/IV) oxides are highly reactive strong oxidants and are often involved in redox reactions with inorganic compounds. Reactivity of Mn oxides is a result of their negative charge, high surface area, the presence of residual Mn(III), and vacancies within mineral lattices (Tebo et al., 2004). There are two dominant morphologies among Mn minerals including chain and layered structures (Post, 1999; Fig. 7). Chain or tunnel structured Mn oxides are arranged as single, double, or triple chains with edge sharing MnO_6 octahedra. The number of chains determines tunnel diameter, surface area, hydration state and accessibility of cations. These chain-tunnel structures are stable and do not collapse or expand regardless of their hydration state. Layered Mn oxide structures are poorly crystalline, forming layers as sheets of Mn octahedra with hydrated cations between each layer, which become incorporated into the mineral structure. The spacing between the Mn layers depends on the presence and size of cations or water within each stacked layer. This structural characteristic allows layered Mn oxides to collapse when dehydrated or expand when hydrated. The ability for Mn minerals to incorporate cations into their mineral structure allows them to sequester some ions by adsorption (i.e., U(VI)) (Webb et al., 2006) and/or co-precipitation through oxidation reactions, influencing bioavailability and distribution of their ions in the environment. For example, Ca^{+}, Mg^{2+}, Ni^{2+}, Cr^{3+}, or Cu^{2+} can all be adsorbed into Mn oxides, while Fe^{2+} and As^{3+} are co-precipitated. However, some metal ions, Cr(VI), and As^{3+} are mobilized through Mn oxidation reactions. The unique ability of Mn oxides to sequester metals has led to engineering applications such as wastewater treatment, soil and sediment remediation, and metal removal and recovery, functioning as catalysts, sorbents and electrical conductors (Tebo et al., 2004; Tebo et al, 2007).

Abiotic manganese oxidation is slow in the absence of a biological catalyst with the homogenous oxidation of Mn(II) to Mn(IV) taking years. In contrast, biogenic oxidation of Mn(II) has a five-fold increase in oxidization rates (Nealson, K.H. et al., 1988; Chapnick et al., 1982). Surfaces, ligands, and other metals have varying degrees of catalytic activity for Mn(II) oxidation. Microorganisms control the redox cycling of Mn in the natural environment, where diverse bacteria and fungi have evolved the ability to

enzymatically catalyze the oxidation of Mn(II/III) to Mn(III/IV) oxides (Tebo et. al., 2007; Nealson, 2006). Other bacteria use Mn-oxides as a terminal electron acceptor during respiration under anaerobic conditions. Microorganisms that can oxidize Mn include *Pseudomonas putida*, *Erythrobacter* spp. and *Bacillus* spp., and Mn-reducing bacteria such as *Shewanella* spp. are ubiquitous in the environment (Nealson, 2006).

Mechanisms of Mn oxidation

Both *P. putida* GB-1 and *Bacillus spp*. SG-1 oxidize Mn(II) to Mn(IV) using a multicopper oxidase (MCO) in two single electron transfers, as evidenced by Mn(III)-pyrophosphate trapping experiments (Geszvain et al., 2013; Soldatova et al., 2012; Villalobos et al., 2003). *P. putida* GB-1 is an aerobe isolated from Green Bay sediments nearly 20 years ago by researchers investigating Mn oxidation. *P. putida* GB-1 oxidizes Mn(II) to Mn(III/IV) with highest activity during early stationary phase (Brouwers et al., 1999). *Bacillus spp*. SG-1 is a gram-positive marine *Firmicutes*, which sporulates during stressful conditions, such as nutrient deprivation. SG-1 Spores are capable of Mn oxidation, which occurs on the surface of the spore within the exosporium (Dick et al., 2007; Francis and Tebo, 2002; Mandernack et al., 1995).

Erythrobacter sp. SD21 oxidizes Mn(II) to Mn(III/IV) using the heme-containing Mn-peroxidase, MopA, which is secreted into the surrounding environment where it may either localize with organic polymers or become loosely associated with the cellular membrane (Anderson et al., 2009; Johnson et al., 2008). *Erythrobacter sp*. SD21 is a gram-negative strictly aerobic marine α-proteobacteria isolated from surface sediments in San Diego Bay.

Recent discoveries have identified a non-enzymatic mechanism in the formation of abiotic and biogenic Mn oxides through the generation of superoxides by microorganisms. Learman et al identified evidence that, under natural environmental conditions the oxidation of Mn(II) by superoxide can occur forming Mn oxides (Learman et al., 2013).

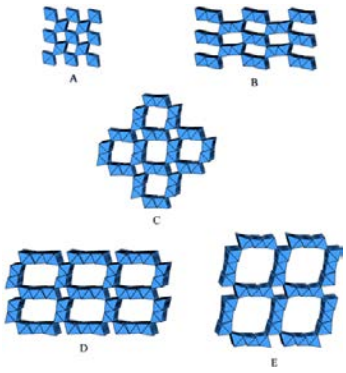


Figure 7. (Right) Examples of Mn oxide mineral structures, A) staring with the single chain tunnel pyrolusite, B) layered birnessite, C) double chain tunnel ramsdellite, and B) the triple chain tunnel todorokite. D) Triple - double chain tunnel, and E) triple chain tunnel (Post, 1999).

Silica

Silica precipitation is an important geological process in geothermal systems where supersaturated hydrothermal fluids emerge from springs leading to the formation of finely laminated siliceous sinters (Konhauser and Jones, 2011). Hydrothermal ground waters originate from deep reservoirs that are in equilibrium with quartz. When groundwater fluids emerge from springs at the surface, they undergo rapid degassing, cooling, evaporation, changes in pH, and mixing with surface waters, collectively these reactions result in enrichment of silica and subsequent super-saturation of silica in hot-springs.

Biogeochemical cycling of silica is known to occur as an active form of mineralization in diatoms, which use silica for frustule formation. However, silicification of biofilms in hot-spring environments has been thought of as a passive process in which microbial biofilms and EPS are merely surfaces for mineral nucleation. Recent studies suggest that silicification may be an active precipitation process directed by templating of minerals at reactive sites in the EPS for protection from UV damage and against predation (Hugo et al., 2011).

Carbon

There is a great need to understand the global biological processes regulating the exchange of carbon between the land, oceans, surface/subsurface waterways and the

atmosphere, to enable for better predictions as to how these processes impact Earth. Microbial communities present in terrestrial ecosystems (i.e., soils, sediments, and groundwater) play an important role in the biogeochemical cycling of carbon as a result of their ability to use simple carbon molecules (CO_2, CO, or CH_4) to synthesize more complex organic carbon molecules, thereby acting as a significant global carbon sink for inorganic carbon and source for organic carbon (Bargett et al., 2008; Bargett et al., 2005).

A majority of biogeochemically cycled inorganic carbon is carried out by autotrophic microorganisms, which convert CO_2 to organic carbon via the Calvin cycle. An important enzyme in the Calvin cycle is ribulose bisphosphate carboxylase (RuBisCO), an enzyme that is unique to autotrophs (Munn, 2004). A second method of inorganic carbon fixation is through the reverse, or reductive, TCA cycle. This cycle uses ferredoxin-like enzymes that result in the formation of acetate. Recent molecular studies have shown that the key enzyme, ATP citrate lyase, is more common than RuBisCO, in some hydrothermal ecosystems (Munn, 2004). While there are several other pathways of carbon fixation these are the two that have been found to be dominant at both Soda Bay and Purple Pool (Hügler and Sievert, 2011).

Carbon has two stable, naturally occurring isotopes: ^{12}C (98.89%) and ^{13}C (1.11%). In geochemistry, $\delta^{13}C$ denotes the isotopic signature of the ratio of the stable isotopes 13C:12C, where proportions of ^{13}C are reported in parts per thousand ‰ relative to the standard Vienna Pee Dee Belemnite, illustrating the fractionation of isotopes per sample. In hydrologic systems, carbon dioxide diffuses out of solution when the pCO_2 of the solution is greater than that of the ambient atmosphere. Under favorable conditions carbon isotopes can be used to understand the biogeochemical reactions controlling alkalinity in watersheds. In total about 112 Tg of CO_2 is biologically fixed yearly by autotrophic microorganisms, with marine microorganisms fixing the majority of inorganic carbon, followed by terrestrial microorganisms. Isotopic fractionation of carbon can be used to determine the source of CO_2 in a groundwater system as being produced biotic or abiotically and to possibly identify different groundwater reservoirs. Atmospheric $\delta^{13}C$ CO_2 has an isotopic composition of -7‰ having little influence on the ^{13}C composition of groundwater. The $\delta^{13}C$ signature from marine carbonate rocks typically has a value 0 ± 5 ‰, while those in subsurface waters are in the range of 5 -

25‰. $\delta^{13}C$ values are indicative of the origin of dissolved inorganic carbon, as negative values indicate a biotic source due to carbon fixation preferentially depleting waters of the lighter isotope of inorganic carbon. In contrast, values that are more positive indicate an abiotic carbon source as waters are enriched with carbon dioxide through dissolution of limestone.

Biogeochemical Cycling of Fe and Mn on Early Earth

The Fe and Mn cycles on modern Earth have been well studied however; we have only recently begun to elucidate the role of microorganisms in the biogeochemical cycling of these transition metals. Microorganisms participated in the cycling of these metals on early Earth, as evidenced by the presence of oxidized Fe and Mn in Precambrian Banded Iron Formations (BIFs) during globally anoxic conditions (Parenteau et al., 2010; Kappler et al., 2005; Konhauser et al., 2002; Hartman, 1984; Cloud, 1965). The origin of oxidized Fe- and Mn in Precambrian BIFs has been debated for decades, with several theories accounting for the appearance of layers, two of which propose metabolic processes of Fe oxidizing bacteria and oxygenation of the atmosphere after the evolution of oxygenic photosynthesis. The most widely accepted theory is the oxygenation of the atmosphere due to ancestral cyanobacteria and the evolution of photosystem II (Cloud 1965, 1973). Recent theories suggest the direct oxidation of Fe(II) by anoxygenic photosynthetic bacteria in the absence of oxygen or chemotrophic bacterial oxidation in the presence of low oxygen or anoxic environments (Parenteau et al., 2010; Straub et al., 1996; Hallbeck and Pedersen, 1991).

Biosignatures

Paleontological evidence demonstrating the role of microorganisms in Fe and Mn cycling on early Earth has been sought for decades. One way to better clarify the microbial role is through the characterization and identification of biosignatures and microfossils. Biosignatures may be fossil evidence for microbial life at a specific time point on early Earth, which can be used to understand not only the presence of microorganisms but also their role in the biogeochemical cycling of Fe and Mn. There are three types of biosignatures in mineralizing environments: microfossils (fossilized

microorganisms), chemofossils (biominerals and biomarkers like lipids), and organosedimentary structures (stromatolites) (Parenteau et al., 2010; Cady and Farmer, 1996). Microfossils form through homogenous or heterogeneous nucleation of nano-particulate minerals on the exterior of cellular surfaces and on EPS, resulting in encrustation, within biofilms. Eventually these biofilms become encased and then entombed. As cell walls degrade, dissolved minerals may have an opportunity to precipitate within the microorganism a process known as permineralization, perfectly preserving the cellular structure (Fortin et al., 1997; Cady and Farmer, 1996). Biomarkers are organic biosynthetic molecules indicative of various microorganisms, for example carbon skeletons or lipids (Parenteau et al., 2010). Biofilms contribute to the formation of organosedimentary structures, or biofacies, however, determining biotic from abiotic mineral formation has proven difficult (Parenteau et al., 2010; Cady and Farmer, 1999). Studies characterizing the mechanisms microorganisms use to construct these structures on modern Earth will allow us to better decipher microfossil formation and therefore, biogenic contributions to the sedimentary rock record of early Earth and on other planetary bodies.

There are environmental considerations that must be taken into account when interpreting microfossil evidence in a modern analog ecosystem. For example, ancient ecosystems undoubtedly differed from modern environments with respect to oxygen concentration, pH, and temperature, to name a few. Insight can be gained by assigning biosignature evidence to extant microorganisms, thereby inferring the physiology of ancestral microorganisms and how their metabolisms may have contributed to the alteration of early Earth. This allows us to better understand these organisms' distribution and ecological impacts on modern Earth.

Scientific Approaches Used In This Dissertation

Microscopy

Various microscopic techniques allowed for the visualization of microbial cells within biofilms, of microbe-mineral associations, microbial cell numbers, and ultra-structural features of minerals associated with cellular membranes and EPS. The use of scanning electron and transmission electron microscopy allowed high-resolution

visualization and characterization of biogenic minerals, and the identification of mineral associated with biofilms using electron diffraction and spectroscopy techniques.

Geochemistry

Geochemical measurements were taken using inductively coupled plasma mass spectroscopy (ICP-MS) to determine dissolved and particulate Fe and Mn concentrations. In addition, measurements of nitrogen and phosphorus were collected using Hach colorimetric assays and fractionation of ^{13}C was measured to determine the source of dissolved carbon dioxide.

Molecular Methods

Microbial diversity and composition was characterized using terminal restriction fragment length polymorphism (T-RFLP) and next generation ultra-high-throughput sequencing of microbial biofilms using the Illumina HiSeq platform. T-RFLP cluster analysis was used to observe spatial and temporal variability of biofilms from both field sites. Characterization of microbial composition was done using small subunit (SSU) analysis of metagenomic sequences.

Biosignatures in Modern Iron and Manganese Depositing Ecosystems

The highly mineralizing environments of modern Fe depositing springs has been recognized as a possible sedimentary analog of ancient Fe deposits much like the ironstone bodies in the Barberton greenstone belt, South Africa (Lowe and Bryerly, 2007; Parenteau et al., 2010). Much like Precambrian BIFs, modern Fe and Mn depositing springs such as Soda Bay cold-springs, in Southeast Alaska, and Purple Pool hot-spring in YNP are thought to share some environmental similarities (Fig. 8): both spring environments have a groundwater supply rich in either dissolved Fe(II) or Mn(II).

Templating of Fe oxide and silicate minerals is reported in the literature as both a passive and directed active process. Extrusion of EPS with reactive side chains is the mechanism microorganisms' use for mineral localization. For instance, Fe oxidizing microorganisms use EPS strands to actively template nano-crystalline Fe-oxide fibers in

close proximity to the cellular membrane in order to harness energy from the proton gradient (Chan et al., 2004).

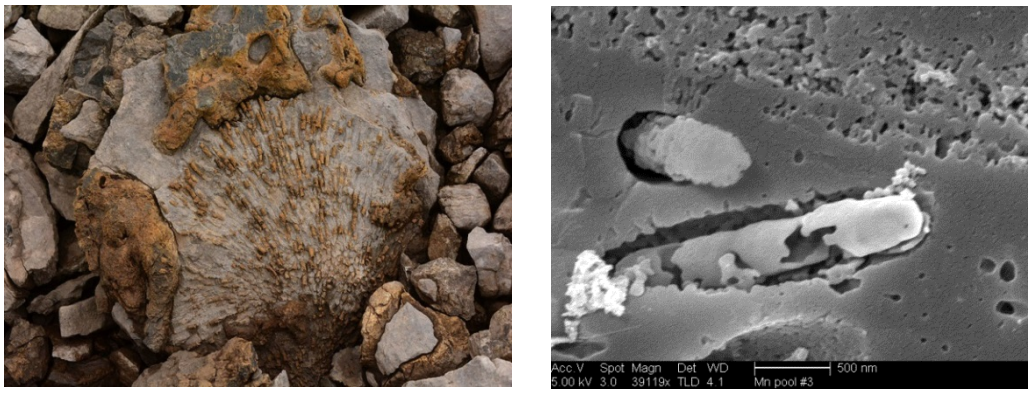


Figure 8. Examples of fossils from Soda Bay and YNP. (Left) Fossilized coral from POW Island, (Right) silica encrusted microfossils from Purple Pool, notice that the cell walls have been preserved but the cellular material inside the cell has degraded.

Geoscience Education

According to the National Science Foundation Native American/Alaska Natives are alarmingly underrepresented across STEM disciplines despite their holistic relationship with the Earth and its inhabitants. The challenge is to effectively build a Geoscience Education Program that couples Western Science with TEK to encourage the participation of Native Americans/Alaska Natives pre-college and undergraduate students in STEM research by creating bi-directional structured pathways that provide opportunities for both Native Americans and Western Scientists, bridging Traditional Ways of Knowing and Western Science perspectives. Participants in the Geoscience Education Program consisted of tribal-led collaborative research projects developed by collaborating with the Hydaburg Cooperative Association (tribal component), and a "role models" initiative working with the Hydaburg School District, in conjunction with an evaluation and dissemination component.

The Center for Coastal Margin Observation and Predictions (CMOP) at Oregon Health & Science University aims to investigate how climate and climate change impact coastal margins. One of the goals of this project was to demonstrate how the science that CMOP engages in on the Columbia River could be applied to other very different river ecosystems that are important to the survival of many indigenous peoples relying on the

health and responsible management of coastal and estuary ecosystems. Understanding climatic influences in coastal ecosystems is imperative for Alaska Native communities that depend on customary and traditional use of natural resources for physical, emotional and cultural sustenance. Community members are concerned about threats to local ecosystems posed by logging, mining, overharvesting, invasive species, fresh and marine water pollution, and climate change. This Geoscience Education Program has contributed to an increased number of high school graduates pursuing college degrees and has been welcomed by the Haida community as an integral component of cultural revitalization (Hugo et al., 2013).

Positive role models are of paramount importance in the education of Native American youths, as tribal members are raised with a strong sense of community and extended family. CMOP has in recent years conducted an experiential geoscience education program (grades 5 - 12) in Hydaburg, Alaska (Bueno Watts and Smythe, 2014) through its CMOP- School Collaboraties that has relied heavily on Native American/Alaska Native scientists as positive role models including:

- providing opportunities for participation of graduate and undergraduate students from all disciplinary backgrounds (with priority given, but not limited to, Native American students) drawn from across the National Science Foundation Science & Technology Center network and,
- creating competitive seed funding opportunities for those students to leverage their Hydaburg experience to design programs that are (a) tailored to their STEM field, (b) require their participation as role models to youth of underrepresented groups, and (c) engage the community in which they are working in culturally appropriate ways.
- teaching STEM coupled with TEK using the Haida language, oral histories and holistic concepts.

Our pedagogical approach for this project relies on a student-centered inquiry-based method emphasizing respect for traditional knowledge and culture. Student projects are driven by community-identified needs, such as river and coastal ecosystems. In this effort, we present western science integrated with TEK as a method to teach how local ecosystems have been utilized and managed in the past and how they are changing in response to natural and anthropogenic inputs. Student activities include both field and

laboratory components and include topics such as watershed, lake and oceanographic studies, bioassessments, and water chemistry experiments to name a few.

The success and longevity of this program is largely due to the inclusion of tribal elders and community members and the support and traditional education that they provide for the students. This program has proven successful for the students from the Hydaburg School District with 70% of high school graduates choosing to receive some form of higher education through college or trade school, (prior to this program college attendance was 5%). Students completing our program have the confidence to achieve higher goals and can relate their abilities to make a positive difference within their community (Fig. 9).

Figure 9. Students presenting their science fair projects at the annual Hydaburg Science Symposium.

Overview of Dissertation

In this chapter I have presented the objectives/questions addressed by my thesis and an overview of the geology of two field sites, an introduction to the biogeochemical cycling of four elements, two of which are the focus of this research, and an introduction to a collaborate STEM research program in an Alaska Native Haida community, using Geoscience education.

Chapter Two introduces and describes the concepts and processes of biological mineral templating using the model cyanobacterium *Calothrix* from the silica-depositing Queen's Laundry hot-spring located in the Lower Sentinel Meadows of YNP. Microorganisms in biofilms are surrounded in EPS, which serve a variety of functions, such as preventing desiccation, building geochemical gradients, protection from

predation, and as templates for mineral nucleation either to prevent mineralization of cell surfaces, which would result in cell death, or to accumulate necessary resources for later usage. Microorganisms have the ability to alter the composition of the reactive side chains associated with EPS, as a response to their environmental conditions, and thus influence the texture or biofabric of rock formations (Hugo et al, 2011). Here we examined the biologically influenced rock formations, or biofacies, associated with *Calothrix* dominated biofilms, using optical and scanning electron microscopy to characterize biofacies formations and fingerprint analysis and clone libraries to observe community diversity of each *Calothrix*-dominated mat. Gaining a better understating of the formation of biofacies will allow us to better interpret ancient geologic formations and the role microorganisms played in those rock formations here on Earth and other planetary bodies.

Chapter Three examines the structural variability of bacteriogenic Mn oxides from different bacterial species from which Mn oxides are produced, examining templating of minerals onto organic substrates, such as cell walls and EPS. Characterization of oxide morphology was elucidated by using electron microscopy techniques, allowing for observations of ultra-structural characterizations of microbe-mineral associations. Investigation of the structural variation of bacteriogenic Mn oxides was accomplished in the laboratory by using known model Mn oxidizing bacterial species; *Pseudomonas putida* GB-1 (Banh, 2013), *Erythrobacter* spp. SD21 (Johnson and Tebo, 2008), and *Bacillus* spp. SG-1 (Dick, 2006), all of which were grown in the presence and absence of dissolved divalent Mn(II). Bacteriogenic Mn oxides were analyzed using EM to characterize microbe-mineral associations at the cellular membrane surface and Mn oxide morphology as they varied between bacterial species. The aim was to further elucidate the mechanism of Mn oxide templating on EPS and lipopolysaccharides, cellular membranes, or other organic substrates. Knowledge gained from this research will be applied to our understanding of environmental Mn oxides and associations with microbial populations.

Chapters Four and Five of this thesis was performed by conducting field studies at two Fe and Mn oxide depositing environments; Purple Pool hot-spring, YNP Wyoming, and Soda Bay, Alaska cold-seeps. Each environment is unique in nearly all aspects with

the exception of the presence of Fe and Mn oxide deposition. Data from Chapter One will be used as a reference for microbe-mineral interactions and templating of both iron and manganese oxides from these complex environments.

Chapter Six compares and contrasts the geology, geochemistry, microbial diversity of the two metal depositing (Fe and Mn) environments, possible future research and directions are discussed.

Chapter Seven describes efforts to increase and encourage Alaska Native students to participate in STEM disciplines, by coupling western science with TEK.

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Chapter 2

Silica Biomineralization Of *Calothrix*-Dominated Biofacies From Queen's Laundry Hot-Spring, Yellowstone National Park, USA

Abstract

Experiments on microorganisms capable of surviving silicification are often conducted to gain a better understanding of the process of silica biomineralization and to gain insights into microbially influenced rock formations and biofabrics like those found in ancient deposits such as the Devonian Rhynie Chert formation. An ideal microorganism for studying silicification is the large sheathed cyanobacterium *Calothrix*, which forms distinctive organo-sedimentary structures or biofacies in the low to moderate temperature regions of hydrothermal springs or columnar stromatolitic structures in aquatic systems. Identifying and characterizing microfossils from ancient cherts allows us to gain a better understanding of environmental conditions and microorganisms present on early Earth as genetic material in ancient deposits is absent due to degradation. This knowledge also provides insight for identifying microfossils on other planetary bodies, such as Mars.

In this study we characterized *Calothrix* biofacies along the outflow apron of Queen's Laundry Hot-Spring in Yellowstone National Park using microscopy and molecular techniques to examine biofacies morphology and phylogenetic diversity. Flow regime has a profound effect on biofacies architecture and temperature along the outflow apron influenced community composition as identified by the observation of five distinct clusters of *Calothrix*-dominated microbial communities, cluster analysis using confirms that biofacies were *Calothrix* dominated.

Introduction

Precambrian cherts typically yield silicified microfossils suggesting that ancient microbial communities present in marine waters and hydrothermal ecosystems became embedded in colloidal amorphous silica and were subsequently entombed and preserved in the fossil record as distinct textures (Westall et al., 1995). Characterization of extant hot-spring environments provides us the opportunity to better understand and interpret

the paleoenvironment in which ancient biofacies formed, allowing us to take a glimpse into Earth's early environments.

The geology of Yellowstone National Park (YNP) strongly influences hydrothermal fluids as meteoric waters interact at depth with acid intermediate volcanic rock, which is high in silica content, thereby enriching hydrothermal fluids with dissolved minerals, and supersaturating them with dissolved silica (Channing and Butler, 2007). Dissolved monomeric silica is precipitated and deposited after the solubility of amorphous silica has been exceeded, e.g., through changes in pH or temperature and super saturation, resulting in the polymerization of monomeric silica forming colloidal silica (White et al., 1956). Silica deposits, referred to as sinter, form rapidly as silica saturated hydrothermal fluids erupt from depth due to evaporation and cooling at the surface. These siliceous deposits in YNP are studied as extant analogues of a period of time reminiscent of early Earth allowing for the study of biogenic silica deposition and microfossil formation (Channing and Butler, 2007).

The term biofacies describes an actively growing microbe-mineral assemblage in which mineral deposition exhibits a specific and unique suite of biological characteristics due to nucleation of minerals onto microbial biofilm/mat surfaces. In contrast lithofacies is a geologic formation displaying biological characteristics, microfossils or chemical signatures that is no longer actively forming. Mineralized *Calothrix*-dominated mats heavily influence the fabrics of low- to mid-temperature siliceous sinters forming distinctive hot-spring-associated rock formations (Jones et al., 2001; Konhauser et al., 2001; Cady and Farmer, 1996; Walter, 1972). The distinctive *Calothrix* biofacies have been instrumental in the interpretation of hot-spring paleoenvironments (Hinman and Walter 2005; Jones et al., 2003; Blank et al., 2002; Campbell et al., 2001; Hugo et al., 2011; Walter et al., 1996). Microbial populations in Queen's Laundry Hot-Spring are exposed to circumneutral geothermal groundwater fluids that are saturated with dissolved silica resulting in a series of abiotic silicification reactions (Benning et al., 2004; Cady and Farmer, 1996). Silica-rich hydrothermal fluids brought to the surface from depth are further enriched in nano-particulate amorphous silica colloids in liquid phase due to evaporation and rapid cooling of spring fluids. Microfossils form as silica minerals nucleate on cell wells and in exopolysaccharides, completely impregnating organic

material; this must occur rapidly for cell and mat morphology to remain intact (Chan et al., 2009; White et al., 1956).

Siliceous sinter deposits form around the perimeter of the hot-spring at the air-water interface where rapid cooling and subsequent evaporation of hydrothermal fluids cause dissolved minerals to precipitate rapidly (Fig. 1) (Hugo et al., 2011; Benning et al., 2005; Rimstidt and Barnes, 1980). Previous studies have shown that *Calothrix* microstructures identified in sinter deposits of extinct hot-springs can be attributed to microbial communities that once thrived prior to hot-springs death suggesting that these microorganisms directly influence the biofabric of sinter deposits in extant hot-springs (Jones et al., 2001; Konhauser et al., 2001; Cady and Farmer, 1996; Walter, 1972). Silicification of *Calothrix* is thought to be a passive process due to the rapid and extreme changes in fluid chemistry gradients from the deep source vent(s) to the shallow outflow apron (Konhauser et al., 2003).

Figure 1. Field photo of the edge of Queen's Laundry Hot-spring illustrating the formation of white siliceous sinter on the perimeter of the spring.

Calothrix are ubiquitous in marine, freshwater, and terrestrial environments. However, little is known about their genetic diversity (Sihvonen et al., 2007). The filamentous cyanobacterium *Calothrix* has been observed to occupy the upper 1-2 mm of microbial mats formed in silica-dominated hot-springs across Yellowstone National Park. *Rivulariaceae* cyanobacteria such as *Calothrix* are morphologically complex possessing specific characteristics that make them unique. This includes tapering trichomes and

terminal heterocysts which are enclosed in a thick fibrillar sheath of microfibrullar, capillaceous structures of exopolysacchaide (EPS) and lipopolysaccharides (LPS) forming large, branched molecules that are insoluble in water making them resistant to dehydration (Hugo et al., 2011; Whitton, 2002; Hoiczyk., 1998; Westall et al., 1995). The multilayered exterior presents mechanical and permeability obstacles for larger molecules making it necessary for alternative transport mechanisms, such as porins, ATP-binding cassette transporters, "Bayer Bridges," and junctional pore complexes. These alternative transport mechanisms may contribute to the silicification of cyanobacteria in hot-spring environments thereby allowing for the formation and preservation of microfossils (Hoiczyk and Hansel., 2000). Monomeric dissolved silica is capable of passing through the outer sheath and accumulating in the periplasmic space. Polymerization occurs as dissolved silica concentrations increase trapping amorphous silica in the periplasmic space which eventually leads to cell death and the formation of a microfossil. The growth pattern of these microorganisms directly influences the texture of rock formations in and around the hot-springs (Cady and Farmer, 1996).

Algal and bacterial biofacies composed of amorphous silica are known to occur around hot-spring environments in YNP. *Calothrix*-dominated mats form stromatolitic textured sinters that are crudely laminated with wavy surface features formed from small domes (pustular mats) or clusters of contiguous domes (terracette ridges) rather than forming the more commonly recognized columnar domes. These biofacies occur in moderate temperature < 30ºC low flow regimes (Walter et al., 1972).

The actively growing surface of the *Calothrix* mat becomes encrusted with silica minerals so that filaments are completely entombed in a silica matrix deeper in the mat. The rate of mineralization is dependent on several environmental parameters such as pH, silica activity, temperature, and presence of water (Braunstein and Lowe, 1996; Walter, 1996; Iler, 1979). Filaments are oriented in the direction of water flow and become fossilized in their orientation due to rapid silica mineralization entombing filaments and EPS making them appear as bundles of filaments. Once completely entombed in silica minerals the sheath is difficult to identify within the rock matrix. Silicified sheaths are more common than the internal trichome as the sheath is more resilient to degradation (Fig. 2) (Jones et al., 2001).

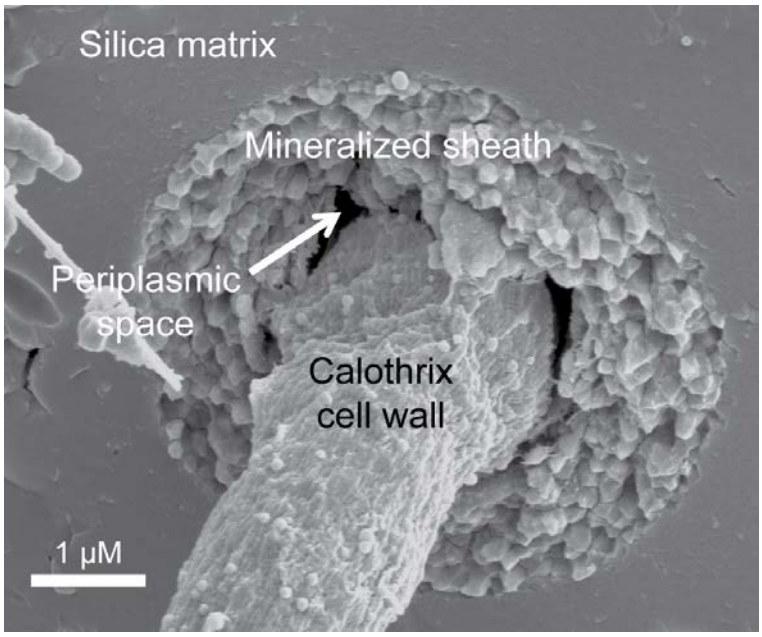

Figure 2. Scanning electron micrograph of entombed *Calothrix* filament. The sheath of the filament is completely mineralized with amorphous silica allowing the cell to remain intact and preserved.

Here we describe the characterization of the biofacies and the microbial communities from three distinct biofacies collected from Queen's Laundry Hot-Spring in Yellowstone National Park. Three distinct *Calothrix*-dominated microbial mats were characterized from specimens collected along the temperature gradient of the outflow apron. This was done as a means to better understand the effects temperature and fluid dynamics have on community diversity and the morphology of each distinct biofacies, and to characterize the process of microfossil formation and preservation in the rock record. Biofacies characterized were: 1) nodular mats forming in shallow pools of thermal fluid located at the top and bottom of the outflow apron; temperatures ~25°C (Fig. 3C), 2) stratiform terracette ridges forming along the length of the outflow apron and wetted by a thin sheet of flowing fluid; temperatures from 50°C at the top closest to the source vent to 25°C at the bottom (Fig. 3B) and 3) pustular mats formed along the length of the outflow apron in shallow gently flowing thermal pools that form between stratiform terracette ridges; temperature of 25°C-50°C from top to bottom of the outflow apron (Fig. 3A).

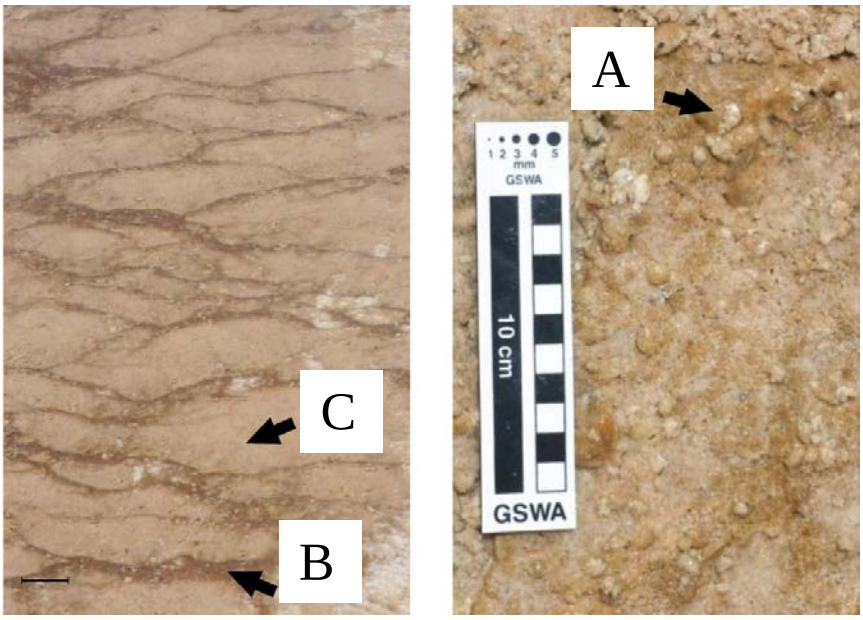

Figure 3. Photo demonstrating three distinct *Calothrix* biofacies found at Queen's Laundry hot-spring. A). Nodular Calothrix mats form adjacent to the outflow channel in shallow pools. B) Stratiform terracette mats form on ridges of micro-terraces along the perimeter of the outflow apron. C) Shallow pools between terracette ridges are where pustular mats form.

Field Site Description

Queen's Laundry is a member of the Sentinel Meadows Group located in the Lower Geyser Basin of Yellowstone National Park, USA at 44°33'48" N, 110°52'13" W (Fig. 4). The perimeter of the outflow apron is dominated by the sheathed filamentous cyanobacterium *Calothrix*, which typically populates neutral to alkaline waters between 25-50°C. This gently boiling and surging hot-spring is comprised of a large deep thermal pool with two source vents discharging approximately 10 L/min of silica-saturated hydrothermal (92˚C) fluids into a narrow stream that meanders around the outflow apron creating a thermal and pH gradient (Hugo et al., 2011; Braunstein and Lowe, 1996). The outflow channel broadens into a large braided channeled apron that gently slopes draining into marshy grasslands (Fig.5).

Figure 4. Map of YNP, Queen's Laundry is located southwest of Madison Junction.

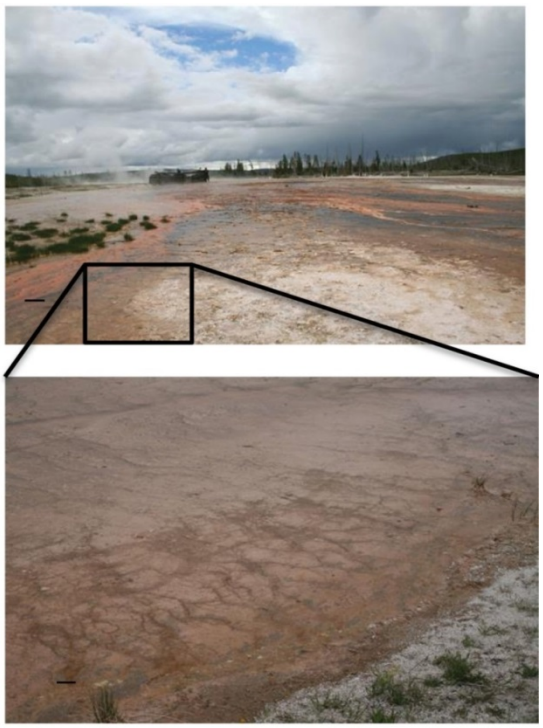

Figure 5. (Top) Overview photo of the outflow channel, looking up from the bottom of the channel. (Bottom) Close-up of *Calothrix* biofacies on the perimeter of the channel, illustrating pustular and terracette biofacies.

Materials and Methods

Sample Collection and Preparation

Sinter and microbial mat specimens (1 cm x 1cm) were collected and immediately fixed in a 3% gluteraldehyde solution of 0.2 µm filtered spring water for microscopy. Samples collected for molecular analyses were fixed in RNALater (Life Technologies,

Grand Island, NY). All samples were stored at 4°C while in the field. Prior to microscopic examination specimens were rinsed twice in 0.1 M cacodylate buffer to remove fixative. *Calothrix* biofacies were examined macroscopically and microscopically characterizing gross morphology of intact mats and the morphology of individual cells.

Microscopy Methods

Stereomicroscope Analysis

Whole intact *Calothrix* mats were examined using a Nikon SMZ800 stereoscope; digital images were acquired using a Leica DFC digital camera. Specimens were first examined intact to characterize morphology, surface features, and growth orientation of filaments. Specimens were then sectioned lengthwise with a scalpel and analyzed in cross-section to characterize the interior of the mat.

Optical Light Microscopy

Highly mineralized *Calothrix* mats required removal of mineral rinds in order to observe cellular characteristics using the optical light microscope (OLM). Specimens were prepared for observation by first removing the silica rind encasing filaments and then by removing the top actively growing portion of the *Calothrix* mat. Sonication of mineralized mats was an effective method of loosening and removing silica rinds that encrusted sheathed filaments. Densely packed filaments cemented in mineral matrices were loosened with the removal of minerals from the sheaths with increasing sonication time. After each 30-second sonication interval the overall mat structure was examined to characterize the integrity of *Calothrix* filaments using 20X objectives under phase contrast using a Leica DMRX optical light microscope and images were acquired with an Apogee CCD camera.

Scanning Electron Microscopy

Calothrix mats from each biofacies were examined using a FEI Siron high-resolution scanning electron microscope (HR-SEM) in which samples were prepared utilizing two dehydration techniques: 1) extensive chemical fixation with osmium tetroxide and dehydration with a graded ethanol series followed by critical point drying,

or 2) chemically fixed with a 2% gluteraldehyde solution rinsed twice in 0.1 M cacodylate buffer and air-dried in a desiccator. Samples prepared using the first fixation technique were rinsed twice in 0.1 M cacodylate buffer soaking for 10 minutes each, post-fixed using a 1% osmium tetroxide solution prepared in 0.1 M cacodylate buffer for 1 hour, after which samples were rinsed twice in 0.1 M cacodylate buffer, dehydrated using a graded ethanol series (50, 70, 90, and 100), and then critical point dried. Dehydrated specimens were mounted on aluminum pins and coated with 100Å Au-Pd.

Genomic DNA Extraction

Samples collected on site were fixed in RNALater (Life Technologies, Grand Island, NY) and stored at 4˚C before being transported back to the lab and frozen at -80˚C. Genomic DNA (gDNA) was extracted from samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer's protocol with the modification that the gDNA was eluted into 10 mM Tris at pH 8. The purity and concentration of gDNA were determined with a Nanodrop ND-1000 spectrophotometer and diluted to ~10 ng/µL for downstream molecular applications.

Community Diversity

Community diversity of Calothrix biofacies was analyzed using terminal restriction fragment length polymorphism (T-RFLP) providing a fingerprint of the community for each sample. The methodology used for T-RFLP analysis has been reported in detail in previous publications (Fleming et al., 2013; Rassa et al., 2009; Davis and Moyer, 2008). This technique accurately resolves populations in microbial communities of low to intermediate richness (Engebretson and Moyer, 2003). Electropherograms are imported into the program BioNumerics (Applied Maths, Austin, TX) where community fingerprints are best-compared using average Pearson product moment correlation (Häne et al., 1993). Community fingerprints were compared in the 50-500 bp range average Pearson product moment correlation and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis combining all eight restriction digests (Davis and Moyer, 2008). The primer set used was 68F-FAM (5' 6-FAM - TdNA dNAC ATG CAA GTC GdK dK CG 3') and 1492R (5' dKGdP TAC CTT GTT ACG

ACT T 3') with identical conditions as previously reported (Rassa et al., 2009). Three replicate PCR reactions were pooled, desalted, and split between eight restriction enzyme treatments using *Alu*I, *Bst*UI, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Msp*I, and *Rsa*I (New England BioLabs, Ipswich, MA). Reactions were visualized with an internal LIZ-500 size standard by capillary electrophoresis on an ABI 3130xl genetic analyzer (50-cm capillary array, POP-6; Life Technologies, Grand Island, NY).

Clone Library

Five replicate SSU rRNA gene PCR reactions were pooled and cleaned with the modification that the forward primer did not contain a 5' fluorescent label. Desalted amplicons were cloned with a CloneJET PCR Cloning Kit following manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). All clones were streaked to isolation and assayed for the correct size fragment using PCR with pJET1.2 forward and reverse primers. Clones were then grown up in Terrific Broth with 100 µg mL-1 ampicillin and were sequenced at Beckman Coulter Genomics (Danvers, MA). Operational taxonomic unit (OTU) analysis was initially determined with 5' reads of the SSU rRNA gene (Tartof and Hobbs, 1987). Sequences were aligned using the ARB-SILVA database with SINA Webaligner (Pruesse et al., 2007), masked, and binned into OTUs based on 97% minimum similarity. At least one clone from each OTU was chosen for full-length sequencing and checked for chimeras using Pintail (Ashelford et al., 2005) and Mallard (Ashelford et al., 2006). Using unambiguously aligned full-length sequences; phylogenetic placements according to maximum likelihood methods were calculated using RAxML version 7.2.6 (Stamatakis, 2006) with the General Time Reversible (GTR) model of nucleotide substitution, optimized substitution rates, and GAMMA model of rate heterogeneity, bootstrap values were calculated using a 1000 replicates.

Estimating Percent *Calothrix* Using T-RFs

Relative proportions of *Calothrix* from each of the three biofacies were calculated using restriction maps from the T-RFLP restriction enzymes. Estimates of community composition were generated *in silico* from *Calothrix* and cyanobacterial sequences found

from clone library analysis. Upon examining the eight restriction enzymes we found that restriction enzyme *Bst*UI had the most specific identifier signature for *Calothrix* by comparison of the terminal-restriction fragments (T-RFs). Comparison of T-RFs showed that peaks at 57 base pairs were shared with the least amount of other cyanobacteria from the *Calothrix* biofacies. To estimate the percent *Calothrix* within the community all bands between 50-500 base pairs were counted for the *Bst*UI digests. The height of the band representing the 57 base pair *Calothrix* T-RF was divided by the sum of the heights from all the bands in the electropherogram above a 3% background relative fluorescence maximum.

Sonication Experiments

Silica encrusted *Calothrix* filaments and associated EPS were sonicated in order to remove mineral rinds. Prior to sonication *Calothrix* mats exhibited a hirsute appearance from mineralization of the EPS around the filaments. Sonication at 30 sec intervals were increasingly effective until 200 sec, when cells began to lyse (Fig. 6).

Table 1. Summary of sonication experiments of intact *Calothrix* mats at various sonication intervals.

Time Point	Mat Description
T0 No sonication	Solid nodular mat, with filaments vertically oriented. In cross-section the top of the mat is pigmented brown transitioning to white silica encrusted filaments that have a 1mm mineral rind.
T1 30 sec	Mineral rind loosens with some large grains separated from the sheaths, minor fractures form, however the mat remains intact.
T2 60 sec	Mineral rind around the sheaths begins to separate from sheaths and large fractures form. Oxygen bubbles trapped within the mat begin to escape.
T3 90 sec	Some filaments begin to lyse, mineral rind continues to loosen and detach from sheaths. Mat has lost about a quarter of its mass from sonication.
T4 120 sec	Filaments are no longer cemented together, silica continues to fracture, moderate cell damage. Mats remain intact with mineral grains continuing to become dislodged and oxygen bubbles continue to escape from the core of the mat.
T5 150 sec	Filaments begin showing signs of considerable damage. The mat has lost about half of its original mass from sonication, there is a compact siliceous core left intact, oxygen bubbles continue to degas.
T6 180 sec	EPS has lost a significant amount of mineral grains; rinds around cells broken and fractured, cell damage evident. Siliceous core begins to break apart.
T7 210 sec	Solution that cells are suspended has an increase in viscosity due to rupture of cells; the cells are highly fractured with minerals grains dispersed in solution. The core is breaking down; the cells have lost enough of the mineral rind for OLM analysis.

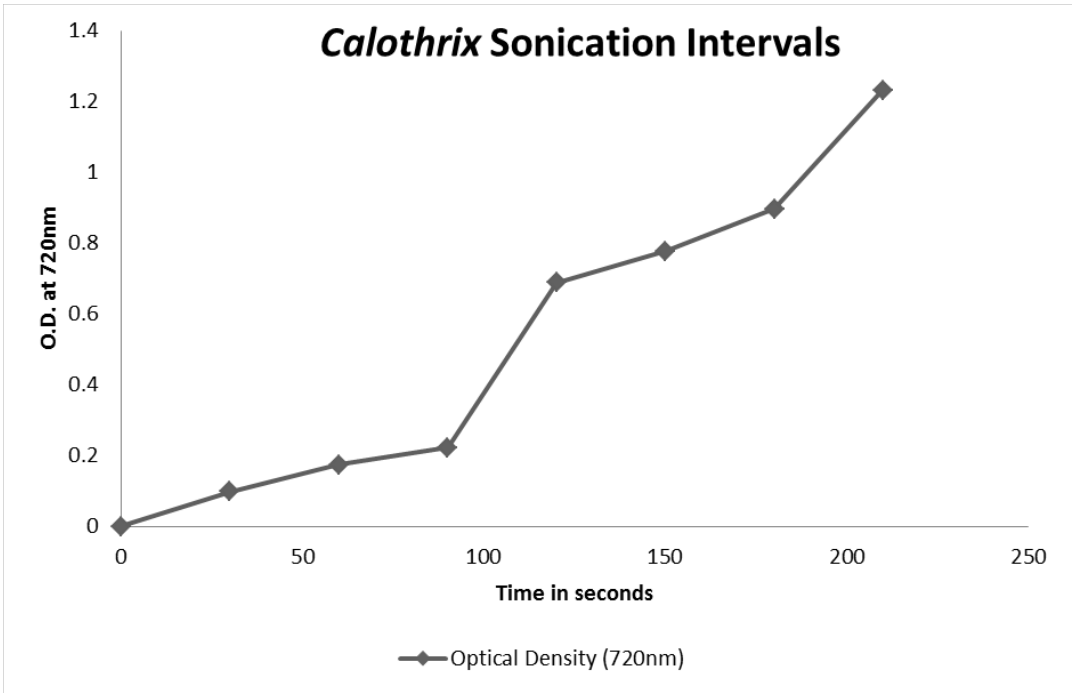

Figure 6. Graph illustrating the loosening and dislocation of silica minerals from Calothrix mats as a function of sonication time. Samples were sonicated at 30-sec intervals, with cell lysis occurring after 200 sec of sonication.

Results

We identified three distinct stages of silicification using microscopic techniques: 1) encrustation, when the sheath of individual filaments is overlain with fragile silica minerals that easily fracture and dissociate when manipulated (Fig. 7A-B); 2) encasement, when cells have a rind of silica minerals forming around individual filaments and EPS developing a thick, tough semisolid outer mineral rind (Fig. 7C); and 3) entombment, of individual filaments occurs when cells are cemented together in a solid silica matrix (Fig. 7D).

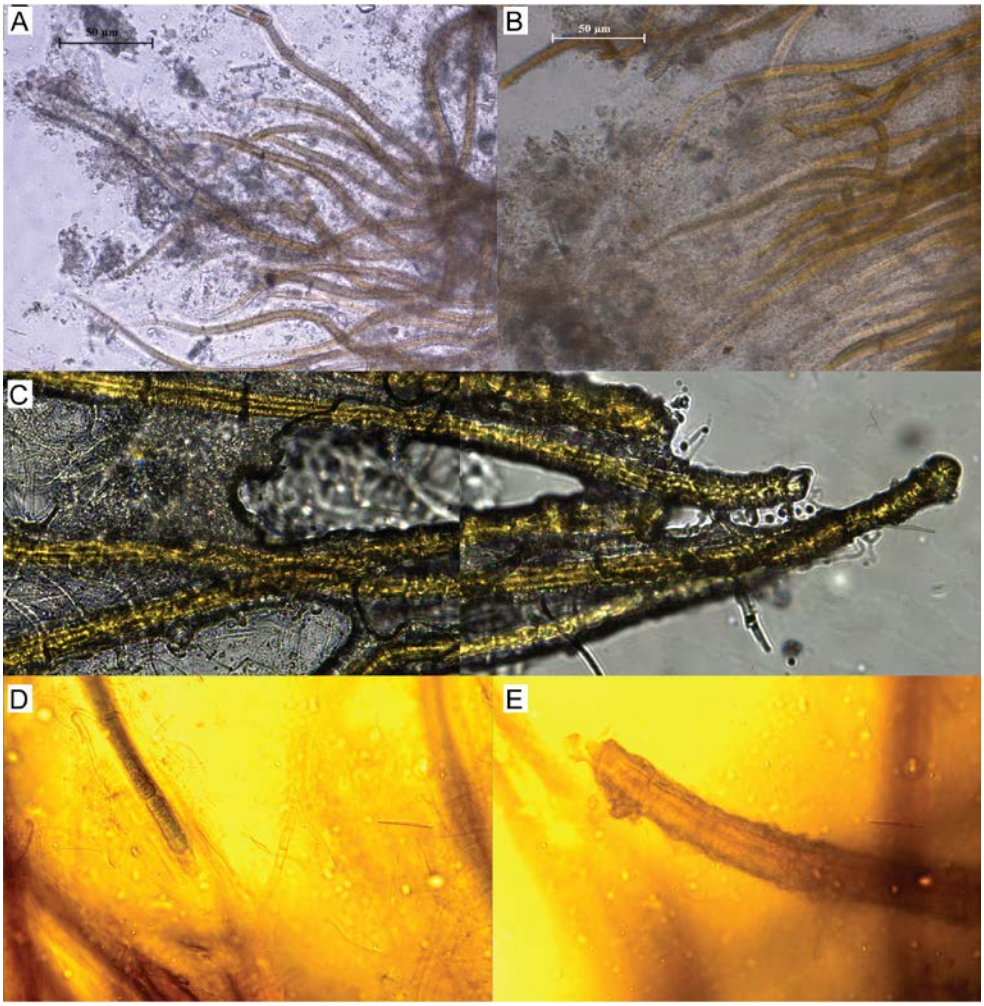

Figure 7. Optical microscope images illustrating the various stages of silicification. A. and B. illustrate primary (A) and advanced (B) encrustation. C) Filaments are cemented together in a solid silica matrix illustrating encasement. D. and E. illustrate entombment, as filaments are not only encrusted in silica but entombed in a solid silica matrix.

Nodular *Calothrix* Biofacies

Nodular *Calothrix* mats formed loose, spherical nodules on the bottom of shallow pools. Nodular mats were pigmented dark green to brown. The dark coloration is due to the presence of the pigment scytonemin in the sheath and is typically found to be synthesized in environments where cyanobacteria are exposed to intense light and UV irradiation (Ehling-Schulz et al., 1997; Dillon and Castenholtz, 2003). Filaments in the nodular mats were oriented vertically from the base of the mat fanning out towards the surface of the mat which was encased in a semi-solid EPS-mineral matrix of amorphous silica (Fig. 8A). Nodular mats were found growing in pooled water around 40-50°C.

Filaments at the surface of the mat were encrusted with silica colloids, filaments in the center of the mat had accumulated more silica in its EPS with the outer sheath becoming semi-solid, and filaments at the base of the mat were entombed in amorphous silica with the extensive outer sheath becoming completely fossilized (Fig. 8 B-D respectively)

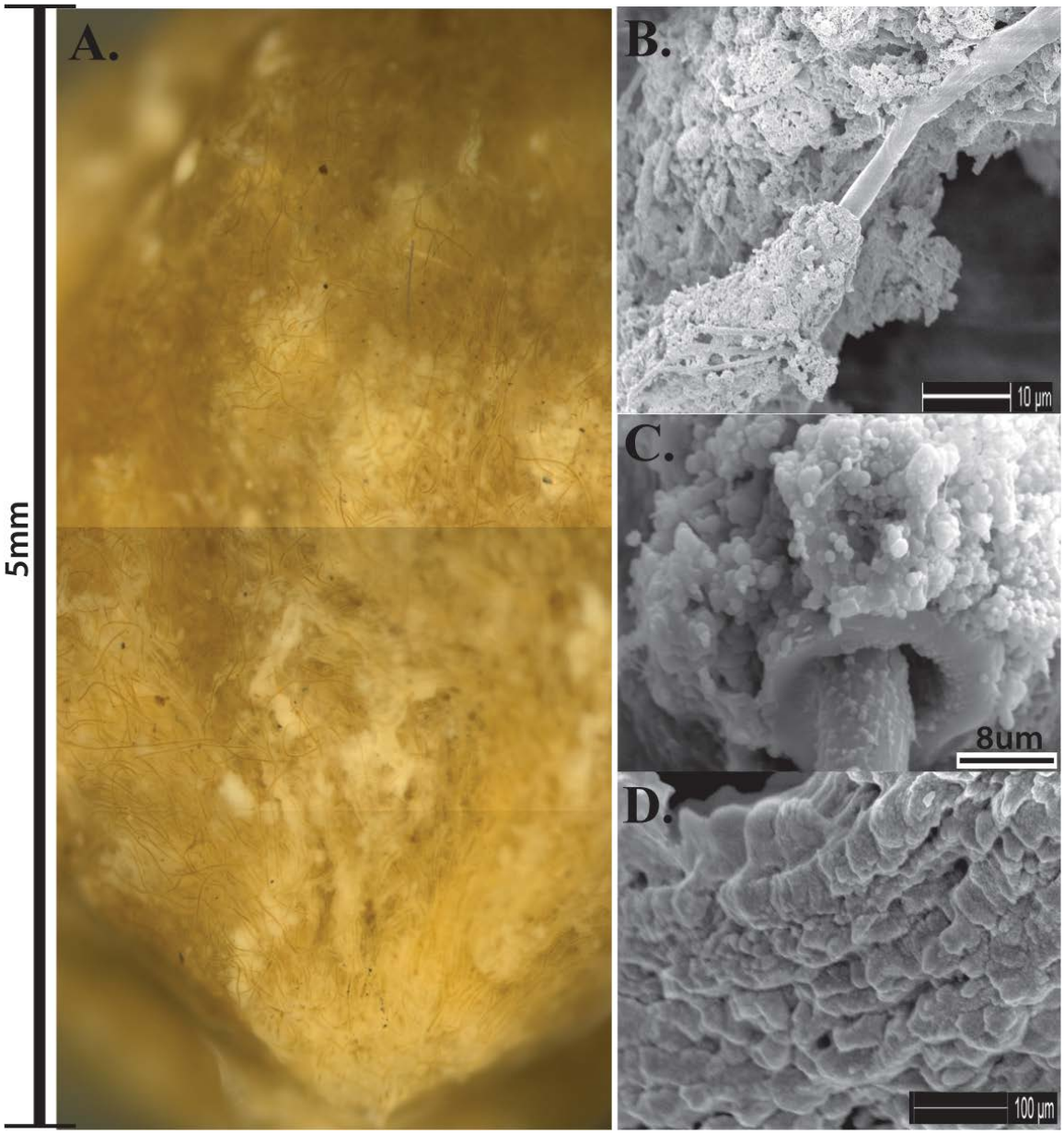

Figure 8. A) Cross-section of *Calothrix* nodular mat, scale bar to the left shows the average width of the mats. The bottom of the mat is highly silicified. Notice filaments radiating from the center of the mat. B) SEM of *Calothrix* filament from the top of the mat having very little silica deposition. C) SEM of *Calothrix* filament from the mat center, the filament is heavily encrusted with amorphous silica. D) SEM of silicified sheath from the mat bottom. Cells have degraded with just the mineralized sheath remaining.

Pustular *Calothrix* Biofacies

Pustular *Calothrix* mats formed attached to the bottom of shallow terracette pools along the length of the outflow apron. Mats had an irregular morphology sitting atop a semi-solid core of compacted silica grains, in which filaments grow radially from the core. Pustular mats were dark brown-green pigmented, forming an extensive mat along the bottom of pools (Fig. 9A). *Calothrix* filaments were cemented together in a semi-solid EPS-silica matrix, with the tapered ends of the filament anchored in the underlying sinter and the filament body and terminal heterocyst directed outwards. The surface of the mat had a hirsute appearance from protruding filaments (Fig. 9B). The mat was comprised of alternating layers of *Calothrix* filaments and amorphous silica, with layers alternating between the tapered and broad ends of the filament (Fig. 9C). Sinter at the base of the mat contained completely entombed intact filaments that were oriented vertically throughout the mat perpendicular to the attachment site at the base of the structure (Fig. 9D).

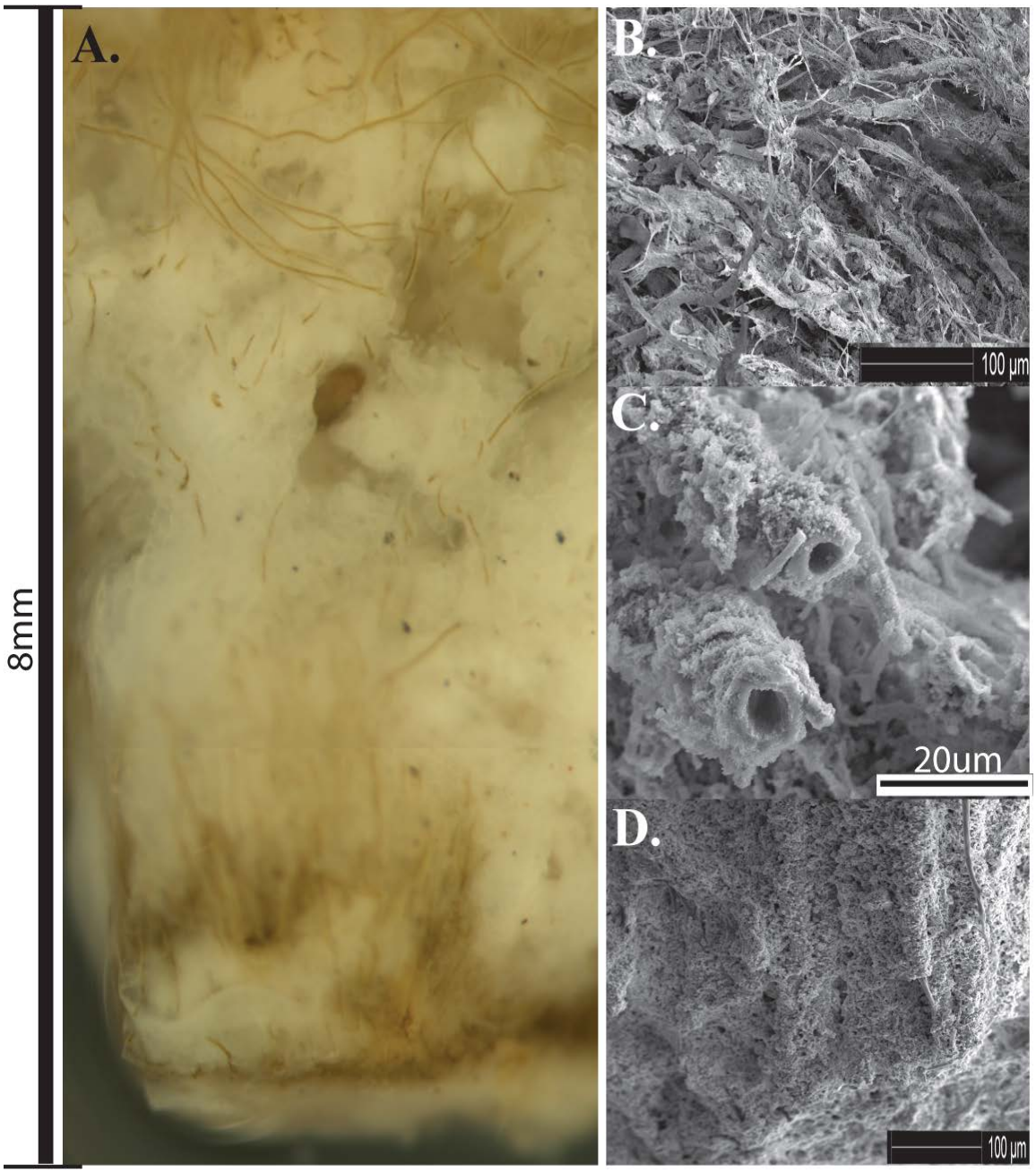

Figure 9. A) Cross-section of *Calothrix* pustular mat. The bottom of the mat is highly mineralized with silica. B) SEM of silica encrusted *Calothrix* filaments and EPS from the top of the mat. C) SEM of filaments from the middle of the mat, filaments are heavily encrusted and encased in amorphous silica. D) SEM of filaments cemented together forming a solid biofacies.

Stratiform *Calothrix* Biofacies

Stratiform terracette mats formed slightly raised ridges that formed a continuous meandering narrow mat across the width of the ridge. Mats form along the length of the outflow apron over a broad temperature range, from 50-25°C. Microscopic observations of cross-sections through terracette mats exposed interwoven layers of filaments oriented vertical to subvertical (Fig. 10A). *Calothrix* filaments exhibited tan to dark brown pigmentation with the actively growing upper portion of the mat overlain with amorphous silica, the second layer was comprised of amorphous silica, under which filaments from the previous summer were entombed in a solid silica matrix at the base of the mat. Filaments at the surface were heavily encrusted with the mat becoming encased in a solid silica matrix rapidly (Fig. 10B – C). Sheaths were fossilized at the base of the terracette structure (Fig. 10 D).

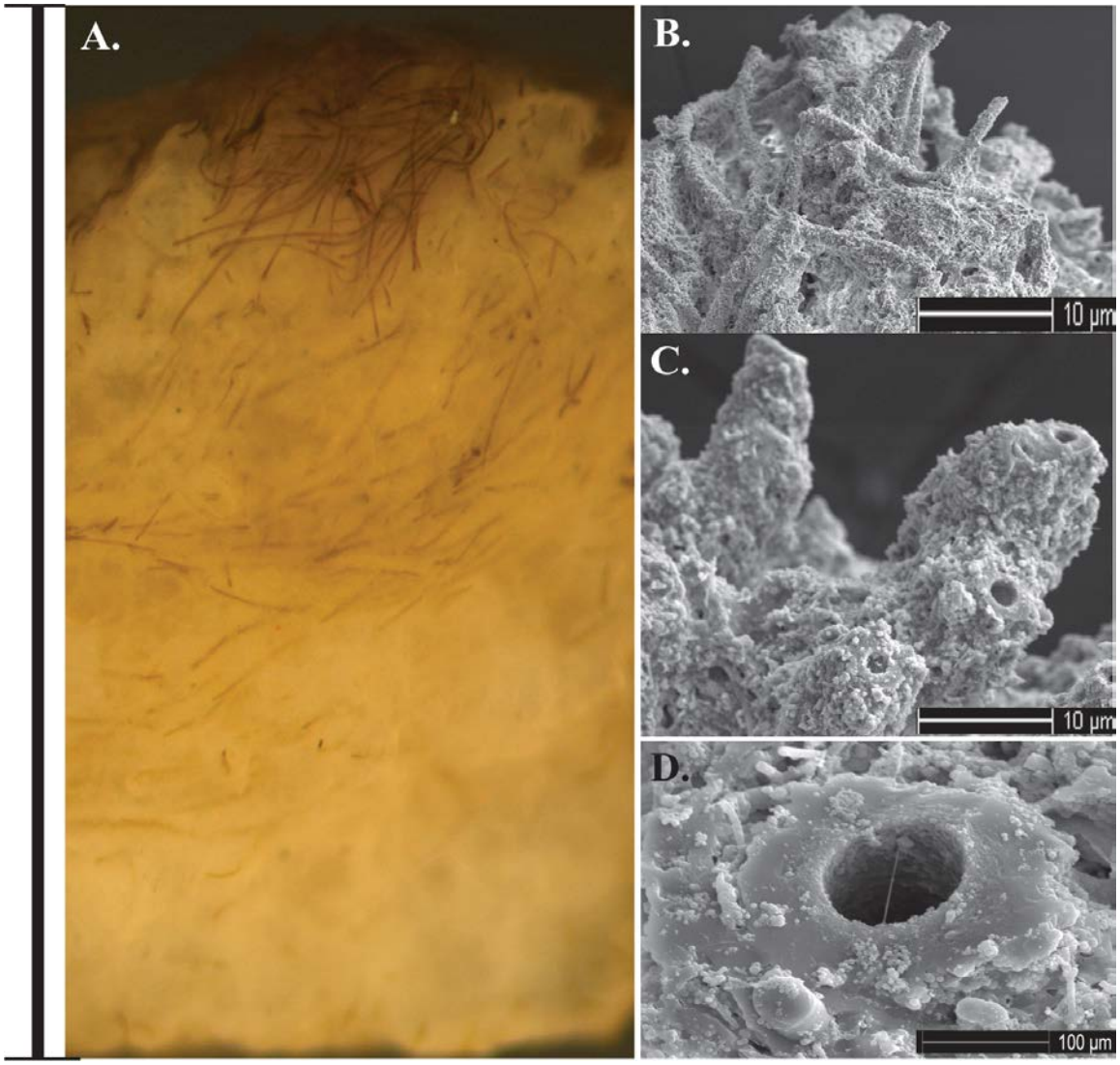

Figure 10. A) Cross-section of *Calothrix* terracette mat. Most of the mat is heavily mineralized with amorphous silica with a thin layer of un-mineralized filaments on the surface. B) SEM of *Calothrix* filaments from the top of the mat, filaments are heavily encrusted with amorphous silica. C) SEM of filaments becoming entombed in a solid silica matrix. C) SEM of a preserved sheath from the mat bottom, cellular material has degraded with just the sheath remaining.

Community Analyses

Clone library and T-RFLP analyses confirmed the presence of *Calothrix* as the dominant bacterial species (or operational taxonomic unit, OTU) in these biofacies samples, making up 46.7% of the community. The next most abundant species was an unclassified cyanobacterium at 11.7% of the community. The phylogenetic placement of the detected *Calothrix* fell within cultivated representatives from the Baltic Sea (98%

similarity; Sihvonen et al., 2007) and an uncultivated cyanobacterium clone from microbialite structures in an alkaline lake (Couradeau et al., 2011). Further, the unclassified cyanobacterium was most closely related (Fig. 11) to an uncultivated clone from a hot-spring in China, with the closest cultivated relative within the GpIV genus of the Cyanobacteria (92% similar to *Halomicronema* spp.). The majority of the remaining members of the community were heterotrophic lineages within the *Bacteroidetes*.

Grouping of biofacies samples by T-RFLP fingerprints showed clustering based primarily by temperature regime and then by biofacies type and the temperature at which these samples were collected (Fig. 12). Clustering of samples was also largely dependent on changes in the relative abundance of secondary heterotrophic microbial communities in agreement with observations made via microscopy. This is seen as a decline in *Calothrix* relative abundance (%) with temperature in Figure 12. Biofacies samples ranged from 75.9% to 8.5% *Calothrix* composition with a mean across all sample sites of 42.7% (Fig.12, Table 1). Terracette biofacies clustered into three communities based on different sampling temperature niches (high 34˚C, middle 30˚C, and low 22˚C). With three clusters of stratiform terracette communities, the diversity of the biofacies was greater than the other two biofacies (55% vs. 83% minimum similarity, respectively).

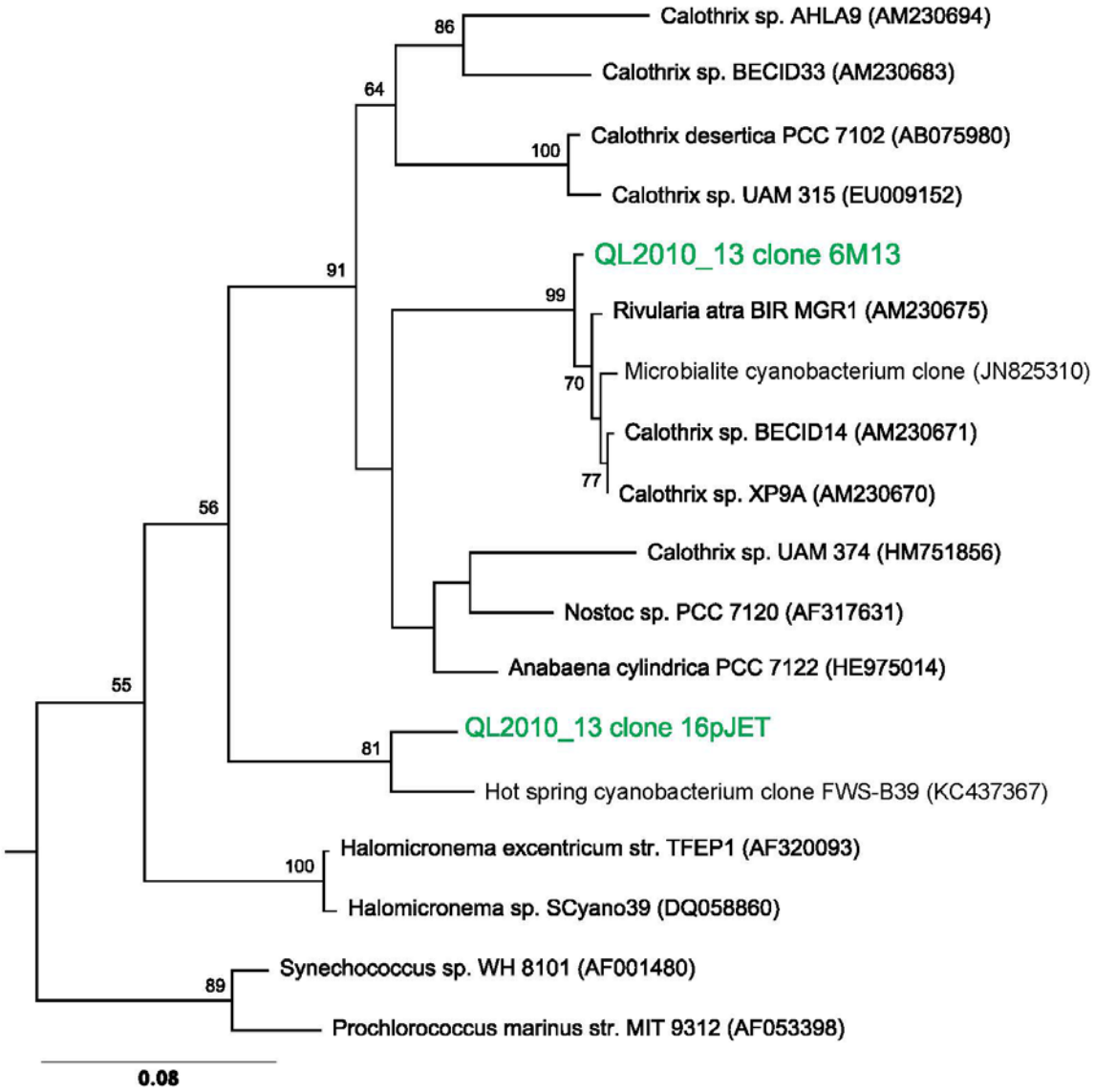

Figure 11. Maximum likelihood phylogenetic tree showing the placement of the two most abundant OTUs from a nodular biofacies (QL2010_13). *Calothrix* clone 6M13 made up 47% of the bacterial community, with the most abundant clone, 16pJET (12%), being an unclassified cyanobacteria. The tree is rooted using *Aquifex pyrophilu*s.

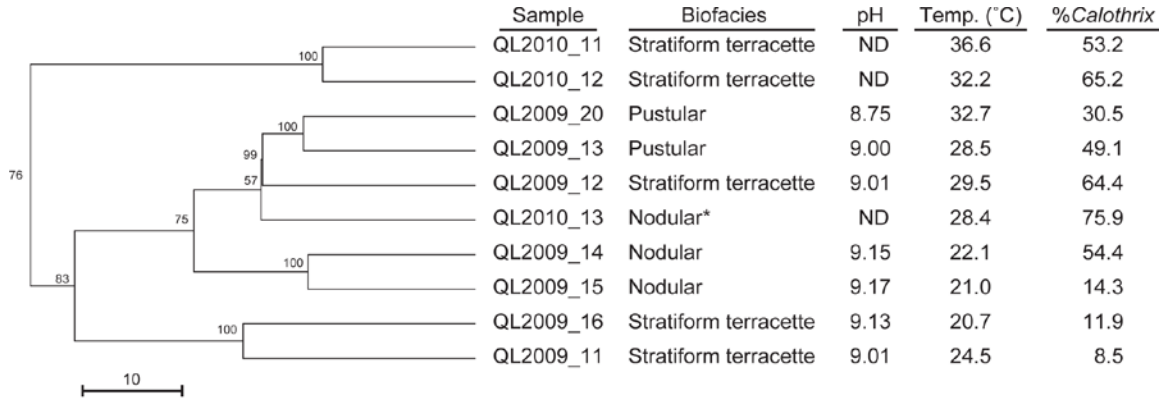

Figure 12. Dendrogram illustrating the cluster analysis of bacterial communities from T-RFLP fingerprints, showing the clustering of our three distinct biofacies at different temperature optima. Starred sample selected for clone library analysis.

Discussion

The extensive fibular sheath around *Calothrix* filaments is thought to play an important role in mineral templating as dissolved silica ions nucleate on the sheath and associated EPS and LPS and thereby preserving microfossils in the rock record (Hugo et. al., 2011). Polysaccharides provide surface area for mineral nucleation and through the addition of active side chains which facilitate mineral nucleation. The addition of reactive side chains to the EPS and LPS challenges the idea of passive mineral nucleation, favoring instead active directed templating of biogenic minerals by *Calothrix* (Bhaskar and Bhosle, 2005). Directed nucleation of silica may serve several purposes for the health of the *Calothrix* filament: the silica rind may offer UV protection, defer predation, and prevent dehydration (Phoenix et al., 2006).

Spontaneous nucleation of silica minerals occurs as mineral rich hydrothermal fluids erupt to the surface into an oxidizing environment and undergo rapid cooling and changes in pH. Our findings suggest that soluble silica ions, similar in molecular structure to water as they both had a tetrahedral coordination with a central oxygen atom, are capable of crossing through the fibular sheath accumulating in the periplasmic space and thereby forming *Calothrix* microfossils. The accumulation of dissolved silica ions in the periplasmic space results in the formation of silica colloids that become trapped, thereby internally mineralizing and preserving the *Calothrix* filaments. Silica precipitates

associated with microorganisms have been found to have an extremely small grain size in a poorly ordered amorphous state (Fortin et al., 1997).

We identified and characterized distinct stages of silicification; encrustation, encasement, and entombment. Close examination of these stages indicate that early silicification of *Calothrix* filaments occurs on the extensive outer sheath with advanced mineralization occurring within the periplasmic space as dissolved silica ions infiltrate and polymerize and thereby fossilize the filaments.

Prior studies of DNA-DNA hybridization showed that fresh water isolates of *Calothrix* spp. were unrelated to marine isolates (Lachance, 1981). However in this study we show that there is a *Calothrix* from a terrestrial freshwater hot-spring that is closely related (98% similarity) to a marine strain of *Calothrix* from the Baltic Sea (Sihvonen et al., 2007). We have also found that within the Queen's Laundry hot-spring microbial community the variation between *Calothrix*-type biofacies allows us to predict environmental conditions (i.e., temperature or pH) in which biofacies form.

Calothrix dominant biofacies are found throughout YNP in the moderate temperature regions of outflow channels like those from this study at Queens Laundry. In this study we identified and characterized three dominant biofacies; nodular, stratiform terracette, and pustular, each forming in specific flow regimes. In Yellowstone *Calothrix* biofacies appear to be constrained to shallow (few cm) moderate temperature (25-50°C) regions, and have not been identified forming in hydrothermal fluids >50°C or in depths greater than a few centimeters. The specific environmental conditions (i.e., flow rate) in which these biofacies form have implications for the interpretation of *Calothrix* biosignatures preserved in ancient cherts. Identification of lithofacies with similar textures allows us to better predict the environment in which lithofacies formed and may serve as effective paleoenvironmental indicators for the environment of early Earth and possibly other planetary bodies.

The ability to characterize the process of silicification in extant hot-spring environments as well as charactering the biofacies indicative of *Calothrix* allows us to better identify *Calothrix* lithofacies from ancient rock deposits and allows us to infer the role that microorganism played in early Earth.

Acknowledgements

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Chapter 3

Morphological Characterization of Bacteriogenic Manganese Oxides From Three Model Manganese (II/III) Oxidizing Bacterial Species

Abstract

To better understand the morphological variability of Mn oxides produced by Mn(II)-oxidizing bacteria, we characterized the morphology of biogenic Mn oxides produced by pure cultures of Mn oxidizing bacteria in the laboratory. This will allow us to better identify biogenic Mn oxides and oxidation products from more complex environmental settings and from ancient geologic deposits. The morphology of bacteriogenic Mn oxides varies as a function of both environmental conditions and bacterial species from which Mn oxides are produced, while the localization of these Mn oxides has been found to be a function of enzyme localization. Biologically-produced extracellular or cell surface associated macromolecules, such as cellular membranes and exopolysaccharides, have been proposed to serve as templates for Mn oxide nucleation since the enzymes responsible for Mn oxidation have most frequently been identified embedded in cellular membranes, and/or associated with the extracellular polysaccharides.

Introduction

Recent studies have shown that bacteriogenic Mn(IV) oxides possess novel nanosheet architectures exhibiting layer thicknesses from a few nanometers (nm) to hundreds of nm in lateral extent, with organic material such as cellular structures and EPS serving as sites of mineral formation (Bargar et al., 2009; Tebo et al, 2004).

To investigate the morphological variability of bacteriogenic Mn oxides the Mn oxides of three model bacterial species were examined. The bacteria *P. putida* GB-1 and *Bacillus* spp. SG-1 oxidize Mn(II) to Mn(IV) using a multicopper oxidase (MCO) in two single electron transfers, as evidenced by Mn(III)-pyrophosphate trapping experiments (Webb et al., 2005; Geszvain et al., 2013; Soldatova et al., 2012; Villalobos et al., 2003). *P. putida* GB-1 is an aerobe isolated from Green Bay sediments by researchers investigating Mn oxidation. *P. putida* GB-1 oxidizes Mn(II) to Mn(III/IV) with highest activity during early stationary phase (Brouwers et al.,1999). *Bacillus* spp. SG-1 is a gram-positive marine *Firmicutes*, which sporulates during stressful conditions, such as

nutrient deprivation. SG-1 spores are capable of Mn oxidation, which occurs on the surface of the spore within the exosporium (Dick et al., 2007; Francis and Tebo, 2002; Francis et al., 2002; Francis and Tebo, 1999; Mandernack et al., 1995). *Erythrobacter* sp. SD21 is a gram-negative strictly aerobic marine α-proteobacteria isolated from surface sediments in San Diego Bay. *Erythrobacter sp.* SD21 oxidizes Mn(II) to Mn(III/IV) using the heme-containing Mn-peroxidase, MopA, which is secreted into the surrounding environment where it may either localize with organic polymers or become loosely associated with the cellular membrane (Anderson et al., 2009; Johnson et al., 2008).

The morphology of biogenic Mn oxides and microbe-mineral associations were observed and characterized using these model microorganisms in a controlled environment. Cleaned Mn oxides and microbe-mineral assemblages were analyzed utilizing electron microscopy (EM) techniques such as scanning electron (SEM) coupled with energy dispersive spectroscopy (EDS) and transmission electron (TEM) microscopy coupled with electron energy loss spectroscopy (EELS) for elemental identification.

Methods

Culture Growth Conditions

All cultures were grown in 500 ml flasks on shaker tables at 200 rpm for aeration at room temperature for two days. Three variant strains of *P. putida* GB-1 were grown in Lept medium (wild-type, increased and decreased EPS mutants). SG-1 and SD-21 were grown in K medium (Geszvain et al., 2013; Johnson et al., 2008; Tebo et al., 2007). Upon formation Mn oxides from cultures were prepared to characterize oxide morphology.

P. putida GB-1 cultures, wild type and mutants, were grown in 250 ml of Lept medium amended with 100 μM $MnCl_2$. *P. putida GB-1_TN56* (mt56) is a mutant with increased EPS production, and *P. putida GB-1_TN215* (mt215)is a mutant with decreased EPS production. Mutants were constructed through transposon mutagenesis by previous M.S. student Thanh Van Ngo (2006).

Bacillus sp. SG-1 spores were produced using a SG-1 plated colony to inoculate K sporulation media, amended with 100 μM $MnCl_2$ (Dick et al, 2007). SG-1 typically reaches stationary phase within 24 hr of inoculation followed by sporulation and Mn oxidation within 7 - 10 days of incubation at 25°C.

Erythrobacter SD21 was grown in 250 ml of K medium amended with 100 μM $MnCl_2$. Mn oxidation occurred after two days in liquid culture and about 5 days plated on 1% agarose K medium plates (Anderson et al., 2009).

Harvesting of Bacteriogenic Manganese Oxides

For SEM analysis a 10 μL aliquot was pipetted onto an aluminum pin and allowed to air-dry. The sample was coated with 20Å Au and examined by SEM. Specimens for TEM were first observed with a wet mount using light microscopy. Specimens were prepared in duplicate. The first suite of samples were prepared by placing a 5 μL aliquot of a stationary phase culture onto a 300 mesh formvar copper coated TEM grid, the second suite of samples were negatively stained with nano-tungsten increasing contrast for better visualization.

After Mn oxidation occurred, cultures were aliquoted into 50 ml falcon tubes and centrifuged (Sorvall RT) at 684 g for 5 min at 4ºC to concentrate cells and Mn oxides. The supernatant was removed and discarded; the remaining pellet was processed to remove all organic material. This was done by first re-suspending the pellet in hexane and vortexing for 1 min; specimens were allowed to sit for 5 min and then pelleted for another 1 min interval at 684 g at 4ºC. Organic material extracted by the hexane remained suspended in the top layer. This layer was decanted leaving the remaining solids, a mixture of Mn oxides and cellular material, at the bottom of the tube. A second rinse in hexane and 1 mL of tetrahydrofuran (THF) allows for further removal of organics from the Mn oxide pellet. The remaining Mn oxides were washed a third time in THF for 5 min and then centrifuged and decanted as above. The remaining material was rinsed a final time in 100% acetone, and processed as above; pellets were air-dried overnight. The cleaned Mn oxides were mounted on aluminum pins for SEM analysis.

δ-MnO_2 Synthesis

δMnO_2 was synthesized for comparison with the biogenic Mn oxides. δMnO_2 was made to reflect structural and surface area changes as a function of environmental changes: 1) δMnO_2 made in 18-MΩ water and rinsed in 18-MΩ water; 2) δMnO_2 made with salt water and rinsed in salt water; and 3) δMnO_2 made in salt water and rinsed in

18-MΩ water. δMnO_2 was made in 500 mL batches using 9.27 g of $MnCl_2 \cdot 4H_2O$, 5 g of $KMnO_4$, 3.5 g of NaOH, 1M NaCl pH 10, 0.01 M NaCl pH 10 in 500 mL 18-MΩ water. The $KMnO_4$ was slowly added to the NaOH solution while stirring. After mixing, a solution of $MnCl_2$ was added very slowly while stirring; black Mn oxides started to form. Mn oxide precipitates were allowed to settle and the supernatant was discarded. The remaining solution of δMnO_2 was placed in 50 mL falcon tubes and centrifuged for 20 min at 4°C at 27K and the supernatant was discarded. The δMnO_2 was rinsed five times in 1 M NaCl; oxides were shaken in NaCl for 1 hr each time. δMnO_2 was placed in dialysis tubing (Fisher Scientific) and dialyzed for 2 days in 1 L 18-MΩ water or seawater. Oxides used for analysis were air-dried and stored at 4°C (Tebo et al., 2007).

Microscopy

Scanning Electron Microscopy of Bacteriogenic Manganese Oxides

Both cleaned Mn oxides and untreated aliquots prepared for SEM and EDS analyses were mounted on aluminum pins and allowed to air-dry. Specimens were coated with 20Å of gold using a Pelco 9100 sputter coater. Images were collected using an FEI Siron high resolution SEM at the Portland State University Center for Electron Microscopy and Nanofabrication, using 3 kV and a working distance of 5.1 mm. EDS was collected using 25 kV.

Transmission Electron Microscopy of Bacteriogenic Manganese Oxides

Specimens prepared for TEM analysis were observed at the Environmental Molecular Sciences Laboratory at the Pacific Northwest National Laboratory in Richland, WA using an FEI high-resolution cryo-TEM, using 2 kV. Specimens were mounted on formvar coated, 300 mesh copper grids (Pelco) and allowed to air dry. Four techniques were used for TEM analysis; i) whole mount of culture using cryo-TEM, ii) whole mount of culture counterstained with nano-tungsten, iii) whole mount treated with 20 μM ascorbic acid and counterstained with nano-tungsten, iv) resin embedded samples that were sectioned for ultra-structural observation of microbe-mineral associations.

The first suite of microbe-mineral samples were analyzed using Cryo-TEM for visualization of samples at cryogenic temperatures (typically liquid nitrogen is about -210°C). This technique allows for the observation of specimens that have not been

stained or fixed, showing them in their native environment, in contrast to dehydration necessary for other EM techniques, which results in conformational changes. Specimens (5 μL/grid) on copper grids were frozen (cryofixation) by plunging them into liquid ethane using a Vitrobot; triplicates of each sample were prepared (Adrian et al., 1984) and observed to characterize Mn oxide morphology.

A second suite of samples were prepared as TEM wet-mounts which were prepared by dispensing 5 μL of culture onto 300 mesh formvar coated copper grids. Two types of specimens were examined i) microbial cells encrusted with Mn(III/IV) oxides, and ii) microbial cells treated with 20 μM ascorbate to dissolve/partially dissolve Mn oxides. Ascorbate treated cells were exposed for 10 min at room temperature, after which cells were pelleted, supernatant was discarded and replaced with culture medium and resuspended. Treated cells were placed on 300 mesh formvar coated grids, samples were allowed to air-dry for 5 min to allow cells to attach to the grid and then gently blotted with filter paper to wick away any fluid, after dry grids were then coated with 50Å of carbon and observed on a Tecnai Spirit TEM at 120 kV.

The third and final suite of samples were prepared for ultra-structural observations of microbe-mineral assemblages by embedding them in EMbed 812 resin (Electron Microscopy Sciences). Cultures were processed after the formation of Mn oxides by centrifuging 2 mL at 14K for 1 min, after which the supernatant was decanted and cell pellets were fixed with 2.5% gluteraldehyde in 0.1 cacodylate buffer pH 7.5, and then gently suspended and dehydrated in a graded ethanol series: 50%, 70%, 70%, and 100% for 5 min each. After dehydration cell pellets were embedded in a graded resin series: 50% resin-ethanol, 75% resin-ethanol, and 100% resin. After the final infiltration step the resin was polymerized in a 60°C oven for 24 hr. Samples were sectioned to a thickness of 50-70 nm and placed on 300 mesh formvar coated copper grids for observation at the University of Delaware, Biotechnology Institutes, Bioimaging Center using a Zeiss Libra 120 TEM. Specimens were imaged at 120 kV. The oxidation state of Mn oxides was measured using EELS, and the identity of Mn oxides was identified using diffraction pattern analysis.

Results

Cultures

P. putida cultures (wild-type and mutants) produced Mn oxides at different rates and formed biofilms with notably different amounts of Mn oxides incorporated (Fig. 1). The biofilm from the EPS over-producing mt56 formed a very dense compact biofilm that was heavily incrusted with Mn oxides, whereas the EPS under-producing mt215 formed a very weak diffuse biofilm that showed moderate Mn oxidation. The wild-type strain formed a slightly diffuse biofilm that was encrusted with Mn oxides. The rate of Mn oxidation varied for each strain with mt56 forming Mn oxides after 24 hr, wild-type after 30 hr and mt215 after 72 hr. Mt56 produced significantly more Mn oxides both in the attached biofilm and within the growth medium; the Mn encrusted bacteria and EPS settled to the bottom of the flask after 72 hr (Fig. 2).

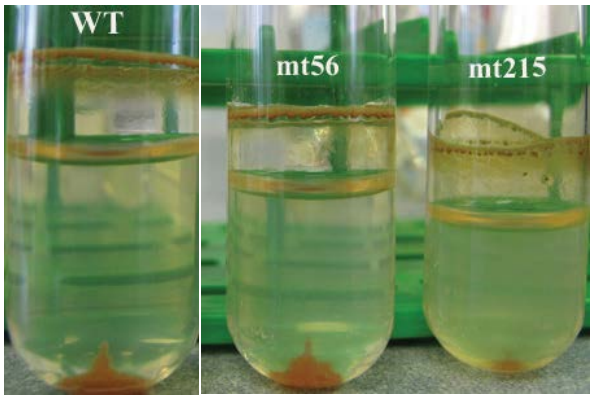

Figure 1. Cultures of *P. putida* (wild type and EPS mutants) after Mn oxidation began during stationary phase. There is a slight increase in Mn oxide particulates in the mt56 with increased EPS production over wild-type (WT), and a significant decrease in the abundance of Mn oxides in the mt215 with decreased EPS production

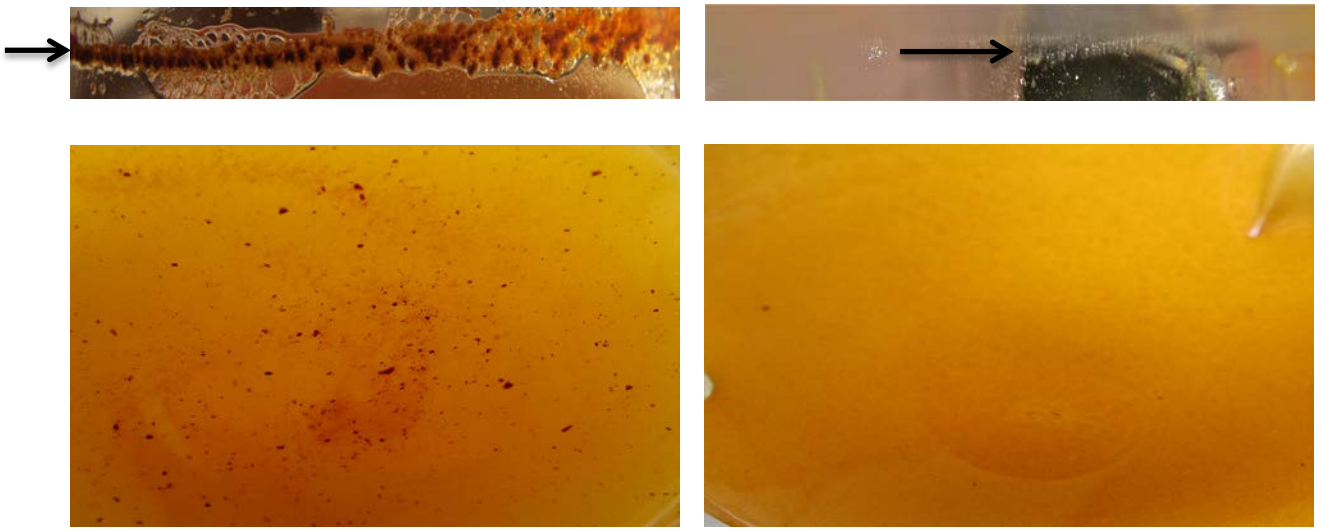

Figure 2. (Top): Close-up of the attached biofilm (arrows) forming at the air interface of culture flasks. (Left) There is a significant increase in both biofilm production and abundance of Mn oxides in mt56 which has an increase in EPS production after 24 hr. (Right) The biofilm at the air interface of the decreased EPS mt215 displays a significant delay in biofilm formation and thus Mn oxidation after 24 hr. (Bottom): Particulate Mn oxides settled on the bottom of the culture flask after 24 hr, (Left) increase EPS mt56, (Right) decrease EPS mt215.

Microscopy

Electron Microscopy

All bacterial strains were analyzed using high vacuum TEM for wet mounts and cryo-TEM for visualization of microbe-mineral complexes in a hydrated state, thereby removing artifacts of dehydration such as the formation of abiotic Mn oxides. In addition, ultra-structural characterization of the bacteriogenic Mn oxide morphology was done using: i) cryogenic TEM which allows for visualization of Mn oxides in a frozen and hydrated state; ii) wet mount visualization; and iii) resin embed and ultra-thin sectioning allowing for visualization of slices through the microbe-mineral complexes. Mn oxides possessed a variety of morphologies as a function of the bacterial strain from which they were produced. Bacteriogenic Mn oxides exhibited two dominant morphologies: platy structures (*P. putida* and *Erythrobacter*) and spines/needles (*Bacillus*).

Pseudomonas Putida GB1

Ultra-thin sections of *P. putida* GB1 were examined by TEM to characterize morphology and possible templating of bacteriogenic Mn oxides. Analysis of microbe-mineral complexes from ultra-thin sections beautifully illustrates the morphology of thin platy nano particulate Mn oxides and their association with the cell walls and EPS (Fig. 3, left). EELS analysis was conducted only to confirm the presence and location of Mn(III/IV) oxides (Fig. 3, center and right). Observations of cells using high vacuum TEM illustrated the Mn oxide encrustation on dehydrated and collapsed cellular and EPS surfaces (Fig. 4, left), cryo-TEM allowed for the visualization of nano-particulate Mn oxides associated with cellular surfaces and dispersed in the EPS matrix (Fig. 4, right).

SEM analysis of Mn oxides associated with wild-type *P. putida* exhibited a thin sheet or platy morphology and appeared to be both incorporated into the EPS as well as on the cell wall (Fig. 5, top?). Mutants with both decreased and increased EPS yielded similar results, however visualization of cells or oxides of mt56 (increase EPS) was difficult as visualization was impeded by the excess of EPS (Fig. 5, center). Even so, when the sample was analyzed using energy dispersive spectroscopy (EDS), Mn(IV) was detected in the EPS matrix (data not shown). Mn oxides in mt215 (decrease in EPS) were clearly visible in the EPS and around individual cells (Fig. 5, bottom). Mn oxides formed on the cell surface and in the EPS produced extremely thin platy morphologies that appeared to be entrained within the EPS. Average thickness of Mn oxides was around 0.0413 μm.

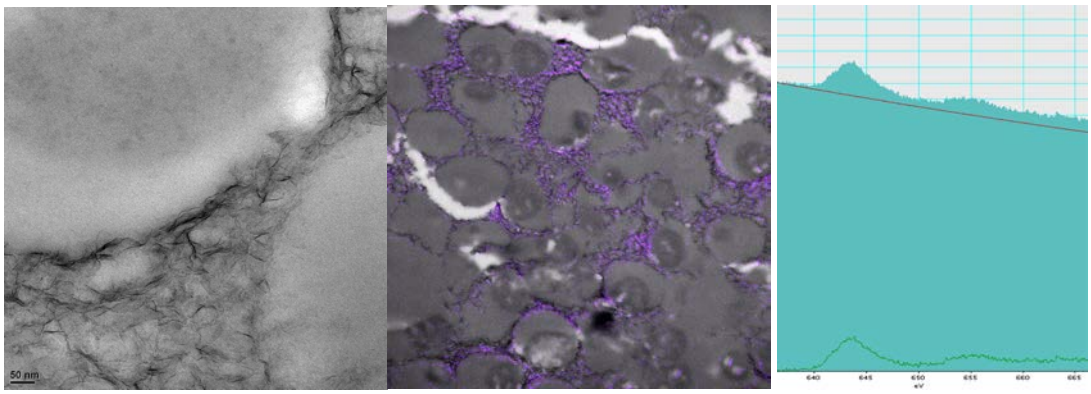

Figure 3. (Left) TEM of *P. putida* with Mn oxides on cellular membranes and in EPS. (Center) False color EELS mapping showing the localization of Mn oxides. (Right) Spectra indicating the presence of Mn oxides, with two characteristic peaks at 644kV and 650kV.

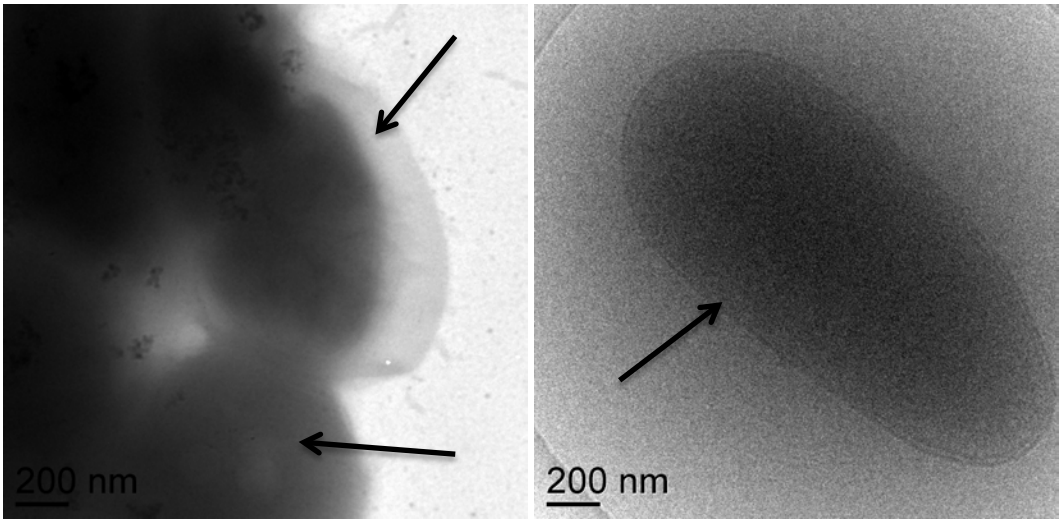

Figure 4. TEM micrograph of a TEM (left) wet-mount of a cluster of cells (arrows) coated with Mn. Mn oxides coated cells make them appear dark. (Right) cryo-TEM of *P. putida* cell in a hydrated state (arrow). The cells appear dark due to the Mn (II/III/IV) encrusting the cellular membranes.

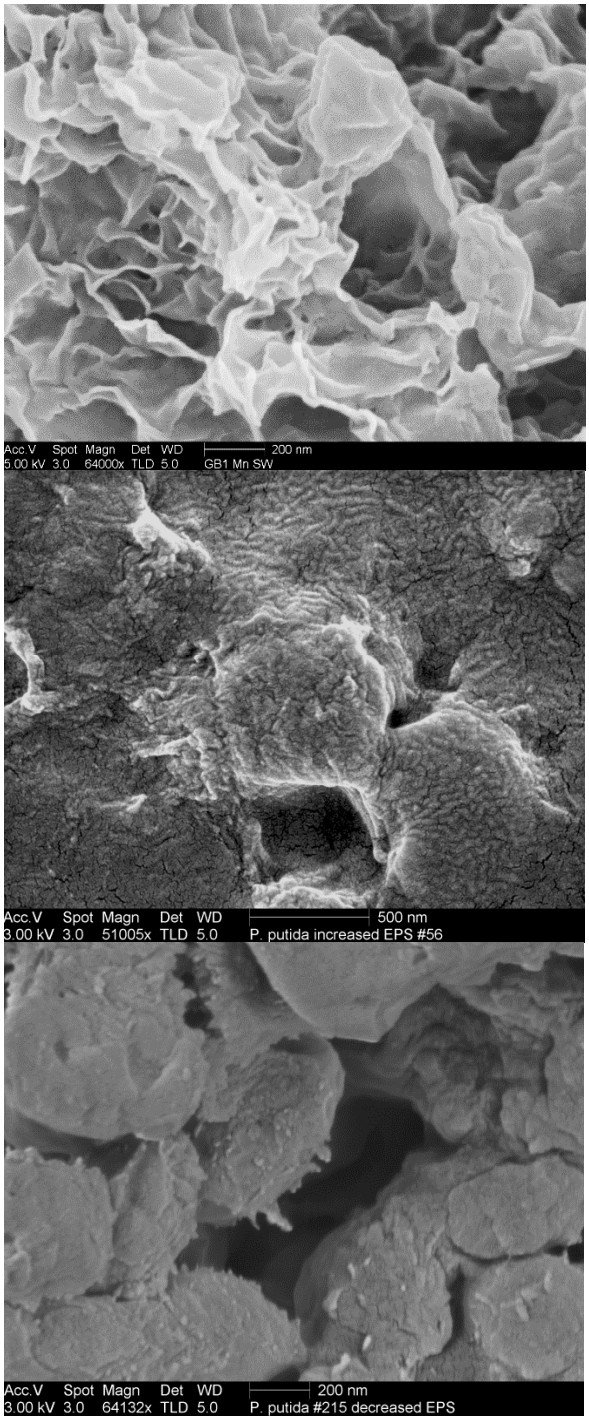

Figure 5. SEM micrographs of *P. putida* wild-type and EPS mutants. (Top) Wild-type illustrating the EPS and cells, visualization of Mn oxides is difficult as oxides are intertwined within the EPS matrix. (Middle) Mt56, increased EPS, visualization of Mn oxides and cells is difficult due to the thick EPS coating the sample. Notice that the EPS did not collapse as much as in the wild-type sample (top). (bottom) Mt215, decreased EPS, Mn oxide encrusted cells are clearly visible due to the lack of EPS.

Bacillus sp. SG1

The morphology of Mn oxides produced in the exosporium of *Bacillus* SG-1 appeared to be long spines that tapered to a narrow point, having a broad base where they radiated from within the exosporium (Fig. 6, left). EDS analysis confirmed the presence of Mn oxides. Spines demonstrated a blunt morphology with an average diameter of 36.81 µm, and protruded on average 183.77 µm from the exosporium (Fig. 6, right).

Cryo-TEM of *Bacillus* SG1 illustrated the dense encrustation of Mn (II/III/IV) bound to the surface of the exosporium (Fig. 7). Observation of wet-mounts clearly illustrated the direct association of Mn oxides with the surface of the exosporium with oxides spines radiating from heavily encrusted spores (Fig. 7, right).

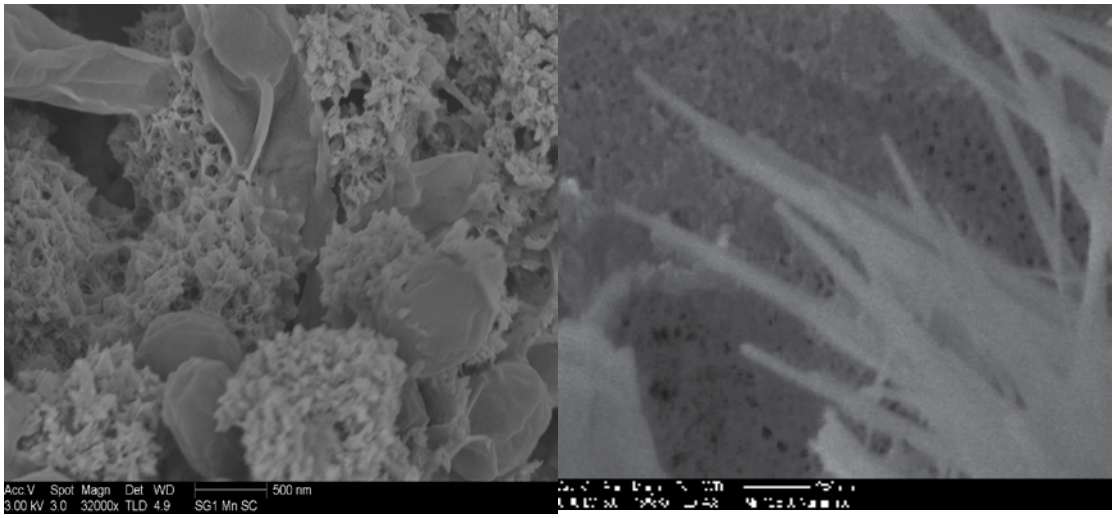

Figure 6. SEM micrographs of (Left) SG-1 spores encrusted in Mn oxides and (Right) the spine morphology of Mn oxides radiating from the surface of the exosporium.

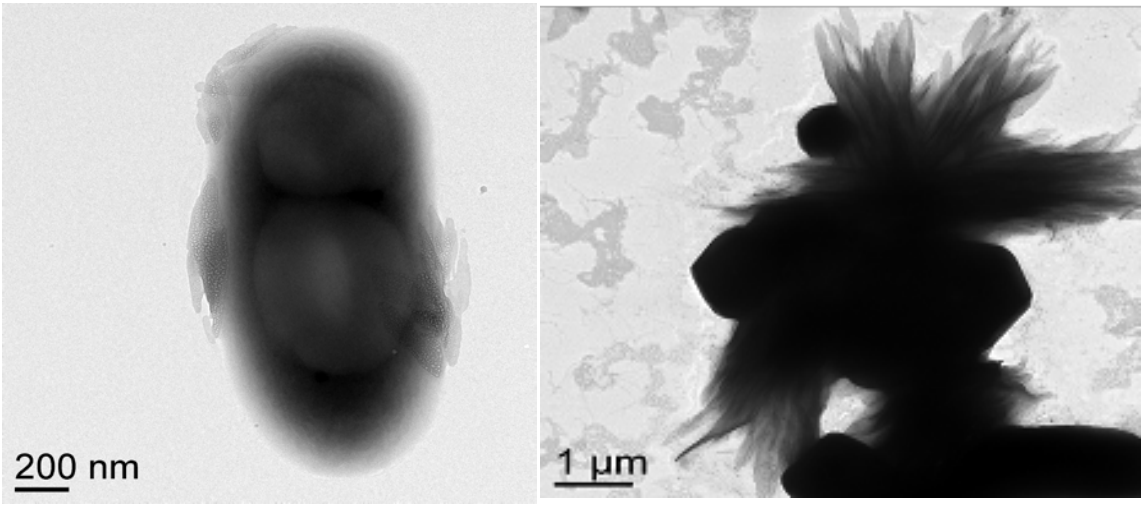

Figure 7. TEM micrographs illustrating (Left) the sporulation of the SG-1 and subsequent encrustation of Mn oxides using cryo-TEM. (Right) TEM of wet-mount illustrating the heavily encrustation of Mn oxides associated with the surface of the exosporium and the needle morphology of Mn oxides radiation from the surface of the exosporium.

Erythrobacter SD 21

Mn oxides formed by *Erythrobacter sp.* SD21 produced an oxide rosette morphology comprised of several large plates with a rugose texture in which oxide plates radiated from a central core (Fig. 8, left). Bacteriogenic Mn oxides appeared visually imperfect with a rugose texture and pores. Oxide plates are not solid structures rather they possess pore spaces within the structure. It is not known whether the pore spaces are produced during processing the samples for analysis. The composition of the Mn oxides was confirmed using EDS (data not shown). Mn oxides averaged 903.9 µm x 1023.4 µm, and the depth of plates averaged 378.8 µm, with a thickness of 21.59 µm. Average measurements were taken using 3 Mn oxide images. Plates were covered with pores that were between 4.3 – 18.39 µm with an average diameter of 11.81 µm (Fig. 8, right).

TEM of *Erythrobacter spp*. SD21 showed the presence of Mn oxides associated with both the cell wall and in the EPS as nano-particulates (Fig. 9, left). Cryo-TEM illustrates the density of nano particulates in the hydrated EPS as a dark shadow around the cell indicating that Mn oxidation occurs both at the cell wall and in the surrounding

EPS (Fig. 9, right). Cells exposed to ascorbic acid showed signs of stress as evidenced by the rapid production of vesicles (Fig. 10).

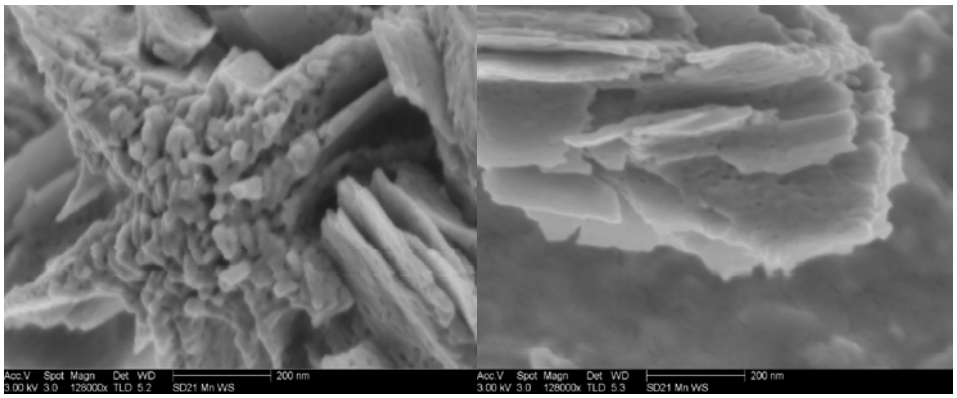

Figure 8. SEM micrographs of Mn oxides produced by *Erythrobacter* SD21. (Left) Top-down view of Mn oxides, illustrating the large platy appearance of oxides radiating from a central core. (Right) Side view of Mn oxide allowing for the observation of pore spaces within the Mn oxide.

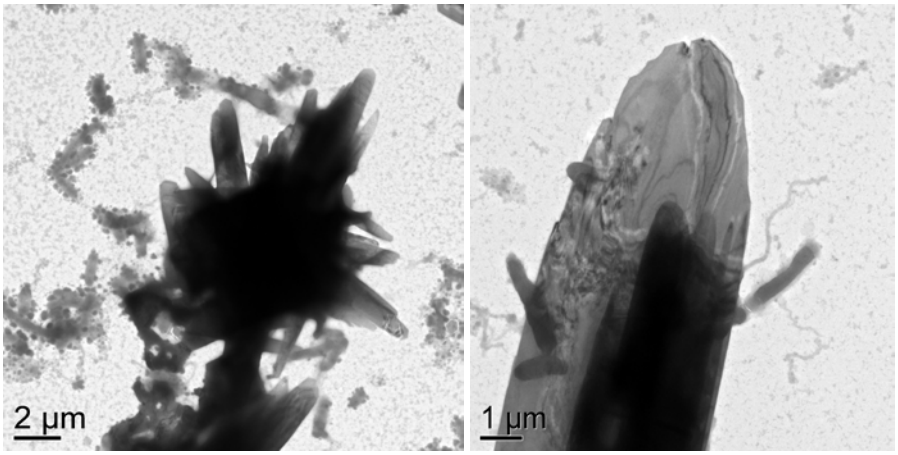

Figure 9. TEM micrographs of wet mounts Mn oxide produced by Erythrobacter SD21. (Left) Micrograph of a Mn oxide rosette with large plates radiating from the center of the core. (Right) Micrograph of Mn oxides, allowing for visualization of the large plate morphology.

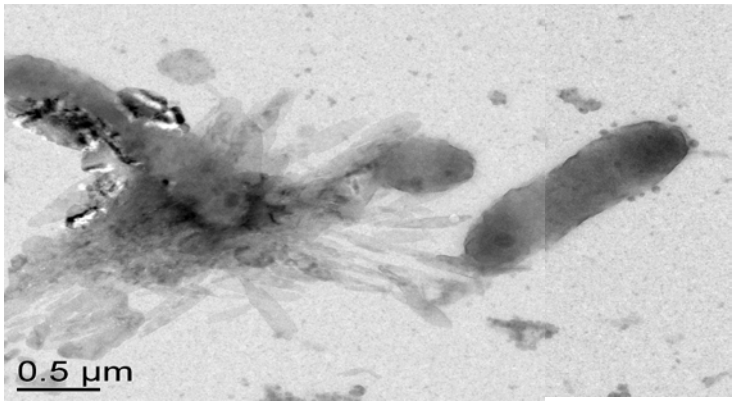

Figure 10. TEM micrograph of a wet-mount of SD21 after brief exposure to ascorbate. The cell on the left is heavily encrusted in Mn oxides that have been thinned allowing for visualization how the Mn oxides are associated with the surface of the cellular membrane.

Discussion

Morphology of bacteriogenic Mn-oxides was characterized using model Mn oxidizing bacteria and a variety of electron microscopy techniques, to better understand the microbe-mineral associations and structural variability of the Mn oxides. The aim of this study was to identify whether the morphology of the Mn oxide was a function of templating onto cellular surfaces, or organic polymers, or merely a function of precipitation with no morphological affects to the Mn oxides. The findings of this study indicate that the factor determining the location of Mn oxidation is the localization of Mn oxidizing activity and presumably the MCO and MopA enzymes rather than the substrate. Organisms that have these Mn oxidizing enzymes anchored in the cell wall, such as MCOs of *Bacillus* and *P. putida* resulted in the formation and subsequent templating of Mn oxides more closely associated with the cell surface while enzymes that were secreted into the surrounding environment and in the cell wall. Microorganisms, such as *Erythrobacter* that have MopA Mn oxidizing enzymes have Mn oxides associated with the both cellular surfaces and in the EPS (Johnson et al. 2008; Tebo et al. 2004).

We found that *P. putida* EPS mutants exhibited drastically different rates of Mn oxidation, with the increased EPS mutant (mt56) exhibiting an accelerated rate of oxidation. This suggests that EPS plays an important role in Mn oxidation however the specific role of EPS in Mn oxidation has not yet been elucidated. The EPS may serve as a

nucleation site for Mn ions, it may have reactive side chains that induce oxidation, or may have Mn oxidase enzymes embedded in the EPS matrix.

The rate of Mn oxidation in *P. putida* mutants suggests that EPS plays an important role in the rate of Mn oxidation, as the mutant overproducing EPS produced Mn oxides more rapidly than both wild-type or the mutant that under-produced ESP.

Acknowledgements

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Chapter 4

Biogeochemistry And Microbial Diversity Of Lotic To Marine Iron And Manganese Depositing Carbonate Cold-Seeps At Soda Bay, Alaska

Abstract

Soda Bay River is a unique watershed providing an opportunity to examine the geochemistry, geomicrobiology, microbial diversity, and biogenic mineral formation of a low-temperature iron (Fe)- and manganese (Mn)-depositing carbonate rich ecosystem.

There is currently no in-depth knowledge regarding the impact of low temperature Fe- and Mn-rich groundwater in lotic or marine ecosystems, the cold-seeps at Soda Bay provides an ideal research site to study Fe-oxidizing bacteria along a lotic to marine ecosystem. Numerous cold-seeps sourced by groundwaters enriched with carbon dioxide (CO_2) and dissolved metals are positioned along the shore of the river along the salinity gradient. Research here has allowed us to better characterize the biogeochemical cycling of elements, biosignature formation and microfossil preservation, as well as provide insight into the evolution of life on early Earth.

Metagenomic analyses of microbial mats from a shallow marine site yielded the first known shallow marine *Zeta-proteobacteria* (Emerson et al., 2007). We identified microbial mats from a Fe-depositing cold-seep effluent composed of diverse microbial communities with the co-mingling of adjacent freshwater and marine bacterial communities capable of both Fe and Mn reduction and oxidation. The co-mingling of freshwater (14%) and marine (74%) Fe-oxidizing bacteria at Soda Bay is a significant finding, as these microorganisms are typically not found in high abundance together leaving a gap our knowledge as to the evolution of neutrophilic Fe oxidizing bacteria in fresh water or marine environments. The high abundance of these microorganisms to co-mingling at Soda Bay is due to the continual percolation of groundwater from seep effluents from below and the overlying seawater covering the cold-seep. Characterizing the relationship of these fresh water and marine Fe-oxidizing microorganisms from this site will provide us the opportunity to better elucidate the ecology and evolution of Fe-oxidizing bacteria.

Introduction

Soda Bay cold-seeps represent a dynamic carbonate-rich groundwater ecosystem where dissolved Fe-, Mn-, and CO_2-enriched fluids erupt from seeps along a lotic to marine system. Reduced metals in the groundwater serve as an energy source for metal-oxidizing autotrophs (and possibly anoxygenic phototrophs). Effluents from cold-seeps located along the shore of the Soda Bay River experience rapid changes in pressure as the groundwater moves from depth resulting in the degassing of CO_2, and changes in pH and oxygen concentration as fluids erupt and are exposed to the oxidizing atmosphere. These chemical changes make abiotic metal oxidation more favorable and more difficult to distinguish abiotic from biotic oxides. Extensive microbial mats form on Fe oxide coated carbonate mounds. Cold-seeps here may serve as a modern analog environment to a specific period of time when banded iron formations (BIFs) formed in the shallow seas atop carbonate platforms such as the BIFs that formed in South Africa during the early Precambrian (Lewy, 2012).

Currently, there is little understanding of the diversity and distribution of metal (Fe and Mn)-oxidizing microorganisms, nor the extent to which these microorganisms contribute to the global Fe and Mn redox cycle nor the mechanisms microorganisms use for metal oxide biomineralization. Biogenic mineral deposition allows for the preservation of microorganisms and their unique oxidation products in geologic deposits, providing the opportunity to identify and characterize microbially derived minerals from the geological record. Fe-oxidizing bacteria are responsible for the formation of biogenic Fe oxides with distinct morphologies, which are easily identifiable (Ghiorse, 1984). These structures include twisted stalks such as those associated with *Gallionella* spp. (Kucera and Wolfe, 1957) and *Mariprofundus* spp. (Emerson et al., 2007) and sheath structures associated with *Leptothrix* spp. (Fleming et al., 2013). Determining the origin of Fe oxides as biogenic is based on oxide morphology (Emerson and Revsbech, 1994).

The high concentration of dissolved Fe(II) from cold-seep fluids leads to the formation of Fe(III) oxide/oxyhydroxide deposition along the salinity gradient. The shallow marine environment in which deposits form make this the ideal environment to compare to the shallow seas from which BIFs formed on early Earth during a period of time when the atmosphere was more oxidizing than reducing. Characterization of the

geochemistry, mineralogy and microbial diversity of both mineral encrusted microbial mats and groundwater from cold-seeps along the salinity gradient at Soda Bay presents a unique opportunity to detect environmental disturbances due to environmental forcing (i.e. climatic, tidal). Understanding the effects environmental disturbances (i.e. precipitation, tidal) have on the ecosystem allows us to better hypothesize about environmental conditions in which ancient Fe deposits formed as well as better predict the response of such ecosystems to global climate change.

Material and Methods

Study Sites

Study sites extend 415 m along the Soda Bay River, in which there are thirteen seeps/springs/vents that were sampled (Fig. 1). Study sites are characterized as freshwater, tidal, and marine, of which eight sites are freshwater (study sites 1-5, 10, Mn and SW), three are tidal (Sites 6-8), and two are marine (Sites, 11 and 13). Microbial mats from the upstream portion of the river are made of light orange, flocculent, amorphous Fe oxides; as the system becomes more saline mats change to a rich dark orange color and are more cohesive and crystalline (Fig. 2).

Figure 1. Google Earth map showing the 415 m transect sampled along Soda Bay River.

Figure 2. Photos of seeps along the salinity gradient, from freshwater to the marine region. (Left) Site 1 freshwater, mats are light orange and flocculent. (Center) Site 7 tidal, mats are thick and cohesive. (Left) Site 8 marine, mats are thin, highly mineralized with a rich orange color.

Geochemistry

Fe and Mn Measurements

Chemical analysis of cold-seep fluids were conducted to measure the concentration of dissolved and particulate Fe(II/III) and Mn(II/III/IV) species using inductively coupled plasma mass spectroscopy (ICP-MS). ICP-MS is an analytical technique using emission spectroscopy to detect the presence and concentration of trace elements by exciting atoms and ions resulting in the emission of electromagnetic radiation at wavelengths indicative of specific elements. The intensity of the emission indicates the concentration of the elements within the sample (Mermet, 2005). Cold-seep fluids were collected and immediately filtered through 0.2 μm polyethersulfone for capturing Mn particulates and Supor© filters for filtering fluids for geochemical analysis. Fluids were acidified with trace metal grade hydrochloric acid to a final concentration of 40 mM, and stored at 4°C. The concentration of particulates from vent fluids were measured by partially digesting particulates collected on polyethersulfone filters. Filters were prepared for ICP-MS by allowing them to digest for 24 hrs in 1 mL of 0.15 μM hydroxylamine hydrochloride, after which the solution was removed and further acidified to < pH 1.8 with 6 M nitric acid and measured on an ICP-MS.

The concentration of dissolved Mn(III) was measured by the addition of a strong Mn(III) ligand, desferrioxamine, as described by Clement et al. (2006) to a 2 mL aliquot

of filtered seep fluids. The Mn(III)-desferrioxamine complex was concentrated from the sample by passing it over a Waters HLB column and eluting the complex with 100% methanol. Samples were measured at a 1:9 dilution with 1 % HNO_3 by ICP-MS.

Nutrient and Alkalinity Measurements

Cold-seep fluid samples were collected for nutrient analysis by first filtering fluids through 0.2 μm polyethersulfone filters and freezing at −20 °C until analyzed. Prior to analysis samples were thawed at room temperature and aliquoted into fifths. Concentrations of nitrate, nitrite, ammonium, orthophosphate, and alkalinity were measured colorimetrically using Hach chemistry kits (Hach, models NI-6, NI-SA, PO-19 and AL-AP respectively) according to manufacturer instructions.

Leucoberbelin Blue

Collected sediment and filter samples were transferred to 1 mL vials to which leucoberbelin blue solution (LBB, 65%, Sigma-Aldrich) was added. LBB (410.5 g mol-1) stock solution was made by dissolving the crystals in Milli-Q water to a concentration between 1 to 4% (24 to 97 mM) and adding 40 mL of either 10 M sodium hydroxide (NaOH) or 21% ammonium hydroxide (NH_4OH) per 10 mL of solution. Working solutions are diluted in 1% acetic acid, to a range between 0.01 to 0.04% (240 to 970 M). LBB is a solution used as a colorimetric indicator to determine the presence of Mn(III/IV) oxides. The presence of Mn(III/IV) oxides is confirmed when the LBB becomes oxidized turning a brilliant blue color (Tebo et al., 2007). Biofilms were tested for the presence of Mn(III/IV) oxides using 5 μL of 0.04% LBB.

CTD Time Series

A remote observatory established at Soda Bay, entailed the deployment of an autonomous Sea-Bird (SBE) 16plus V2 SeaCAT sensor, deployed adjacent to Site 6. The high-accuracy sensor measures conductivity, temperature, and pressure using an optional recorder designed for long-duration, fixed-site deployments**.** The 16plus V2 has internal batteries and the capacity to store data for later collection on its internal memory, which can be output in engineering units or raw HEX. Battery endurance varies, depending on

the sampling scheme and uses nine alkaline D-cells to provide power for 3.55 x 10^5 data points at pre-programmed intervals. For this study, data was collected every 30 min.

Isotopic Carbon Fractionation

To determine the source of inorganic carbon from cold seep fluids, effluents were collected in duplicate and analyzed for carbon isotopic fractionation ($\delta^{13}C$) (Doctor et al., 2008; Li et al., 2009). Samples were collected in 140 ml acid-rinsed glass bottles and sealed with rubber stopper to prevent atmospheric exchange of gases and stored at 4°C until analyzed. Glass bottles were filled by gently pushing fluids through a combusted 25 mm glass fiber filter in a Swinnex filter holder using a 60-ml syringe. Saturated mercuric chloride, 0.5 mL, was added to halt microbial activity. Fluid samples were analyzed at the University of Arizona, Environmental Isotope Laboratory, in Tucson, AZ.

Bioassessment

Bioassessments were conducted along the salinity gradient as a means of gauging the impact of excess dissolved CO_2 and heavy metals on the food web within this ecosystem. Bioassessment involved the characterization of macroinvertebrates allowing for the evaluation of the health of the watershed. Macroinvertebrates we expect to find in rivers on Prince of Wales (POW) Island as indicators of a healthy river are; stoneflies, mayflies, caddisflies. Macroinvertebrates are excellent indicators of water quality due to their limited mobility confining them to a specific environment for most of or all of their life cycle. They demonstrate a range of responses to pollution and their short life span allows for changes in watershed health to be detected rapidly. Macroinvertebrates also play an important role in the food web, particularly for anadromous fish such as salmon. Bioassessments were conducted using a D-loop net with 900 µm mesh to capture macroinvertebrates dislodged from the streambed. Macroinvertebrates were sorted, identified, and counted using an identification key to measure the health of the river.

Cell Numbers

Enumeration studies were carried out to determine the presence and quantity of microbial cells within the spring effluents. Cell counts were done by staining 1 gram (wet

weight) of mat sample with Syto-13 green fluorescent nucleic acid stain. Sediments were stained and cells enumerated using the same technique to quantify cells associated with Fe and Mn oxides; data was compared and contrasted to the effluent enumeration data. Enumeration was done by incubating samples with stain for 30 min in the dark, after which samples were twice rinsed in 10 mM HEPES buffer pH 7.2 removing unbound stain. Cell counts were conducted using Zeiss Axio Imager M1 laser scanning confocal microscope, using 10X magnification. Digital images were taken of 10 random fields and analyzed using ImageJ (http://imagej.nih.gov/ij/) image processing and analysis freeware. Cells were enumerated by sharpening the image to increase the contrast of stained cells after which the image was inverted to black and white. Contrast of cells was further enhanced by setting a standard threshold for all images, in which particles less than 10 pixels were removed, resulting in a final binary image used for enumeration and for batch analysis on all images to reduce threshold bias per image (Blackburn et al., 1998). Samples were placed on glass slides and covered with a coverslip for observation.

Molecular Methods

Genomic DNA Extraction

Microbial mats were collected, immediately preserved in RNA Later (Life Technologies, Grand Island, NY) and stored at 4˚C before being transported back to the lab where they were frozen at -80˚C. Genomic DNA (gDNA) was extracted from mats using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer's protocol with the modification that the gDNA was eluted into 10 mM Tris pH 8. gDNA concentration was determined using a Nanodrop ND-1000 spectrophotometer and diluted to ~10 ng/µL for downstream molecular applications.

Community Diversity

Community diversity of microbial mats along the salinity gradient was resolved using terminal restriction fragment length polymorphism (T-RFLP). The methodology used for T-RFLP analysis has been reported in detail in many previous publications (Fleming et al., 2013; McAllister et al., 2011; Rassa et al., 2009; Davis and Moyer, 2008). T-RFLP resolves populations from mixed microbial communities of low to intermediate

richness (Engebretson and Moyer, 2003) and is a reliable method for detecting changes in community compositions (Hartmann and Widmer, 2007).

Bacterial small-subunit ribosomal RNA (SSU rRNA) genes were amplified from the purified gDNA using the primer set 68F (5' 6-FAM - TdNA dNAC ATG CAA GTC GdKdK CG 3') and 1492R (5' dKGdP TAC CTT GTT ACG ACT T 3'), with identical conditions as previously reported (McAllister et al., 2011). Three replicate PCR reactions were pooled, desalted, and equally partitioned between 5U of eight restriction enzymes *Alu* I, *BstU*I, *Hae* III, *Hha* I, *Hinf*I, *Mbo* I, *Msp* I, and *Rsa* I (New England Biolabs, Berverly, MA) for digestion in a total volume of 30 mL at 37°C, with the exception of *BstU*I which was incubated at 60°C. After digestion, fragments were desalted using Sephadex G-75 (Amersham Biosciences, Uppsala, Sweden) and dehydrated. Fragments were then resuspended in 15 μL of formamide and 0.33 μL GeneScan 500 LIZ internal size standard (Life Technologies), and denatured by heating for 5 min at 95°C. Capillary electrophoresis was used to separate fragments using an ABI 3130XL genetic analyzer with a 50 cm capillary array using POP6 polymer (Applied Biosystems).

Fluorescently labeled 5' terminal-restriction fragments (T-RFs) were sized against Genescan LIZ-500 internal size standard using Genemapper v3.7 (Applied Biosystems). Restriction fragments between 50 to 500 nucleotides were used for analysis. Electropherograms were imported into BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) for analysis. Fragments were sized using the average Pearson product moment correlation and unweighted pair group method with arithmetic mean (UPGMA, Applied Maths) cluster analysis of all -restriction digests using relative fluorescent proportions from each electropherogram. Peak detection was limited to peaks between 50 and 500 bp with a height of at least 3% of the maximum value of the fingerprint. At Soda Bay characterization of both temporal and spatial variability of biofilm diversity from cold-seeps along this system were collected using T-RFLP analysis.

RuBisCO Survey Using a Clone Library

Clone libraries were constructed using three replicate PCR products from gDNA from sample Site 6 using identical PCR conditions and the two sets of primers for amplifying RuBisCO Form I and II used for assimilation of inorganic carbon. The primer

set for Form (II) RuBisCO 484F-GGN CAN ATC ATC AAR CCN AA and 1146R-TTN TCR AAG AAR CCN GGN A identifies the *cbbM* gene. For Form I RuBisCO primers were, TIGF- CAR CCN TTC MWR CGB TGG and TIGR-GTN CCD CCD CCR AAY TG to identify the *cbbL* gene (Davis, 2014).

PCR was performed in triplicate 20 μL reactions in a master mix containing 400 μM dNTPs, 0.5 μM each primer, 20 ng of template DNA, 10 μg molecular biology grade BSA and 1 μL Phire polymerase. Amplification of DNA was done on a thermocycler starting with a 98°C hot start for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec each, 60°C for 20 sec, and 72°C for 30 sec, with amplification completed with a final extension step at 72°C for 60 sec. Amplicons were excised from the 1.5% agarose gel and purified with a GeneJET gel extraction kit (Thermo Fisher) per manufacturer's protocol. PCR amplicons were assayed for size and purity using gel electrophoresis.

Aliquots of gDNA were cloned into the pJET1.2-cloning vector using the CloneJet PCR cloning kit (Thermo-Fisher). Clones were streaked on LBamp plates for isolation and allowed to grow overnight at 32°C, after which colonies were assayed for plasmid insertion using PCR amplification with pJetF and pJetR primers. Amplicons were assayed by again running a 1% agarose gel electrophoresis against a 1-kb ladder size standard. Reaction mixtures yielding expected DNA fragment sizes were used, with the remaining fluorescently labeled amplicons desalted using Montage PCR centrifugal filter devices (Millipore). Clones with inserts were end-sequenced with pJetF and pJetR primers. Vector sequences were discarded while sequences of interest were trimmed to include 400 bp from the end of the 68F primer. Sequences were aligned using the automated sequence alignment tool FinchTV. Operational taxonomic units (OTUs) were defined as groups of at least two clones with a minimum of 97% similarity grouped using the furthest neighbor algorithm calculated with the program ARB (Ludwig et al., 2004).

Quantitative PCR

Non-degenerate QPCR primers were designed for carbon fixation and *Zeta-proteobacteria* genes by using PCR-cloned sequences and cloned into pCR4-TOPO *E. coli* vector using the TOPO TA cloning kit (Life Technologies, Carlsbad CA). RuBisCO is used in carbon fixation via the Calvin Benson Bassham (CBB) cycle (Tabita et al.,

2007). The *cbbM* primer was used to amplify PCR products from Soda Bay samples collected July and September 2014. Functional *cbbM* genes were quantified using absolute quantitation for gDNA against linearized plasmid standards. QPCR assays were run in a 96 well plate format on a Step One Plus Real Time PCR System (Life Technologies). The degenerate primer pair for RuBisCO used was 7.3F 5'-GCT TTG GCA AAT GGT TCA GG-3' and 7.3R 5'-ACC GAC TTC TGG AAA GTA TTG G-3', while the primer pair Zeta542F 5'-GAAAGGDGCAAGCGTTGTT-3' and Zeta658R 5'-TGCTACACDCGGAATTCCGC-3'. Samples were run in triplicate using 2X Power SYBR Green Mastermix (Life Technologies). All assays were run using 0.3 μM of forward and reverse primer pairs in a total reaction volume of 20 μL. One nanogram of gDNA template was run for each unknown sample using absolute quantitation against a 10-fold dilution series of one nanogram linearized plasmid (10^{-1}–10^{-7}). Samples were run with negative controls at 95°C for 10 min (initial denaturation), 40 cycles of 90°C for 15 sec (denaturation) and 50-60°C for 1 min (annealing) (Jessner et al., 2015).

Metagenomic Analysis and Community Diversity

One sample was analyzed using a next-generation sequencing (NGS) approach. The gDNA from a cold-seep located in the estuary was processed at University of Delaware Biotechnology Center. Reads were trimmed for quality with rare reads removed using the program Khmer (https://github.com/ctb/khmer). Contiguous sequences were assembled using the de novo assembler, MetaVelvet (Namiki et al., 2012) program based on Velvet (Zerbino and Birney, 2008).

Metagenomic data was analyzed using a novel iterative mapping method, based on the expectation maximization (EM) algorithm (Dempster et al., 1977) that accurately reconstructs full-length SSU sequences from the microbial community. This method, referred to as expectation maximization iterative reconstruction of genes from the environment (EMIRGE), uses raw reads and quality scores outputting data of the most probable consensus sequences after several comparative iterations (Miller et al., 2011).

Colony Assay using PCR

Microbial colonies from enrichments cultures were collected and assayed using PCR amplification to determine if colonies were bacterial or archaeal. Universal primer pairs were used for PCR amplification, for bacteria 68F and 1492R and for archaea 27F and 1492R. To each reaction tube 5 µL of gDNA was added to 45 µL master mix. Amplification was done using a Biorad thermocycler, denaturation 95°C, 30 seconds hybridization: 55°C, 1.5 minute, elongation: 72°C, 2 & 7 minutes for 30 cycles.

Biogenic Fe oxidation using Enrichment Studies

Enrichment studies using Modified Wolfe's Minimal Medium (MWMM) in slush tubes for both heterotrophic and autotrophic microorganisms were conducted to grow Fe oxidizing bacteria. Colonies were harvested and partitioned into three aliquots. The first aliquot was frozen in 50% glycerol at -80°C, the second aliquot was transferred to fresh media for continued growth, and the third aliquot was processed for molecular analyses. Enrichments were grown at 10°C, as close to the environmental temperatures as possible, in Hungate tubes prepared in an anaerobic chamber. Media was saturated with N_2 gas to eliminate oxygen from media. Hungate tubes were prepared by first placing a 2% agar plug containing 625 mg zero valent Fe shavings at the bottom of each tube. Emplaced plugs were degassed overnight in the anaerobic chamber after which they were overlain with 0.01% agarose slush MWMM. Microbial mats were suspended in HEPES buffer and tubes were inoculated vertically using sterile glass pipettes. Autotrophic and heterotrophic enrichments were grown in conjunction with negative controls to allow for the identification of abiotic oxidation gradients (Emerson and Moyer, 2002).

Microscopy

Microscopic analysis of microbial mat samples was conducted to identify biogenic Fe oxides and to characterize the overall morphology of microbial mats. Electron microscopy (EM) allows for micron scale features of mineral forms, crystal growth rates, oxidation state, microbe-mineral interactions, spatial patterns of distribution and composition of mineral grains as a function of microbial populations and local geochemical processes.

Mineralogy of Cold-Seep Deposits

Metamorphic greenschist is the dominant rock-type of SW POW Island (Dusel-Bacon, 1996), many of the green schist rocks found in the region are coated with a thin (0.1-2 mm) purple mineral layer. Rock specimens collected from Soda Bay were prepared as 30 µm thin-sections without a coverslip to investigate the Fe and Mn mineralogy of the rocks. Thin section specimens were analyzed using petrographic microscopy, X-ray Absorption Near Edge Structure (XANES), and EM techniques. XANES analysis was conducted at Stanford Synchrotron Radiation Lightsource, Menlo Park, CA.

Petrographic Microscopy

Petrographic microscopy of thin-sections allows for characterization of mineralogy of oxides and visualization of templating of oxides onto microbial surfaces. Thin-sections were observed using a Zeiss petrographic microscope to characterize the morphology of Fe- and Mn minerals and the depth at which minerals formed.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) coupled with Energy Dispersive Spectroscopy (EDS) allowed for characterization and elemental analysis of Fe oxides associated with microbial mats. Collected samples were fixed using 2% gluteraldehyde in 0.2 µm filtered spring water and stored at 4°C until air-dried for SEM analysis. Samples were gold coated using a Pelco 91000 sputter coater with a gold target. Specimens were analyzed using a FEI Sirion High-Resolution SEM (HR-SEM) equipped with an Oxford INCA EDS system at the Center for Electron Microscopy and Nanofabrication, at Portland State University, Portland, OR. Specimens were observed at 5 kV and at a working distance of 5 mm; digital images were captured using a CCD camera.

Transmission Electron Microscopy

Nano-scale cellular and mineralogical features were observed using a Zeiss Libra 120 TEM at Delaware Biotechnology Institute BioImaging Center, Newark, DE. Samples were prepared for TEM analysis by first fixing them in a 2% gluteraldehyde-filtered spring water solution, after which samples were rinsed twice in 10 M HEPES buffer at

pH 7.2 and then dehydrated using a graded ethanol series (25%, 50%, 75 %, and twice in 100%). Samples were embedded in EMbed 812© (EMS, Hatfield, PA.) resin per manufactures instructions for medium resin and sectioned to 50 - 70 nm thickness using a ultra-microtome allowing for TEM analysis and subsequent characterization of ultra-structural features of microbe-mineral associations on cellular and EPS surfaces. TEM analysis provides information about crystal structure of Mn-oxides and allows for imaging of crystallographic structures at an atomic scale. Digital images were taken using a using a low accelerating voltage reducing the potential of damage to soft materials.

Fluorescence Microscopy

Fluorescence of Syto-13 stained microbial mats were examined using a Zeiss Axio Imager M1 laser scanning confocal microscope with probe-appropriate excitation wavelengths and LSM 5 Pascal Version 4.0 image-acquisition software (Carl Zeiss, Oberkerchen, Germany). Observations were made to characterize the texture of mats along the salinity gradient and to determine the presence of microorganisms associated with Fe oxides. Samples were prepared by adding Syto-13 to a 1 ml aliquot of mat suspended in HEPES buffer.

Results

Geochemistry

Chemical Composition of Vent Fluids

The pH values of cold-seep fluids along the river ranged from 6.0 to 6.7 and temperatures were between 10°C and 12.5°C. Both pH and temperature remained stable spatially (Table 1). Under these conditions, the bicarbonate ion is one of the dominant anions with dissolved calcium and magnesium species being the dominant cations in the groundwater. The concentration of ionic species in groundwater is generally greater than in surface waters indicating extensive dissolution of the underlying carbonate rocks.

Precipitation played a significant role in the concentration of dissolved chemical species in the groundwater as a decrease in metal concentrations occurred after above average precipitation. Average annual precipitation in Southeast Alaska is 358.14 cm; however 2014 was one of the warmest and wettest years since 1985 (NOAA,

climate.gov) (Fig. 3), the increase in regional precipitation (Feb – July), resulted in a surge in groundwater percolation of meteoric waters. The recharge event resulted in the mobilization of excess pools of dissolved chemical species from the karst aquifer.

Bioassessment

It has been long known that streams with a heavy particle load or high concentrations of Fe particles demonstrate low abundance and diversity of macroinvertebrates (Wellnitz et al., 1994). Bioassessments conducted along the Soda Bay River revealed that there is only one macroinvertebrate present in the river, an aquatic worm, due to its tolerance to low oxygen, elevated CO_2 and particle loads, which suffocate most macroinvertebrates. Typically, lotic ecosystems on POW are occupied by a variety of macroinvertebrates such as mayflies, stoneflies and caddisflies indicating that the water in these ecosystems is cold and oxygenated with low particle loads.

The low diversity and abundance of macroinvertebrates at Soda Bay explains the Traditional Ecological Knowledge from the local Alaska Native Community, who describes this site as having “bad” water. Salmon populations are dependent on macroinvertebrates for sustaining hatchlings, without which the salmon run for that river completely collapses much as it has at Soda Bay.

Table 1. Temperature and pH of cold-seeps along Soda Bay River, from the fresh water (Sites 3, 10, & Mn), tidal Sites 6 & 8), and marine (Sites 11 & 13) of the system

Site	Water Type	pH July	T July
Site 3	Fresh	6.57	12.7
Site 10	Fresh	6.38	10.1
Site 6	Tidal	6.23	12
Site 8	Tidal	6.21	10.6
Site 11	Marine	6.3	12.1
Site 13	Marine	6.07	10.6
Site Mn	Fresh	6.08	12.7

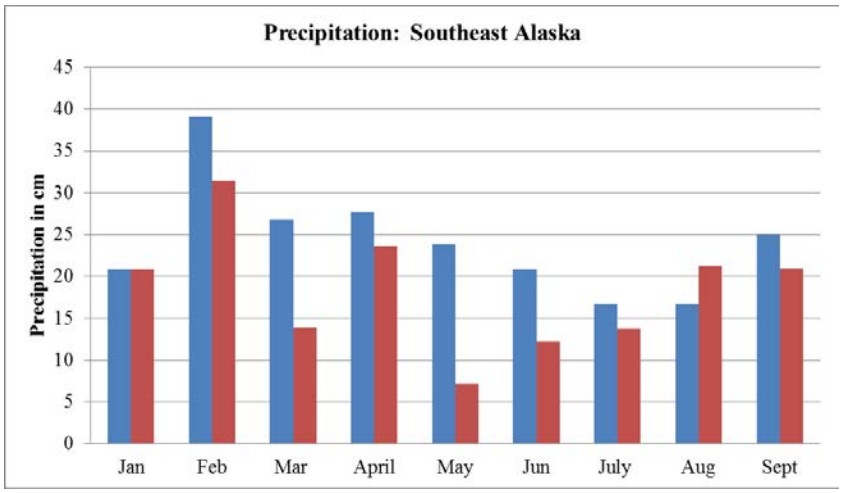

Figure 3. Average and 2014 monthly rainfall in Southeast Alaska. The red bars are the annual average; the blue bars are the precipitation from 2014.

CTD time series

Data from the Sea-bird sensor CTD sensor was downloaded and analyzed two months after the deployment in May 2014. This short-term data set allowed us to make fine scale observations of the tidal flux in the lower portion of the river. During high tide, seawater moves upstream along the bottom of the river, as it is denser due to the increase in salinity and cooler temperatures. From May to June 2014, the temperature was clustered around 14°C ± 2°C with an increase in salinity as seawater moved upstream. Temperatures from June to July 2014 showed both an increase and a wider range in temperature, averaging 12°C to 20°C (Fig. 4).

The results showed that river discharge was elevated in June-July after the recharge event and that tides were slightly elevated as well. In the June-July time series the increase in salinity was not evident until the tide was above 0.5 m. In the May-June time series there was a constant background of a salinity of 9 psu at 0.5 m. This suggests that the river discharge was high enough to push back the incoming saltwater in the early stages of the incoming tide. Results here coincide with the increased precipitation recorded during the spring, showing an increase in river discharge and subsequent flushing of the microbial mats from cold-seeps in the river.

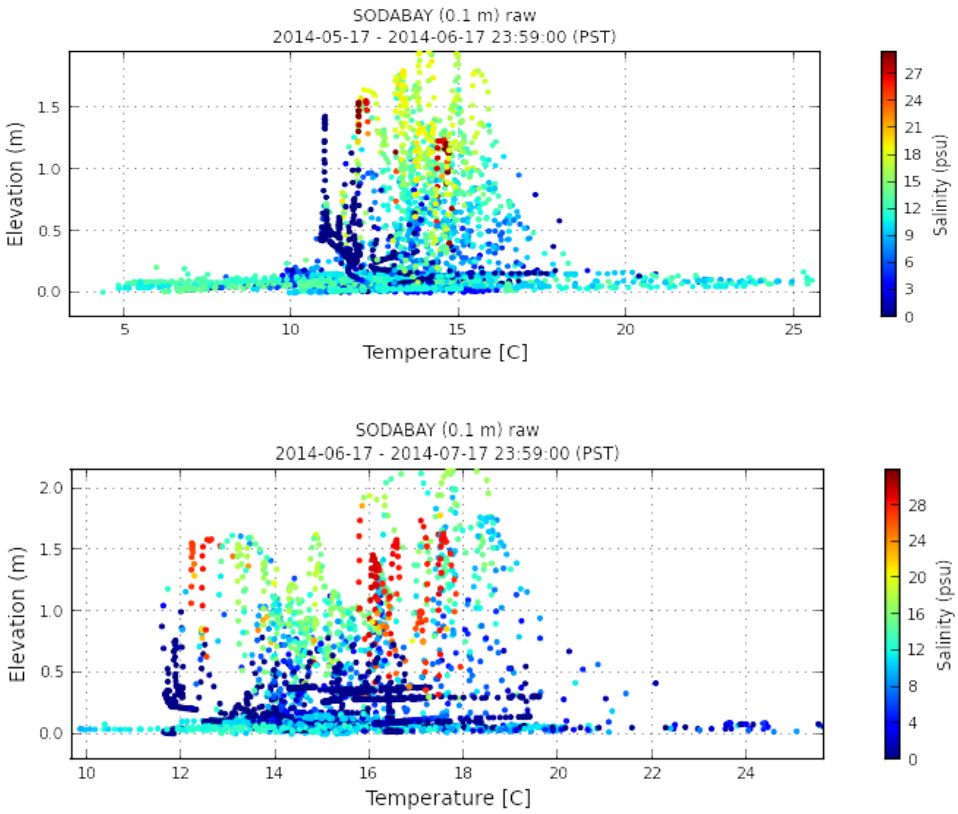

Figure 4. Sea-bird CTD sensor data. (Top) May-June notice there is less salt-water intrusion during high time due to high river discharge (red). (Bottom) June-July there was less river discharge allowing salt water to move up stream during tidal events.

Fe and Mn Concentrations

Dissolved Fe(II) (dFe) concentrations from cold-seeps along the Soda Bay River were elevated compared to most surface waters. For example in natural surface waters, the amount of Fe present is typically between .012 to 1.18 µM (Vuori, 1995; Förstner et al., 1979). However, in Soda Bay cold-seeps dFe concentrations ranged from 48.36 µM up to 1175 µM over both spatial and temporal scales (Fig. 5). Study sites located in the marine region consistently demonstrated elevated particulate Fe (pFe) concentrations. Fluid samples collected during the recharge event had elevated dFe concentrations, with a decrease in dFe during the rebound period. pFe concentrations were elevated during the May 2014 recharge event. pFe was particularly high in the marine region of the system due to flocculation of Fe by major salts in sea water (Eckert and Sholkovitz, 1976).

Dissolved Mn (dMn) was the dominant Mn form at all cold-seeps along the system; however, dMn concentrations were elevated in the tidal region of the system. Particulate Mn (pMn) was significantly lower in the marine portion of the system (Fig. 6). The measured increase in pMn from Site 3 is an artifact of sampling as some sites were too shallow to sample resulting in excavating a small trench to pool fluids.

Mn(III) concentrations were low across the entire system when sampled in the spring during the groundwater recharge event (May 2014) and during the rebound period (September 2014) in the fall; averages were between 0.05 to 0.65 µM (Fig. 7, Table 2). Mn(III) concentrations were highest at cold-seeps located in the tidal region of the system after the recharge event.

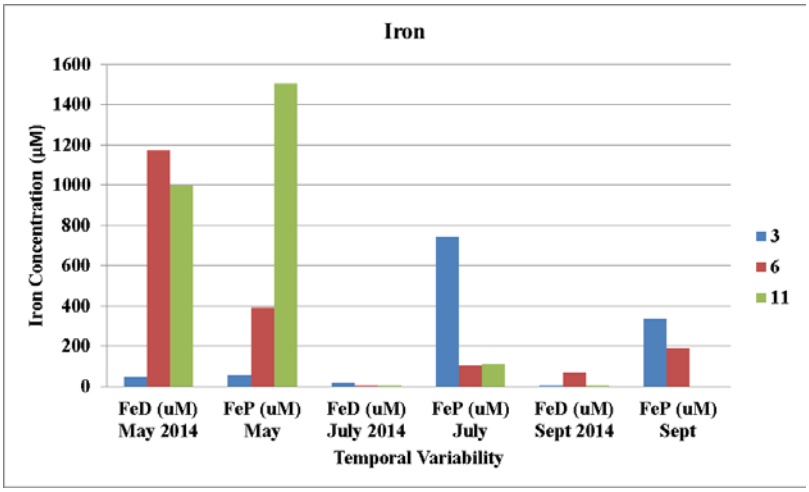

Figure 5. Graph demonstrating dFe and pFe concentrations from fluid samples collected at three time points in 2014. Samples were collected from three representative study sites, freshwater (Site 3), tidal (Site 6), and marine (Site 11).

Table 2. Summary of Fe- and Mn dissolved and particulate temporal concentrations from 2014.

	May 2014					July 2014					Sept 2014				
Site	**FeD (uM)**	**FeP (uM)**	**Mn(III) (µM)**	**MnD (µM)**	**MnP (µM)**	**FeD (uM)**	**FeP (uM)**	**Mn(III) (µM)**	**MnD (µM)**	**MnP (µM)**	**FeD (uM)**	**FeP (uM)**	**Mn(III) (µM)**	**MnD (µM)**	**MnP (µM)**
3	48.36	59.78	0.344	5.1	0.24	18.89	744.39	#N/A	1.87	111.29	0.73	338.22	0.39	5.07	3.44
6	1174.98	393.03	0.05	58.43	1.91	2.84	102.92	#N/A	50.72	10.61	68.33	192.98	4.65	20.95	2.54
11	998.63	1507.43	#N/A	35.22	5.41	1.95	113.4	#N/A	26.84	9.21	6.79	#N/A	0.37	6.16	#N/A

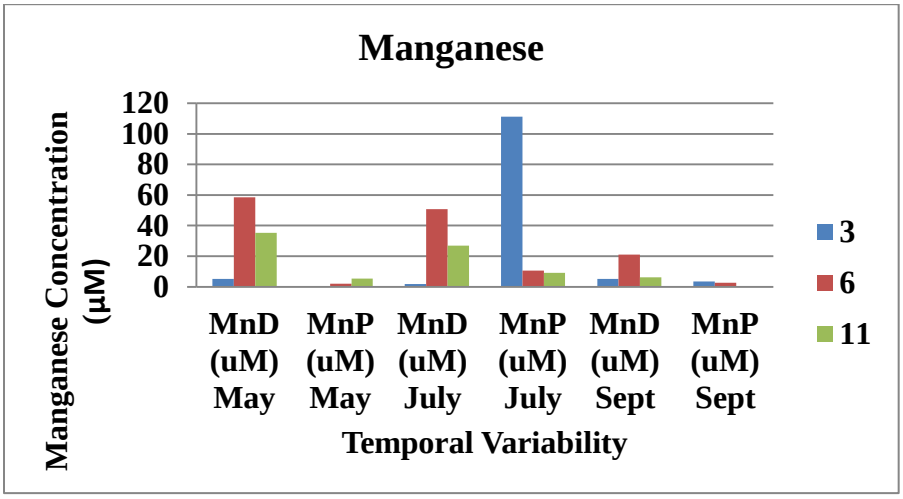

Figure 6. Graph demonstrating dMn and pMn concentrations from three representative freshwater (Site 3), tidal (Site 6), and marine (Site 11) study sites. The increase in MnD along the salinity gradient is evident, as is the significant decrease in MnP during the recharge event.

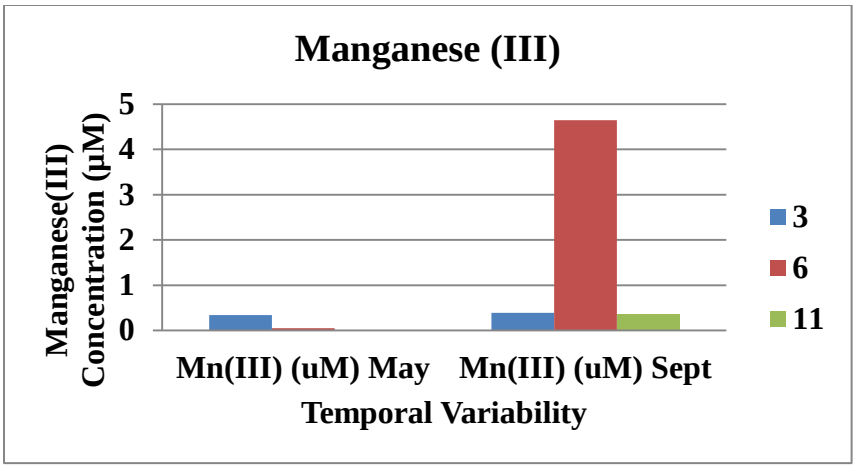

Figure 7. Graph demonstrating Mn(III) concentrations from three representative freshwater (Site 3), tidal (Site 6) and marine (Site 11) study sites during the groundwater recharge event (May 2014) and during the rebound period (September 2014).

Sediments from all sites, except the upstream freshwater Site 3, demonstrated a positive reaction when assayed with LBB. Surface sediments from Site 6 did not react to LBB, however subsurface sediments, 5 mm depth, had a positive reaction (Fig. 8). Suspended particulates concentrated on filters and assayed with LBB demonstrated a positive reaction indicting the presence of Mn(III/IV) oxides in cold-seep fluids.

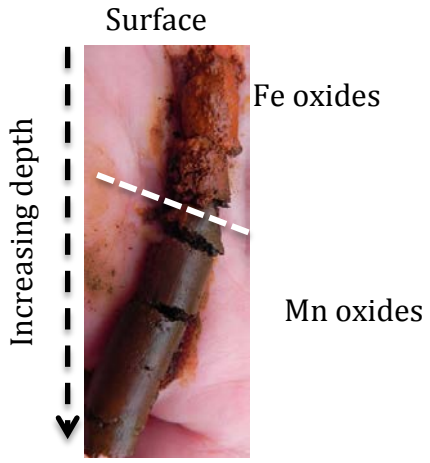

Figure 8. Core of mineral mound from Site 6 illustrating the occurrence of Mn at shallow depths.

Isotopic Carbon Fractionation

Cold-seep fluids had a positive $\delta^{13}C$ signature indicating that dissolved inorganic carbon in the groundwater originated from the limestone capping this region and not from a biological source, which would have a negative $\delta^{13}C$ signature (Table 3).

Cold-seep fluids had a less positive $\delta^{13}C$ signature after the recharge in May 2014, as fluids in the groundwater aquifer were enriched with dissolved inorganic carbon due to an increase in dissolution of limestone (Groves and Meiman, 2013). After the recharge event the system returned to a steady state conditions; this is referred to as rebounding of the system. Further analysis will show what the equilibrium for the system is, however more data is needed to make that assessment.

Table 3. Table of $\delta^{13}C$ fractionation along Soda Bay River.

Site	$\delta^{13}C$ May	$\delta^{13}C$ July	$\delta^{13}C$ Sept
3	3.2	1.7	no data
10	3.7	2.8	no data
6	1.4	0.8	1.9
8	2.8	2.6	1
11	0.5	0.7	no data
13	no data	1.6	0.9
Mn	0.9	3.3	no data

Cell Numbers

Enumeration studies were used to estimate cell density as a function of groundwater discharge. Cell enumeration revealed relatively low cell counts from microbial mats collected during the spring recharge event, with cell densities from 2.0 x 10^3 cells per gram in freshwater mats to $3.8x10^4$ cells per gram from mats collected from the tidal/marine region of the system. Cell counts of mat samples collected in the fall, during the rebound period, increased at each site. Cell densities averaged from $2.0x10^4$ in the freshwater mats to $3.7x10^4$ cells per gram in the tidal/marine mats (Fig. 9). This method has limitations as most cells are encrusted with minerals making staining and subsequent enumeration difficult.

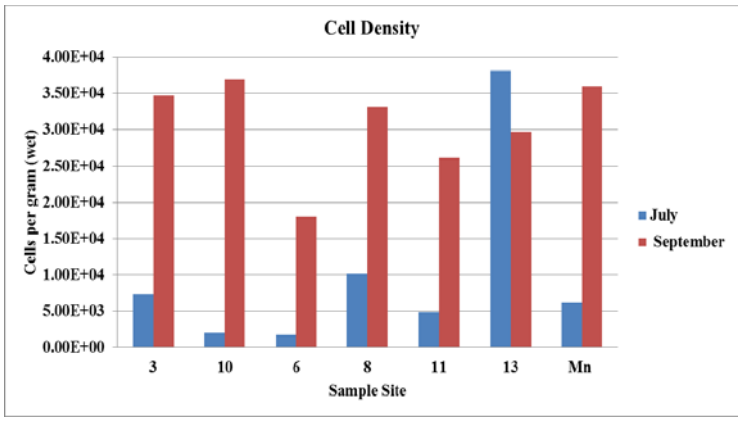

Figure 9. Graph indicating a decrease in cell density per gram (wet) during the July recharge event and increase in cell density during the September rebound period. With the exception of Site 13 located in the estuary.

Molecular Microbial Ecology

Community Diversity Along Salinity Gradient

Temporal results from T-RFLP analyses of samples collected twice annually over four years from the entire ecosystem demonstrated five phylogenetically distinct clusters. Clusters one, two and three are predominantly from marine and estuarine habitats (blue), while clusters four and five are freshwater habitats (green) (Fig. 10). Closer examination of the temporal data, revealed a clear bifurcation between fresh water and marine/tidal ecosystems. However, occasionally an anomalous result with a fresh water sample clustering within the marine data set was evident. There are a few possible reasons for this: Co-mingling of freshwater and marine microbial communities, climatic forcing due to increased precipitation washing out communities, or transport in subsurface groundwater conduits suggesting that the entire ecosystem is connected.

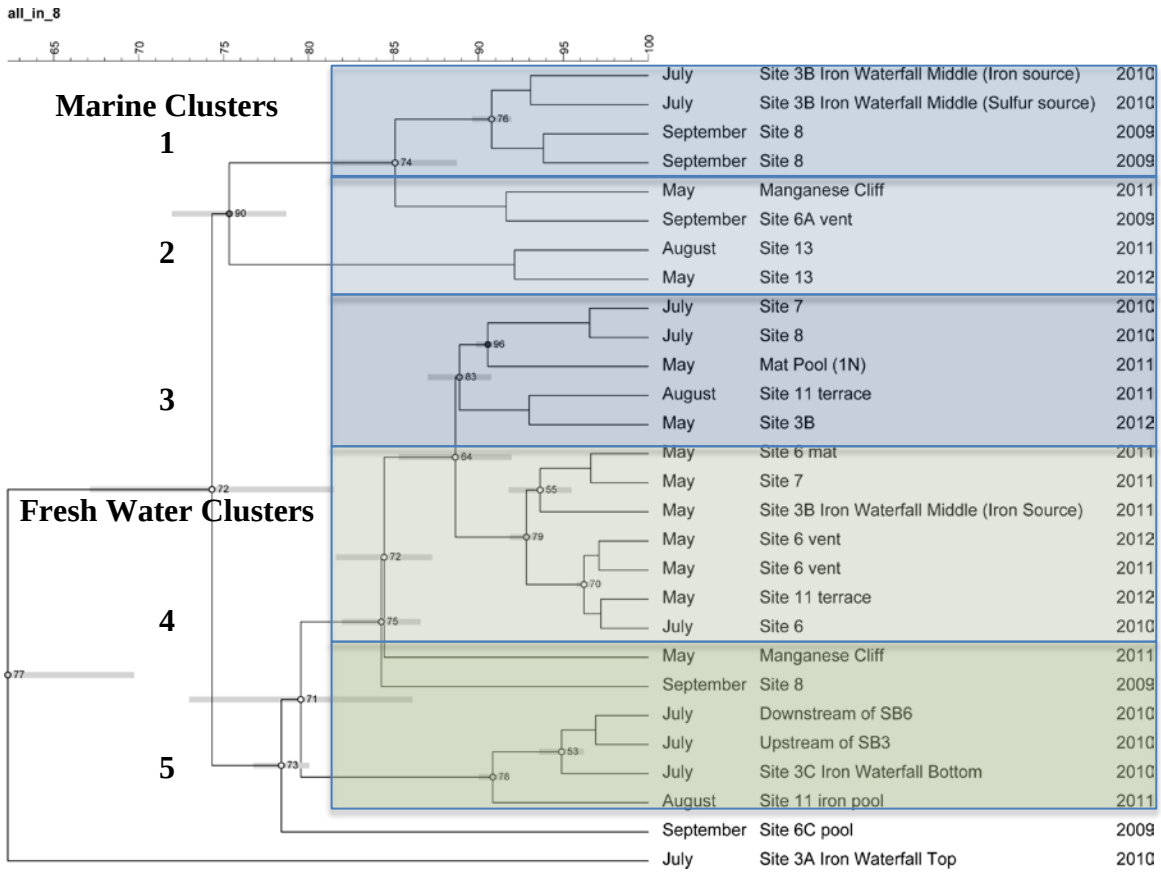

Figure 10. T-RFLP of cold-seep microbial communities from mats along the salinity gradient. Marine sites colored blue, fresh water sites colored green. Grey horizontal bars represent the margin of error for each cluster.

Quantitative PCR

QPCR to quantify *Zeta-proteobacteria* along the salinity gradient at Soda Bay revealed their presence in very low abundance in both freshwater and tidal cold-seeps with a higher abundance present in the marine region at Site 13 (Fig. 11).

QPCR for the Form II RuBisCO *cbbM* gene detected the gene at all sites along the system, however cbbM genes were most abundant from microbial mats in the marine region of the system at Sites 11 and 13 with 7 x 10^2 to 7 x 10^3 copies per gram (Fig. 12). Study Sites 6 and 8, which experience moderate tidal influence, had lower copy numbers of *cbbM* genes present, while none of the mats from freshwater region showed the presence of *cbbM* genes.

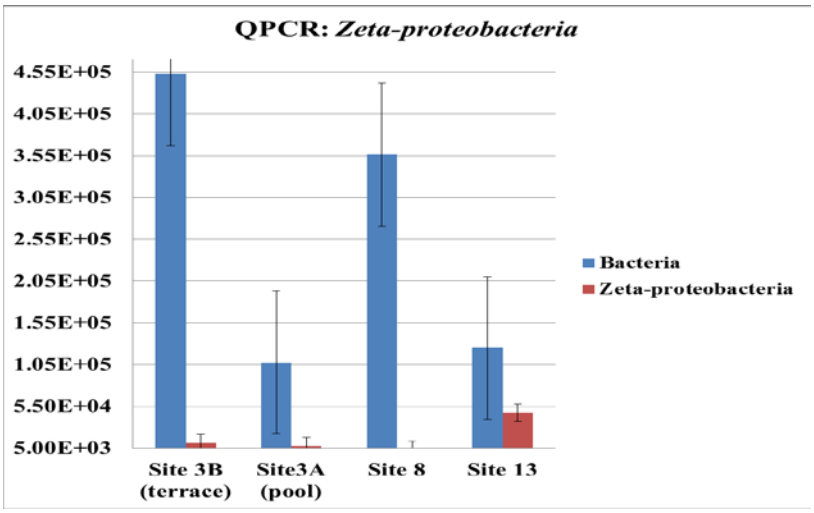

Figure 11. QPCR results of SSU phylogenetic genes for Bacteria and *Zeta-proteobacteria* (red) from cold-seeps along the salinity gradient of the Soda Bay River.

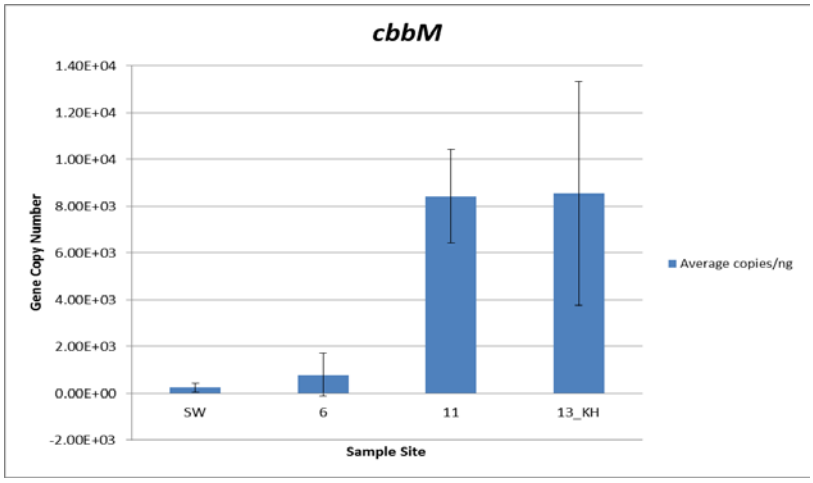

Figure 12. QPCR results of RuBisCO cbbM gene copy number in mats from a freshwater site (SW), a tidal Site (6) and two marine Sites (11 & 13), *cbbM* genes are more abundant in the marine sites.

Metagenomic Analysis of Site 13

Metagenomic analysis was done on microbial mats collected in July 2011 from Site 13 located in the marine region of the system. Using EMIRGE we found that Site 13 was extremely phylogenetically diverse with the apparent co-mingling of both fresh water and marine Fe-oxidizing bacteria (Fig. 13). Four percent of the population was comprised of archaea with the remaining 96% bacteria.

We identified both potential chemotrophic and heterotrophic microorganisms capable of completing a microbial Fe cycle, with both organisms previously identified as

Fe oxidizers and reducers present within the biofilm community. Neutrophilic Fe-oxidizing bacteria identified were from both fresh water and marine genera, *Gallionella*, *Leptothrix*, *Thiobacillus*, and *Methylophaga*, and in the division *Zetaproteobacteria*. There was one Fe reducing bacterium identified from the metagenome, *Albidiferax*, which is known to exist in both fresh water and marine environments (Lu et al., 2013).

Mn oxidizers and reducers identified were all chemotrophs known to occur in both fresh water and marine environments: *Erythrobacter SD21*, *Roseobacter*, *Pseudomonas putida*, and one Mn reducer was identified *Shewanella*.

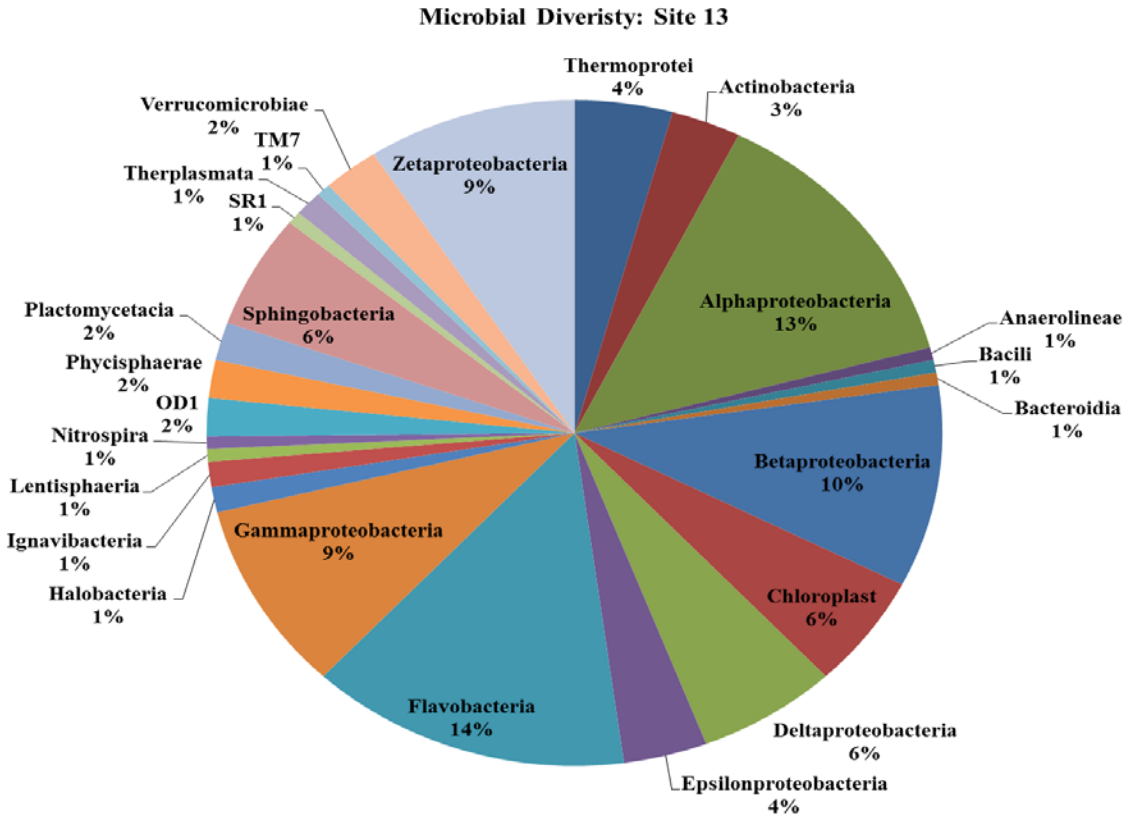

Figure 13. Graphic distribution of microbial diversity from Site 13 using EMIRGE.

Enrichment cultures

Enrichment cultures inoculated with samples from all sites along the lotic to marine ecosystem were conducted to determine the presence of microorganisms capable of producing biogenic Fe oxides and to identify the probable mode of carbon assimilation. Fe-oxidizing bacteria produced two colony morphologies; they produced

bands by growing in one plane that possessed optimal oxygen or Fe concentrations or in clumps that slightly extended vertically along the oxygen gradient. Uninoculated controls were used to observe abiotic Fe oxidation and to compare and confirm the presence of biogenic Fe oxides from enrichments.

PCR using universal small subunit primers for archaea and bacteria SSU ribosomal RNA genes were done on each colony/band to determine the presence of archaea or bacteria. There were no amplicons produced using the archaeal primer pairs, however, all samples yielded a bacterial amplicon, indicating that Fe oxidation in the enrichments was likely bacterial.

Microscopy

Mineralogy

Microscopy of petrographic thin-sections of core samples collected along the Soda Bay salinity gradient was done to observe mineral textures from cold-seeps. The texture and composition of a rock deposit relays important information about the environment in which the deposit formed. Petrographic examination of cold-seep core samples provided a glimpse into the current environmental conditions at Soda Bay. Core samples collected and analyzed in 2010 could be correlated with the occurrence of the climatic phenomena known as El Niño and La Niña, allowing us to observe the environmental impacts on the geochemistry through observing changes in the composition and morphology of minerals deposited and microbial ecology of cold-seeps along the Soda Bay ecosystem.

Changes in the composition of mineral deposition is most evident in core samples collected from Site 6, a rapidly flowing deep seep that is located in the moderate tidal region along the bank of the river. This site is submerged under about 1.5 m of seawater during high tidal events such as those that occur during the winter. During low tide, this site sits about 3 m above the river. Minerals formed during high tide are fine grained due to the rapid dilution and flushing of vent fluids as seawater covers the vent. Fine-grained salt minerals become entrained in the mineral matrix allowing us to identify tidal events and season (fall/winter). Minerals formed from seep fluids are large grained and composed primarily of Fe and Mn oxides and carbonates. During the spring/summer

when there are relatively low tides and low precipitation mineral grain sizes are larger. Observed changes in the composition of minerals deposited and the alternating pattern of iron deposition from Site 6 core samples captured what appears to be a three-year climatic cycle and environmental conditions from examination of (Fig. 15). Iron deposition occurs in the spring/summer when tides are shorter in duration allowing for the growth of large Fe minerals, in contrast to the small grain sizes of Fe oxides in the winter when tides are stronger and last longer preventing growth of Fe oxides. Using the temporal banding pattern of iron and carbonate minerals from core samples we can then date the bottom of the core as representing 2007 – 2008, during which time there was a very weak La Niña event occurring. The second portion of the core represents 2008-2009, which experienced a moderate La Niña event, resulting in an increase in precipitation during the spring/summer. There is a slight increase in the grain size of the Fe oxide mineral due to the increase in vent fluid discharge. The Fe oxide has a less dense structure with more visible pore spaces. The increase in discharge also changed the composition of the minerals deposited with an increase in Mn oxides. The top portion of the core represents, 2009-2010, when a moderate El Niño event was occurring. There was less precipitation resulting in formation of relatively large Fe oxide minerals.

The core sample from the estuarine Site 8 show minerals that are fine grained due to the long duration of tidal events in which the cold-seep is submerged under seawater for longer time intervals, decreasing the time from Fe oxide formation to occur (Fig. 14).

The Mn mound has two mineral formations: i) a ferro-manganese breccia, and ii) a manganese carbonate crust. The breccia is composed of large ferro-manganese grains in a carbonate matrix. The surface of the ferro-manganese grain shows weathering from dissolution by the slightly acidic vent fluids.

XANES analysis of rock formations from Soda Bay show a rock composed of mixed valent Fe and Mn. The reduced metals in these rocks may serve as energy sources for microorganisms. X-ray mapping of the green schist illustrates the low concentration of Mn present in the +2, +3, and +4 oxidation states (Fig. 16). Fe is present in high abundance also in both reduced and oxidized forms. The highest concentration of Fe oxide was present on bedrock surfaces due to weathering.

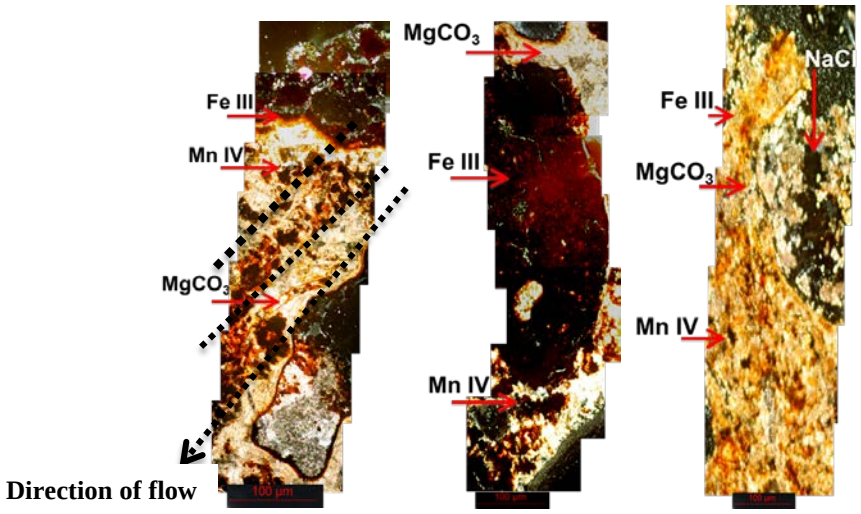

Figure 14. Petrographic thin-sections of cores from seeps along the salinity gradient. (Left) Oblique thin-section from fresh water site, ferromanganese minerals are encased in a magnesium carbonate matrix. Mineral layering shows subtle changes in geochemistry, dashed line indicates flow direction. (Center) Thin-section from tidal site, note the ferromanganese minerals are larger. This is due to the ability of Fe- and Mn to precipitate from the seep during low tide allowing minerals time to grow. (Right) Thin-section from marine site, notice the granular texture due to small mineral size. Minerals here do not have much time to grow as they submerged in seawater most of the time preventing accumulation of precipitates. Salt inclusions are present in these samples (arrow).

Figure 15. Thin-section of core from the tidal Site 6. Minerals are composed of Fe and Mn oxides and carbonates. Core samples from this site captured a three year climatic cycle and environmental conditions

The top portion of the core represents, 2009-2010, when a moderate El Niño event was occurring. During the spring/summer when the sample was collected there are relatively low tides and decreased precipitation allowing mineral grains to grow larger.

The middle of the core represents 2008-2009, which experienced a moderate La Niña event, resulting in an increase in precipitation during the spring/summer. There is a slight increase in the grain size of the Fe oxide mineral due to the increase in vent fluid discharge. This increase in precipitation resulted in an increase in the discharge in mineral saturated fluids. The iron oxide has a less dense structure with more visible pore spaces. The increase in discharge also changed the composition of the minerals deposited with an increase in Mn oxides.

At the bottom of the core represents 2007 – 2008. During this time there was a very weak La Niña event occurring. The minerals formed during this time have a regular alternating pattern of deposition.
Layers are dated by the rate of mineral formation with less iron deposition in the winter months when the tides are higher and the site remains under water most of the time.

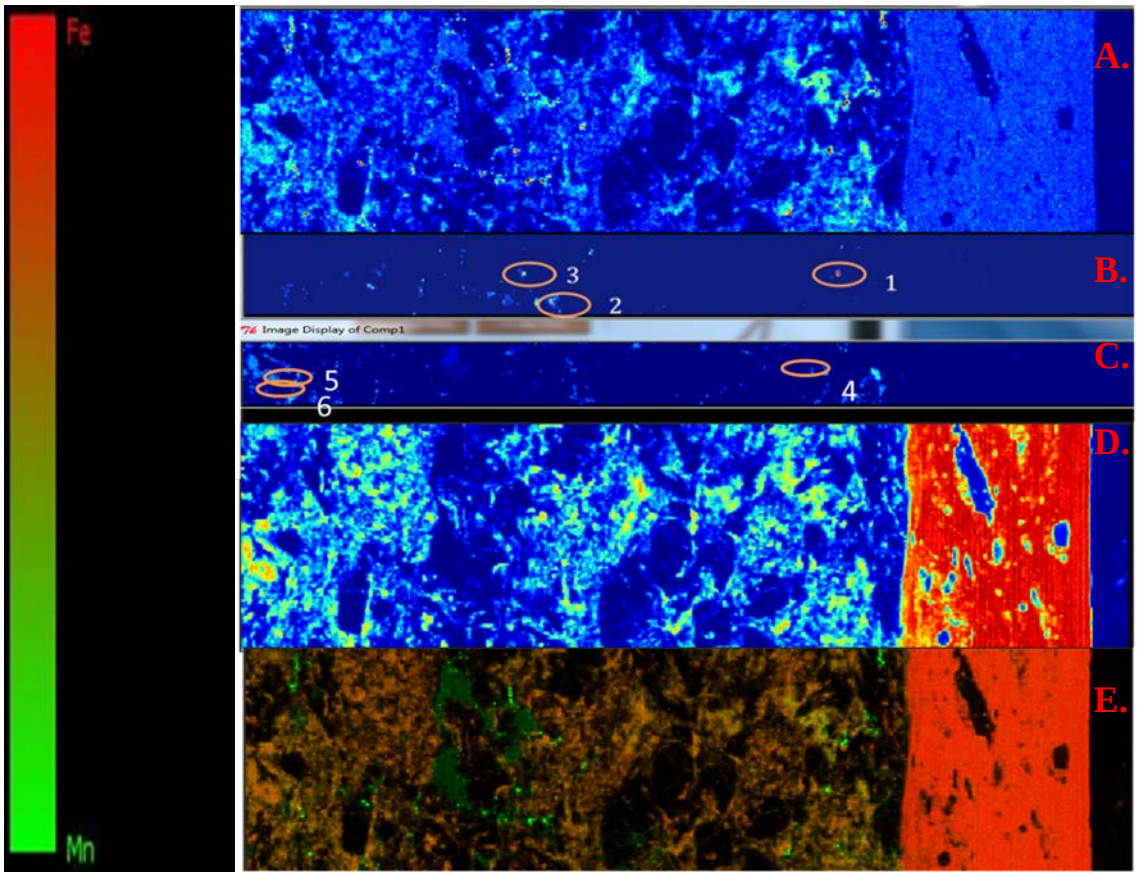

Figure 16. XANES of metamorphic green schist parent rock underlying the carbonate cap from POW. To the left is a color scale indicating the presence and intensity of Fe (red) and Mn (green). A) X-ray map of rock showing the distribution of Mn(II/III/IV) (green). B) Numbers 1 - 3 indicate oxidized Mn. C) Numbers 4 - 6 indicate the presence of reduced Mn. D) X-ray map showing the distribution of Fe. Rocks are composed of more Fe, the strong signal (red) to the right of the figure is the surface exposed to the environment. E) X-ray map showing distribution of both Fe and Mn.

Scanning Electron Microscopy

SEM coupled with EDS of microbial mats allowed for the characterization of the mineral morphology, the microbe-mineral associations, and the elemental composition. We found that the effects of Mg^{2+} ions influenced the morphology of the calcite minerals formed, resulting in magnesium carbonate ($MgCO_3$) minerals that deviated from the typical calcite rhombohedra growing along the long axis forming a trigonal trapezohedron (Fig. 17A). This modification indicates that there is a high concentration of Mg^{2+} ions since small amounts of Mg^{2+} ions (Mg^{2+}/Ca^{2+} = 1:10) are not able to exert such effects (Kim et al., 2012). Microbial cells were attached to the surface of the $MgCO_3$

minerals and showed signs of mineral dissolution (Fig. 17B-D). Fe and Mn oxides formed in conjunction with carbonate minerals (Fig. 17?). Microbial cells were heavily encrusted with Fe and Mn oxides due to rapid mineral deposition.

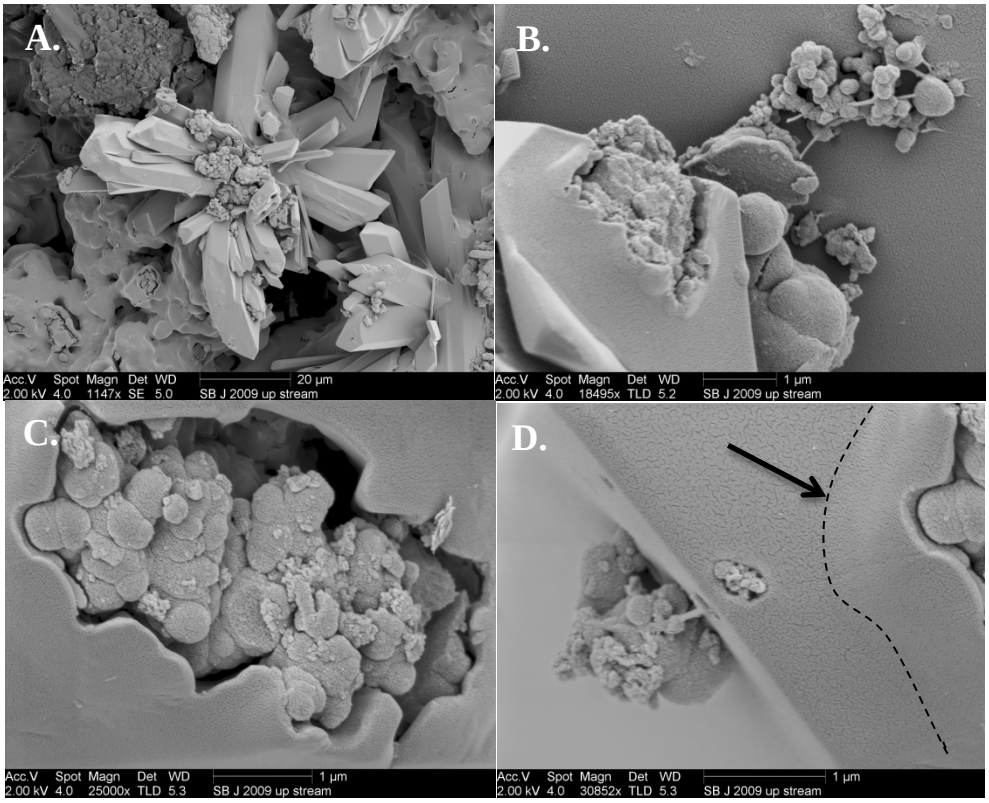

Figure 17. SEM micrographs of A) magnesium carbonate crystals and B-C) attached microorganisms. D) Microorganisms attached to grain surfaces appear to be dissolving mineral grains as evidenced by the textural changes of the mineral where microbial cells are attached (arrow).

Transmission Electron Microscopy

TEM analysis of ultra-thin sections allowed for the characterization of the various stages of microbial fossilization from the initial stage of mineral precipitation to formation of a microfossil (Fig. 18). TEM revealed a variety of Fe oxides associated with microorganisms. Nano-particulate Fe oxides were identified on the outer surface of cell walls and in some instances inside the periplasm, preserving the structure of the cell envelope and thereby forming a microfossil (Fig. 18A). The stages of microfossil formation began with the formation of nano particulate Fe oxides on the cell or in the

EPS (Fig. 18B), with time more nano particles form and the cell becomes completely encased in nano particulate Fe oxides (Fig. 18C). As cells begin to die cell walls become leaky, allowing dissolved Fe(II) to infiltrate the cell and Fe oxides begin to accumulate inside the cell preserving what remains of the cell wall (Fig. 18D). Eventually the cell is replaced with oxides forming a bona fide microfossil.

The identification of biogenic Fe oxide stalks (Fig. 18E) suggests the presences of known Fe oxidizing stalk forming bacteria such as *Gallionella* spp. and *Mariprofundus* spp. Biogenic Fe oxides were identified in EPS surrounding microorganisms, in which an extensive and intricate net of nano-particulate Fe oxides formed around a centrally located microorganism. The microorganisms observed were either cocci or filaments. Fe oxide minerals that were not associated with microorganisms or EPS had a solid smooth appearance (Fig. 18F).

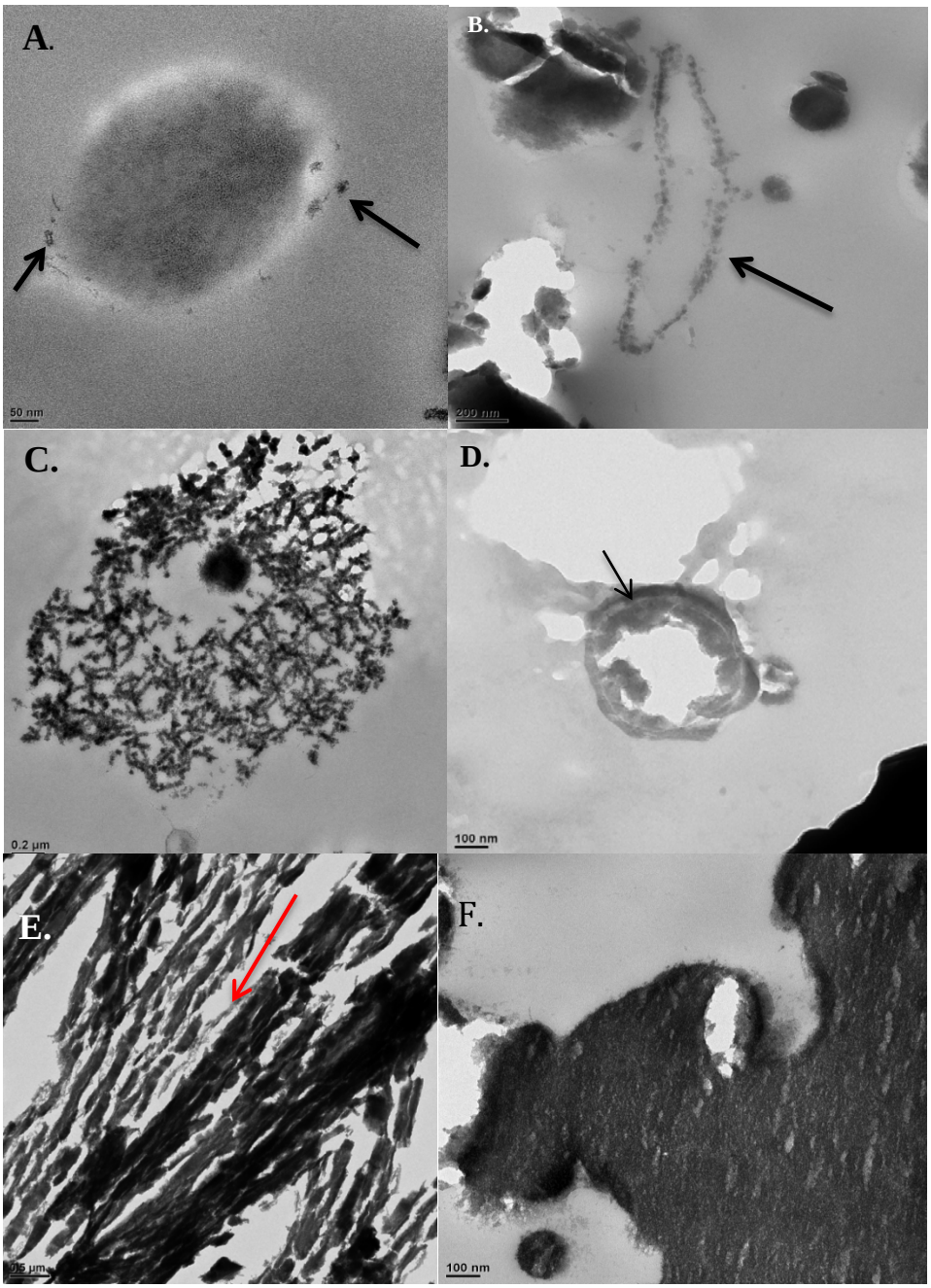

Figure 18. TEM micrographs of a microbial mat from Site 13, illustrating the progression of biomineralization and microfossil formation. A) Microbial cell in the initial states of being encrusted in nano-particulate Fe oxides (arrows). B) A cell that being encased in Fe oxides. C) A encrusted cell and EPS matrix. D) A microbial microfossil that is completely preserved, the white ring is the cell wall (arrow). E) TEM of Fe oxide stalks produced by Fe oxidizing bacteria notice EPS (arrow). F) Abiotic Fe oxide demonstrating the difference in mineral texture from that of biogenic Fe oxides.

Optical Light Microscopy

Optical light microscopy of Fe oxide mats indicated that Fe oxides were morphologically similar to a variety of Fe oxide sheath and twisted stalk structures typical of Fe oxidizing bacteria (Fig. 19). Two twisted stalk morphologies were identified, i) those with a single cell associated with a single stalk and ii) those that have two daughter cells located at the growing end of the stalk which splits when the cell divides forming two stalks.

Fluorescent microscopy using the fluorescent nucleic acid dye Syto-13 showed that microorganisms were closely associated with both Fe- and Mn oxides from all sample sites and that the mats have distinct textures (data not shown). The mat from Site 13 was comprised primarily of microbial cells associated with a loose Fe oxides comprised of long iron oxide stalks. Mat samples from sites with no tidal mixing (e.g., Site 3) were composed of fine textured minerals with loose clumps of iron oxides held together by EPS. Samples formed in regions with moderate tidal influence formed a coarser mat with large mineral grains incorporated into the EPS matrix.

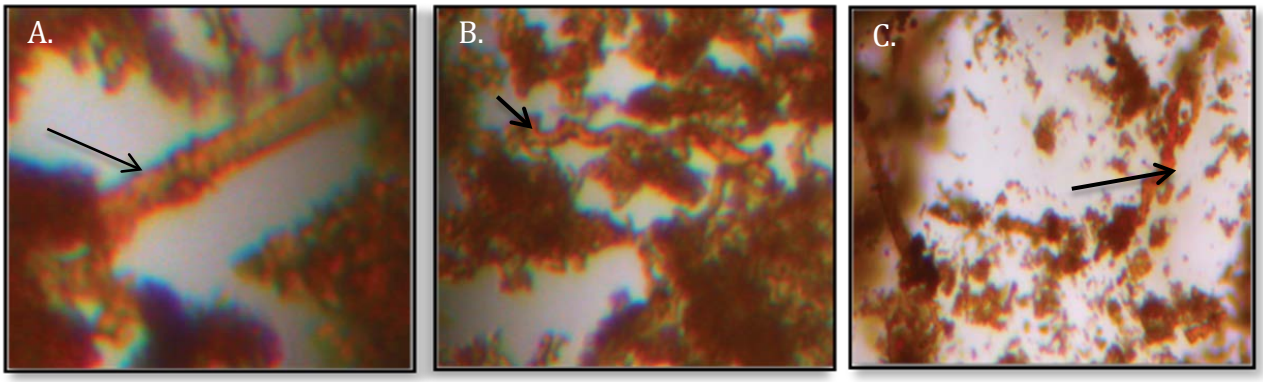

Figure 19. Confocal micrographs demonstrating the various morphologies of biogenic Fe oxide stalks and sheaths. A) Fe oxide sheath, B – C) twisted stalks (arrows).

Discussion

The Soda Bay River watershed is a dynamic lotic to marine ecosystem that provides a good opportunity to investigate Fe oxide biomineralization and how it responds to changing environmental conditions. There is a rapid and robust change in the composition of the groundwater geochemistry in response to inputs from precipitation

and runoff of surface waters, which brings about changes in the geochemistry, microbial diversity, and Fe oxide deposition on short time scales. Where the Soda Bay River enters the marine waters (Site 13) we discovered the first known shallow neutrophilic Fe-oxidizing *Zetaproteobacteria*. We also discovered mixed communities of marine and fresh water Fe-oxidizing bacteria from the microbial mats at this Site. Other studies reporting co-mingling of neutrophilic Fe-oxidizing bacteria found less than one percent of the population were freshwater bacteria (Fleming et al., 2014). The ability of these microorganisms to co-mingle is due to the physical mixing of marine and fresh waters. The continual percolation of groundwater from seeps allows for the presence of freshwater Fe-oxidizing bacteria and the overlying seawater allows for the presence of marine Fe-oxidizing bacteria. Soda Bay cold-seeps are enriched in inorganic carbon and dissolved metals making it anomalous relative to what is typically found in most oligotrophic groundwater and marine ecosystems.

Climatic data (USGS) and data from the Sea-bird sensor recorded an increase in precipitation for Southeast Alaska when samples were collected in 2014. Sensor data recorded an increase in river discharge during the spring when there was an increase in precipitation and the subsequent decrease in river discharge during the late summer as the system rebounded. These findings were important for analysis of the geochemistry and cell numbers measured during this period as the groundwater system responds rapidly and increased discharge strongly influences the concentrations of dissolved and particulate metals and cell numbers in the effluents. Studies of subsurface microorganisms and their influence on geochemical transformations had been over-looked until it was shown that microorganisms are active and relevant for geochemical processes. They are involved in weathering and formation of minerals and store important quantities of carbon, nitrogen and phosphorus in their biomass. They are responsible for a majority of turnover of energy and matter in the subsurface and thus have a profound impact on groundwater chemistry.

We found that dissolved Fe(II) and Mn(II/III) are the dominant metal species in cold-seep fluids, which may serve as potential energy sources for the microorganisms that inhabit microbial mats, associated with seeps. In the marine region of the system, the concentration of FeD decreases and particulate Fe oxides dominate due to flocculation of

Fe upon mixing with seawater. This system is predominantly a Fe-depositing system due to the enrichment of Fe from the underlying metamorphic green schist. However, layers of Mn oxides were identified at shallow depths at the oxic-anoxic interface.

Molecular analyses examining the carbon cycle at Soda Bay revealed the presence of several carbon cycling pathways, with microbial mats composed of chemoautotrophs,. Groundwater fluids are supersaturated with dissolved CO_2 from the dissolution of limestone capping POW Island providing an inorganic carbon source, while surface waters from the nutrient rich muskegs provide organic carbon.

Microbial diversity was observed using both SSU genes from metagenomic data and from T-RFLP fingerprinting of mixed microbial populations over spatial and temporal scales (Liesack and Dunfield, 2004). T-RFLP cluster analysis revealed a bifurcation with two major clusters corresponding to fresh water and marine microorganisms (Fig. 10). EMIRGE analysis of SSU genes shows a complex highly diverse microbial community from the marine Site 13 cold-seep. Microbial mats were comprised of bacteria, 96%, and archaea, 4%. Site 13 experiences steep chemical gradients due to the rapid changes in salinity and metal concentrations due to tidal flux, which probably accounts for the diverse microbial community found there.

Characterization of the geochemistry, microbial diversity and the process of Fe biomineralization from cold-seeps along the salinity gradient of this ecosystem have allowed us to add to our understanding the mechanisms for biomineralization, the preservation and identification of microfossils from ancient geologic deposits, and the microorganisms involved in the geochemical cycling of Fe. Our findings of microfossil formation from an Fe depositing ecosystem provides insight as to the environmental conditions in which biosignatures are preserved allowing us to hypothesize as to the environmental conditions in which ancient BIF deposits formed in shallow seas on an early Earth and to better understand the role microorganisms played. The potential for microfossil preservation is high at Soda Bay due to the elevated concentrations of dissolved Fe(II) and Mn(II/III) and the presence of Fe and Mn oxidizing bacteria. EM analysis allowed us to characterize the progression of microfossil formation and for the identification of biogenic Fe oxides.

Form II RuBisCO has a simple structure in comparison to Form I RuBisCO: it consists of only large subunits, having different catalytic characteristics, and only functioning well at low oxygen and high CO_2 concentrations, conditions that reflect the ancient earth atmosphere. Those characteristics suggest that the more complex Form I derived from Form II (Jesser et al., 2015; Tabita et al., 2007; Tabita, 1999). Form II occurs in some chemolithotrophs and phototrophs.

Our findings have allowed us to characterize the biogeochemistry of cold-seeps at Soda Bay observing spatial and temporal variability along a lotic to marine ecosystem, microscopic characterization of biosignature formation and microfossil preservation.

Acknowledgements

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Chapter 5

Biofilms From A Manganese Depositing Hot Spring In Yellowstone National Park

Abstract

Microorganisms that produce biofilms in hydrothermal springs experience environmental extremes (pH, temperature, and nutrient availability) that directly influence microbial biodiversity, gene expression, three-dimensional biofilm architecture, and biogeochemical metabolic processes. Prior studies of hot-spring mineral deposits suggest that biofilms have a direct influence on the texture and formation of mineral deposition just as the environment has a direct and profound influence on the complexity of the biofilm community in which distinctive microbe-mineral assemblages or biofacies are formed. The fate and behavior of redox-reactive chemical species is closely linked with the metabolic processes of microorganisms. The microbial diversity of hyperthermophilic microorganisms inhabiting a circumneutral (pH 6.91 to 7.82) near-boiling Mn-depositing hot-spring in Yellowstone National Park was studied to better understand its influence on biogeochemical cycling of inorganic carbon and metals as well the preservation of biosignatures from these types of environments. In an effort to gain a better understanding of the effect biofilms, have in a Mn depositing hot-spring we studied biofilms attached to natural and artificial substrata. This study was carried out by conducting *in situ* experiments allowing us to characterize the community composition, rate of biofilm formation and mineralization, community composition, and how the microbial communities influence the biogeochemical cycling of Mn(II/III/IV) in this hot-spring. Microorganisms present in biofilms from this ecosystem belong to known bacteria including *Aquificales*, *Proteobacteria*, and *Deinococcus-Thermus*; the most abundant archaea were the *Crenaraechota*. Some of the microorganisms from the biofilms were unrelated to any previously cultured organisms.

Introduction

Reduced minerals present an ideal source of bioavailable chemical energy for microorganisms (Marusenko et al., 1987) and the abundance of reduced chemical species in hot-spring fluids offers an ideal environment for growth of thermophilic microorganisms. The chemical composition of hydrothermal fluids is driven by the local geology. In Yellowstone National Park (YNP) superheated pressurized groundwater interacts with the bedrock at depth dissolving minerals, and enriching hydrothermal fluids. These fluids are then saturated with

reduced chemical species, which precipitate resulting from the rapid fluctuations in temperature, pH, pressure, and oxygen concentration once fluids erupt from hot-springs.

Prior studies of YNP hot springs indicate that microbial biofilm communities in the near-boiling springs are closely associated with mineral deposits. The morphology of these nano-particulate minerals vary in different regions of the spring as a function of temperature, concentration of dissolved minerals in hydrothermal fluids, physical properties of flow (flow rate, fluid depth, surging vs. splashing) and composition of microbial community. One structure that typically forms in hot springs are spicules, which are mineral deposits that form elongated columnar structures, typically a few millimeters in diameter and several millimeters in length. Spicules are frequently colonized by thin biofilms encrusted in fine mineral grains from which these biofilms gradually become entombed and thereby directly influence the biofabric and morphological formation of the rock.

Biofilms are characterized as organized consortia of prokaryotic micro-colonies forming three-dimensional architectural structures attached to surfaces. Biofilms are held together by semi-solid hydrated extracellular polymeric substances (EPS) (Crang et al., 1988; Dolan, 2002). EPS matrices are hydrated structures, composed of up to 97% water, and serve a variety of functions within the biofilm by providing surface attachment, diffusion gradients, channels for waste and nutrient products, functional side chains for mineral nucleation, and temporary protection from dehydrating environments. EPS is composed of various polyanionic molecules such as proteins, glycoproteins, glycolipids and small amounts of extracellular DNA (eDNA) (Dykstra, 1993; Krümbein et al., 2003; Dolan, 2002), in which the composition provides specific chemical and physical properties thought to be directly influenced by environmental conditions of the biofilm community inhabiting this microenvironment. The three-dimensional biofilm architecture affects the porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability of the biofilm (Stoodley et al., 1999). EPS also traps and binds minerals, along with other environmental organic and inorganic molecules incorporating them into the biofilm architecture (Krümbein et al., 2003; Hugenholtz et al., 1998; Jörgensen, 1992; Crang et al., 1988). The aim of this study was to characterize microbial diversity, Mn oxidation in a hot-spring, biofilm formation and its role in Mn deposition along a temperature gradient in a variety of microenvironments within one hot-spring.

In situ microcosm experiments present an opportunity to investigate the rates of mineral deposition and biofilm formation onto substrate surfaces. Microcosm studies were conducted by deploying sterilized glass slides secured in aluminum trays along the temperature gradient. Immediately upon submersion into the hydrothermal fluids, a conditioning film begins to form on the exposed surfaces of the glass slides. The conditioning film is composed of EPS and dissolved organic and inorganic molecules, which readily adsorb onto surfaces and chemically modify surfaces by changing surface charge, hydrophobicity and surface roughness. These adsorbed molecules form the scaffold for the three-dimensional biofilm architecture. EPS composition is mediated by enzymes secreted by individual microorganisms within the biofilm community in response to environmental conditions; hence, the biofilm architecture is specific for the environment in which it is formed (Kostankioti et al., 2013). The conditioning film subsequently influences the rate and extent of biofilm attachment and maturation by increasing surface roughness and by decreasing shear forces allowing for the attachment of microorganisms (Sutherland, 2001). Biofilms form at inert solid/liquid interfaces, where dissolved gases and nutrients are easily obtained from the surrounding fluid environment. Biofilm attachment is not only affected by the substrate surface charge but also low Reynolds number hydrodynamics as bulk-flow of fluids cross the biofilm are governed by inertial forces that drive transport of dissolved compounds (Dolan, 2002; Jörgensen, 1994).

There are several stages to biofilm formation, and the transition from a planktonic to sessile lifestyle is complex. Biofilms form due to changes sensed in the environment and require the involvement of multiple regulatory pathways, which signal changes in gene expression with the up-regulating of factors for a sessile lifestyle, such as genes that trigger EPS production. Once microorganisms become irreversibly attached, they continue to grow, expanding laterally across the attachment surface as they mature. With continued maturation, biofilms begin to expand vertically forming micro-columns and spicules, composed of microbes, EPS, minerals, and other molecules from the environment.

LWCGNN050 (reference designation in Yellowstone Coordination Network database), referred to as Purple Pool, is a small persistent gently surging hot-spring that deposits Mn oxides with temperatures ranging from 92.2°C in the main pool at the source vents to 75.3°C in the shallow outflow channels. This spring is located in the Lower Geyser Basin, of the White Creek Group Thermal Complex. The spring is relatively shallow (~100 cm) in the main pool and shoals

to 10 cm in the bifurcating outflow channel. Here we present results of microbial colonization studies conducted in Purple Pool at four sites along a temperature gradient (Table 1) (Fig. 1),where glass sides were deployed in aluminum slide trays (Fig. 2).

Table 1. Summary of temperatures, and pH from each sample site.

Sample Site	Temperature (°C)	pH	Fluid flow (high/low sheer)	Site Description
Vent 1	92.2?	6.91	high	Source vents from which hydrothermal fluids gently surge into the main pool from groundwater.
Vent 3	78.2	7.64	low	
Outflow 1	75.3	7.89	high	Shallow (25 cm) streams.
Outflow 3	72.2	7.64	low	

Figure 1. Field photo of Purple Pool (A-C) Source vents, (C-D) are outflow channels. A & B) High temperature Vents 1 & 2. C) Low temperature Vent 3. D & E) Outflow 1 & 2, respectively.

Figure 2. Glass slides deployed in the hot-spring to allow for microbial colonization.

Material and Methods

Collection and Fixation

Subaqueous rock samples and glass slides were collected using a chisel and/or tweezers (respectively). Glass slides were incubated in a slide tray in the spring from 1 to 48 hours and triplicate samples were preserved in RNAlater© solution for molecular analysis, 70% ethanol for optical microscopy, and in glutaraldehyde vapor to preserve the three dimensional architecture of the attached biofilm thereby reducing mechanical alterations from fluid shear during transport. Samples were stored at 4°C until processed in the laboratory. Vapor fixation was done by placing cotton saturated with 25% glutaraldehyde into the bottom of falcon tubes with samples. Chemical fixation allows for the preservation of DNA/RNA molecules, individual microbial cells, and the overall structural integrity of the biofilm architecture. With the knowledge that there are always induced artifacts, the ideal fixative should halt all cellular processes. Mechanical alterations from fluid shear prevent observation of the true biofilm architecture. Biofilm alterations that may occur include: 1) loss of biomass due to sloughing, 2) changes in cellular integrity, such as swelling and collapse and 3) overall quantity and integrity of EPS matrices affecting the three-dimensional biofilm architecture. Fixation stabilizes the structural organization of the attached biofilm allowing ultra-structural relationships to be preserved in satisfactory condition, and enhancing image contrast.

Microscopy

Optical Light Microscopy

Biofilms attached to glass slides were analyzed using a Zeiss Axioplan optical microscope with probe-appropriate excitation wavelengths. Leica Version 4.0 image-acquisition software (Leica Microsystems) was used for visualization and image capture. Cover slips were not used to prevent disturbing the attached biofilm while the topside of each slide was marked with a glass cutter to maintain proper orientation during analysis. A series of consecutive images were taken starting at the leading edge and ending in the middle of the slide. To enhance visualization of EPS, biofilms where prepared using cationic stains with a protocol modified from Erlandsen et al. (2000). Slides were sectioned into thirds by using a glass cutter, after which each third prepared for analysis using the cationic stains ruthenium red, alcian blue, and no stain for visualization of EPS and the fluorescent nucleic acid stains 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Benecia, CA) and syto-13 (Life Technologies) for total counts. DAPI fluoresces blue and was used at a final concentration of 25 $\mu g\ ml^{-1}$ with an excitation at 485 nm, while Syto-13 was used at 10 $\mu g\ ml^{-1}$ with an excitation at 488 nm.

Cation Staining of Polysaccharides

The cationic stains alcian blue and ruthenium red were used to stain biofilms attached to glass slides. Each of the stains have specific shape, size, and charge properties (Table 2). Alcian Blue is a large planar molecule, 4 nm, with a +4 charge, polyvalent water-soluble basic dye, exhibiting an intense blue color due to the copper center within the molecule. The molecule binds to and stains half-ester sulfur and carboxylated acid mucopolysaccharides and glycoproteins. Interaction with acidic functional groups results in the formation of an insoluble precipitant (Passow and Alldredge, 1995). Ruthenium Red is a small spherical molecule, 1 nm, with a +6 charge. This dye stains capsules and O-specific muco- and lipopolysaccharides. Stained sections were incubated for 2 hrs in a 0.15% staining solution that was made using 10 mM HEPES buffer Slides were rinsed in 10 mM HEPES two times to remove unbound stain and air-dried. Each stain binds to specific EPS side chains thereby allowing us to infer the chemical modifications in the EPS as a function to temperature and time.

Table 2. Cationic stain properties.

	Alcian Blue	Ruthenium Red
Shape	Planar	Spherical
Size	4 nm	1 nm
Charge	+4	+6

Petrographic Microscopy

Petrographic microscopy of thin-sections allows for characterization of mineralogy of oxides and visualization of templating of oxides onto microbial surfaces. Thin-sections were observed using a Zeiss petrographic microscope to characterize the morphology of Fe- and Mn minerals and the depth at which minerals formed. Biofilms attached to rock surfaces and fixed with glutaraldehyde vapor were prepared as 30 μm petrographic thin-sections, allowing for visualization of biofilms attached to rock surfaces (Spectrum Petrographic, Vancouver WA). Petrographic microscopy of thin sections was conducted to characterize the texture of mineral deposits and observe the distribution of Mn oxides.

Scanning Electron Microscopy (SEM)

SEM coupled with Energy Dispersive Spectroscopy (EDS) was used for the characterization and elemental analysis of Mn oxides and microbe-mineral associations. Specimens were analyzed on a FEI Sirion SEM. Fresh and older mineralized assemblages of rock were sampled for composition of morphology and ultra-structural features to observe whether significant differences were evident as a function of environmental conditions, such as temperature or pH. SEM provides a means of examining nano to micron scale features of mineral forms, crystal growth rates, oxidation state, microbe-mineral associations, spatial patterns of distribution, and composition of mineral grains as a function of microbial populations and local geochemical processes. SEM microscopy was conducted at the Center for Electron Microscopy and Nanofabrication, at Portland State University, Portland, OR. Specimens were coated using a Pelco 91000 sputter coater with a gold target to improve specimen conductivity. Specimens were observed at 5 kV and at a working distance of 5 mm.

Transmission Electron Microscopy (TEM)

Nanoscale cellular and mineralogical features were examined using a Zeiss Libra 120 TEM. Specimens were embedded in resin and sectioned via ultra-microtome to 50-70 nm sections, allowing for TEM analysis and subsequent characterization of ultra-structural features of microbe-mineral associations on cellular and polysaccharide surfaces. TEM analysis provides information about crystal structure of Mn-oxides and allows for imaging of crystallographic structures. Digital images were taken using a using a low accelerating voltage to reduce the potential of damage to soft materials. Electron energy loss spectroscopy (EELS) analysis was done to confirm and map the presence of Mn oxides. EELS analysis entails exposing a sample to a beam of electrons with a known, narrow range of kinetic energies. Some of the electrons will undergo inelastic scattering, losing energy in which the amount of energy loss can be measured using an electron spectrometer and interpreted to identify the element that is present. Samples were analyzed at Delaware Biotechnology Institute BioImaging Center, Newark, DE.

Enumeration and Particle Counts

Particle count analysis was conducted using ImageJ (http://imagej.nih.gov/ij/index) image analysis and processing freeware, to quantify the relative number of particles in the biofilm as a function of time showing maturation of biofilms as a function of time. Analysis was done using glass slides from specific time intervals. For each slide five consecutive phase contrast images, at 100x magnification, were used. Counts were determined for each sample by taking the average of the total counts for each of the five images. Particles were defined as anything larger than 10 pixels, such as microbial cells, mineral grains, and mineralized EPS attached to glass slides. Images were prepared for count analysis by first removing background noise by setting a standard threshold and batch processing images to reduce threshold bias per image. The threshold was set by removing all particles less than 10 pixels, resulting in a final binary image used for particle counts (Blackburn et al., 1998) (Fig. 3).

Enumeration studies were conducted to determine the presence and quantity of microbial cells within biofilm communities attached to substrates deployed in the spring effluents. This was done by staining sections of the biofilm with Syto-13 for 30 min after which unbound stain was rinsed away with HEPES buffer. Stained filters were visualized using epifluorescent microscopy. RNA fluoresces at an excitation/emission of 591⁄514, and DNA fluoresces at an

excitation/emission of 488⁄509. Mat and sediment specimens were stained and cells enumerated using the same technique to quantify cells associated with Mn oxides. Data was compared to the effluent enumeration data.

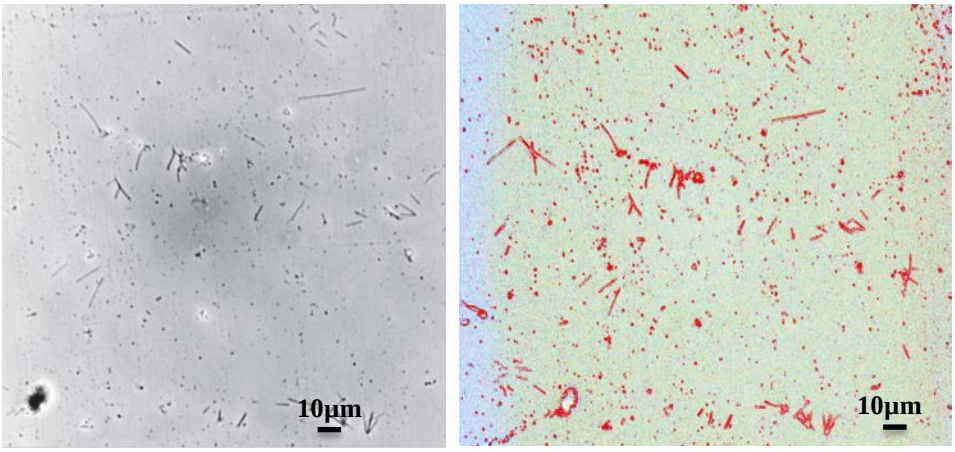

Figure 3. (Left) Phase contrast image of biofilm attached to glass slide before threshold is set, (Right) the same image after threshold has been set using ImageJ for particle counts of red objects.

Geochemistry

Chemical analysis was conducted using inductively coupled plasma mass spectrometry (ICP-MS) on cooled acidified hot-spring fluids from four sites along the system, to determine the concentration of total dissolved Mn(II) present in the water flowing from the source vent(s) and along the outflow apron. Hydrothermal fluids were collected and filtered using a 0.2 µm polycarbonate filter to remove particulates and microorganisms, fluids were stored at 4°C prior to analysis. Bulk fluid samples were split for chemical analysis of nutrient data and major and minor ions. Measured concentration are reported as total concentrations for Mn, Fe, NH_4, NO_3^-, NO_2^-, and Mo. Dissolved nutrient data was collected using an auto-analyzer; and metal concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS.) Auto-analyzer samples were filtered a second time using 0.45 µm combusted glass fiber filters and acidified using trace metal grade HCl and then diluted using ultra-pure Milli-Q water prior to analysis. Samples analyzed on the ICP-MS were acidified prior to analysis ultra-pure hydrochloric acid to a pH of 2 and stored at 4°C until analyzed.

Leucoberbelin Blue

Collected sediment and filter samples were transferred to 1 mL vials to which leucoberbelin blue solution (LBB, 65%, Sigma-Aldrich) was added. The LBB (410.5 g mol-1) stock solution is made by dissolving the crystals in Milli-Q water to a concentration between 1 to 4% (24 to 97 mM) and adding 40 mL of either 10 M sodium hydroxide (NaOH) or 21% ammonium hydroxide (NH_4OH) per 10 mL of solution. Working solutions are diluted into 1% acetic acid, to a range between 0.01 to 0.04% (240 to 970 M). LBB is a colorimetric solution used to determine the presence of Mn(III/IV) oxides. The presence of Mn(III/IV) oxides is confirmed when the LBB becomes oxidized turning a brilliant blue color (Tebo et al., 2007). Biofilms were tested for the presence of Mn(III/IV) oxides by pipetting 5 μL of 0.04% onto biofilms.

Molecular Methods

DNA Extraction

Samples collected on site were fixed in RNALater (Life Technologies, Grand Island, NY) and stored at 4˚C before being transported back to the lab and frozen at -80˚C. Genomic DNA (gDNA) was extracted from samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer's protocol with the modification that the gDNA was eluted into 10 mM Tris at pH 8. The purity and concentration of gDNA were determined with a Nanodrop ND-1000 spectrophotometer and diluted to ~10 ng/μL for downstream applications.

Community Diversity

Community diversity of biofacies was analyzed using terminal restriction fragment length polymorphism (T-RFLP) providing a fingerprint of the community for each sample. The methodology used for T-RFLP analysis has been reported in detail in many previous publications (Fleming et al., 2013; Davis and Moyer, 2008; Rassa et al., 2009). This technique accurately resolves populations in microbial communities of low to intermediate richness (Engebretson and Moyer, 2003) and has also been shown to be reliable for detecting changes in synthetic community compositions (Hartmann and Widmer, 2008). Electropherograms are imported into the program BioNumerics (Applied Maths), where community fingerprints are best-compared using average Pearson product moment correlation (Häne et al., 1993). Community fingerprints

were compared in the 50-500 bp range average Pearson product moment correlation and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis combining all eight restriction digests (Davis and Moyer, 2008). The primer set used was 68F-FAM (5' 6-FAM - TdNA dNAC ATG CAA GTC GdKdK CG 3') and 1492R (5' dKGdP TAC CTT GTT ACG ACT T 3'), with identical conditions as previously reported (Rassa et al., 2009). Three replicate PCR reactions were pooled, desalted, and split between eight restriction enzyme treatments using *Alu* I, *BstU* I, *Hae* III, *Hha* I, *Hinf* I, *Mbo* I, *Msp* I, and *Rsa* I (New England BioLabs, Ipswich, MA). Reactions were visualized with an internal LIZ-500 size standard by capillary electrophoresis on an ABI 3130xl genetic analyzer (50-cm capillary array, POP-6; Life Technologies, Grand Island, NY).

Clone Library

Five replicate SSU rRNA gene PCR reactions were pooled and cleaned as previously described (McAllister et al., 2011), with the modification that the forward primer did not contain a 5' fluorescent label. Desalted amplicons were cloned using the CloneJET PCR Cloning Kit following manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Clones were streaked to isolation and assayed for the correct size fragment using PCR with pJET1.2 forward and reverse primers. Clones with the correct insert were grown up in Terrific Broth with 100 µg mL-1 ampicillin and sent for sequencing at Beckman Coulter Genomics (Danvers, MA). Operational taxonomic unit (OTU) analysis was initially determined with 5' reads of the SSU rRNA gene (Tartof and Hobbs, 1987). Sequences were aligned using the ARB-SILVA database with SINA Webaligner (Pruesse et al., 2007), masked, and binned into OTUs based on 97% minimum similarity. At least one clone from each OTU was chosen for full-length sequencing and checked for chimeras using Pintail (Ashelford et al., 2005) and Mallard (Ashelford et al., 2006) software. Unambiguously aligned full-length sequence phylogenetic placements were calculated using RAxML version 7.2.6 (Stamatakis, 2006) with the General Time Reversible (GTR) model of nucleotide substitution, and the GAMMA model of rate heterogeneity.

Quantitative PCR: Community Composition

Quantitative polymerase chain reaction (QPCR) a real-time PCR technique was used to amplify and simultaneously quantify bacterial-archaeal ratios. The method used was for the

detection of products using sequence-specific DNA probes consisting of oligonucleotides labelled with a fluorescent reporter allowing for detection only after hybridization of the probe with its complementary sequence to quantify messenger RNA (mRNA) and non-coding RNA.

Bacteria to archaea ratios were estimated by using a 5' nuclease Q-PCR assay developed by Takai and Horikoshi (2000). Probes were labeled with 6-FAM on the 5' end and Iowa Black FQ quencher at the 3' end (Integrated DNA Technologies, Coralville, Iowa). Reactions were done in triplicate in 30 mL reaction tubes containing 20 ng gDNA, forward and reverse primers at a concentration of 800 nM, probe concentration of 200 nM, and 1X universal master mix (Applied Biosystems), with the addition of 1 unit of Platinum Taq and 1X ROX (Invitrogen) to optimize signal to noise of reactions. A series of plasmids were diluted from 1 to 10^{-6} beginning with a 10 ng/mL stock solution and a negative control (Davis and Moyer, 2008).

Metagenomic Sequencing and Analysis

Extracted gDNA was sequenced using Illumina sequencing at the OHSU Massively Parallel Sequencing Shared Resource Core (http://www.ohsu.edu/xd/research/research-cores/mpssr). Sequencing consisted of one lane of paired-end 100bp reads on a HiSeq 2000 sequencer, along with one lane of large-insert mate pair reads to aid in de novo assembly. Metagenomes where compared to identify dominant biofilm phylogenies.

Metagenomic reads were trimmed for quality and rare reads were removed using the program Khmer (Crusoe et al). Contiguous sequences were assembled using MetaVelvet (Namiki et al., 2012) which is an extension of the Velvet assembler used for de novo assembly from short sequence reads (Zerbino and Birney, 2008). Contigs were assembled into scaffolds using Bambus2 (Koren, 2011) along with manual blastX queries after which metagenomes were compared using MEGAN4 (Huson et al., 2011). The metabolic potential of each community was observed in MEGAN4 using KEGG and SEED classification. Custom Blast databases for the samples were constructed to find putative functional genes (e.g. for manganese oxidase, carbon fixation, nitrogen fixation).

Metagenomic data was analyzed using a novel iterative mapping method, based on the expectation maximization (EM) algorithm (Dempster, et al., 1977) that reconstructs full-length SSU sequences. The method used was the Expectation Maximization Iterative Reconstruction of

Genes from the Environment (EMIRGE). EMIRGE uses raw reads and quality values to output the most probable consensus sequences after several comparative iterations (Miller et al., 2011).

Results

Geochemistry

Hydrothermal fluids are essentially devoid of dissolved oxygen due to high pressures and temperatures experienced as depth. In the main pool the hydrothermal fluids had a dissolved Mn(II/III) concentration of 2 μM decreasing to 1 μM in the outflow channel (Table 3, unpublished data). Biofilms formed at Vents 1 and 3 possessed the most particle counts and were experiencing high fluid shear. The high silica content of the hydrothermal fluids is a result of superheated fluids interacting with silica-rich rhyolitic rocks. The shallow depth of outflow channels may be allowing slow oxygenation of hydrothermal fluids through mixing.

Geochemical analysis revealed a hot-spring system that is abundant with trace metals and inorganic carbon (Fig. 4). The concentration of essential metals, those necessary for growth of microorganisms, was highest in the high temperature region of the spring, decreasing in the outflow channel (Fig. 5), the essential metals molybdenum and tungsten are important for enzyme function of thermophiles (Schmitz et al., 1992).

Dissolved inorganic nitrogen, in the form of reduced ammonium, has been measured in hydrothermal fluids across YNP, with micromolar concentrations along the Firehole River, where Purple Pool is located. It has been found that ammonium concentrations can persist for several meters in surface fluids draining from hot-spring outflow aprons (Holloway et al., 2011)**.** Measurements of nitrogen species along the temperature gradient of Purple Pool, from the high temperature source pool to the lower temperature outflow apron, showed that ammonium concentrations were elevated at OF 3, this site also demonstrated slightly elevated nitrate, nitrite, and orthophosphate concentrations (Fig. 6).

Table 3. Purple Pool spring fluid chemistry from Nancy Hinman University of Montana (unpublished). Concentrations are shown as 50x there normal concentrations.

Element	Micromolar
Be	0.5473
Sc	1.79
Mn	1.14
Zn	1.42
Ga	0.0413
As	11.8
Se	0.0241
Rb	1.25
Sr	0.0447
Mo	0.135
Sb	0.322
Cs	1
Ba	0.543
W	0.892
Hg	0.1346
Tl	0.00166
Si	3.8
SO4 2-	39.5

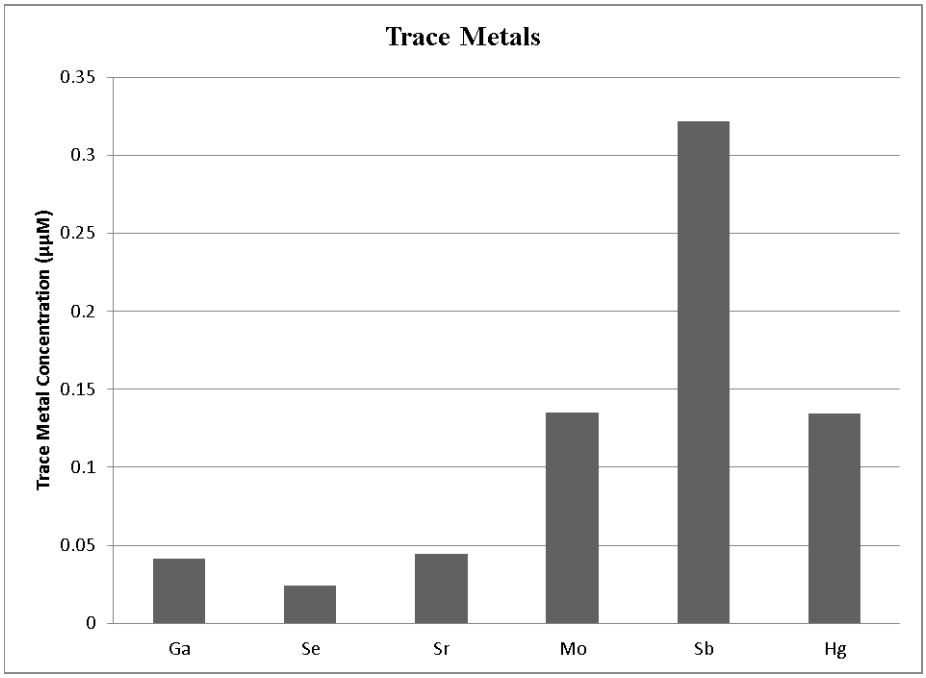

Figure 4. Micromolar concentrations of trace metals from Purple Pool.

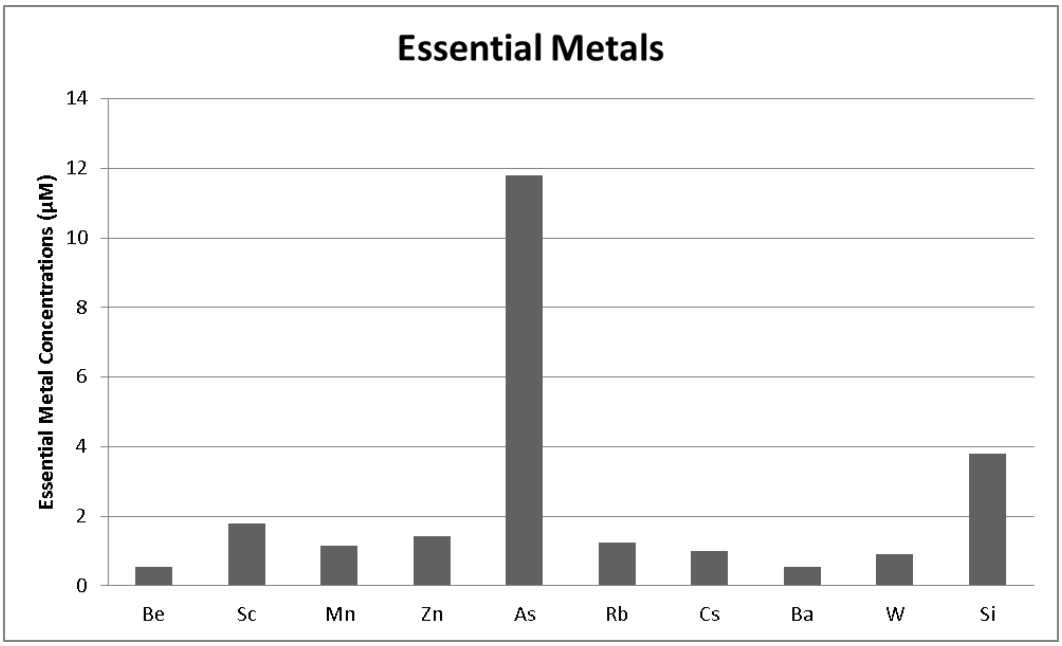

Figure 5 . Essential metal concentrations from Purple Pool.

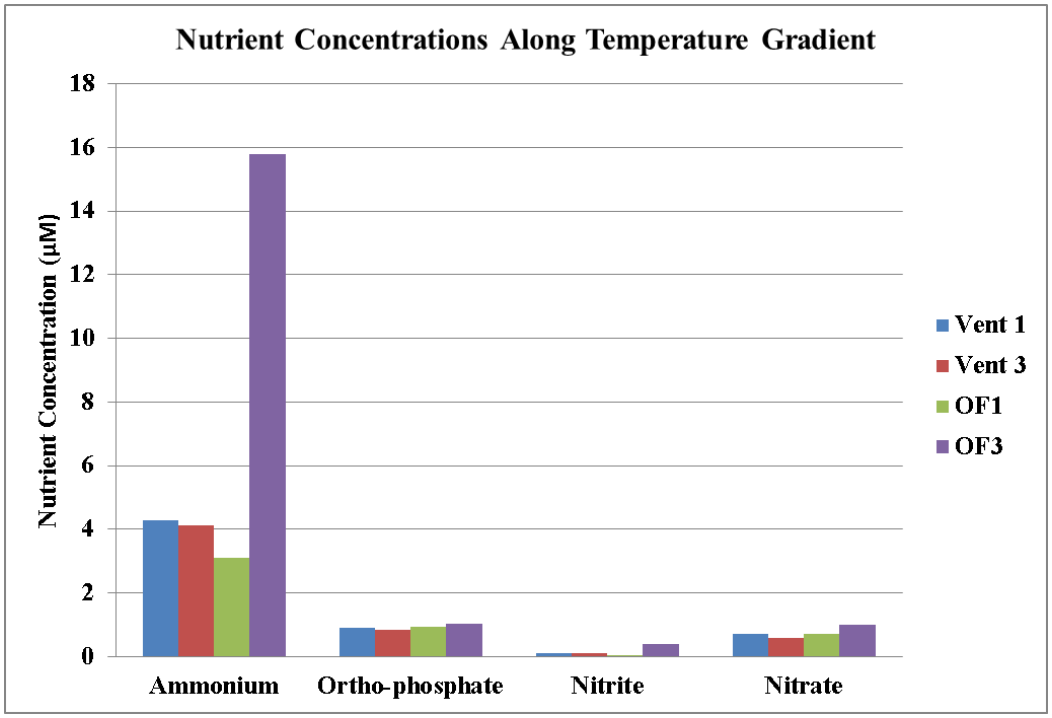

Figure 6. Summary of auto-analyzer nutrient data along the temperature gradient of Purple Pool.

Microscopy

Microscopic examination of Mn-oxide mineral deposits from Purple Pool suggests a biogenic origin for mineral deposition. Petrographic thin-sections reveal vertical growth of Mn-oxide columns and show webbed mineral encrusted EPS (Fig. 7A) and Mn encrusted filaments (Fig. 7B). In addition, SEM analyses show columns of filamentous microorganisms a few microns in diameter, clustered together into larger columns forming the scaffolding for the Mn-oxide spicules. Characterization of biofilm formation using cationic and fluorescent stains was done using glass slides, while natural samples of biofilms growing on rock surfaces, were used for both SEM and TEM analyses.

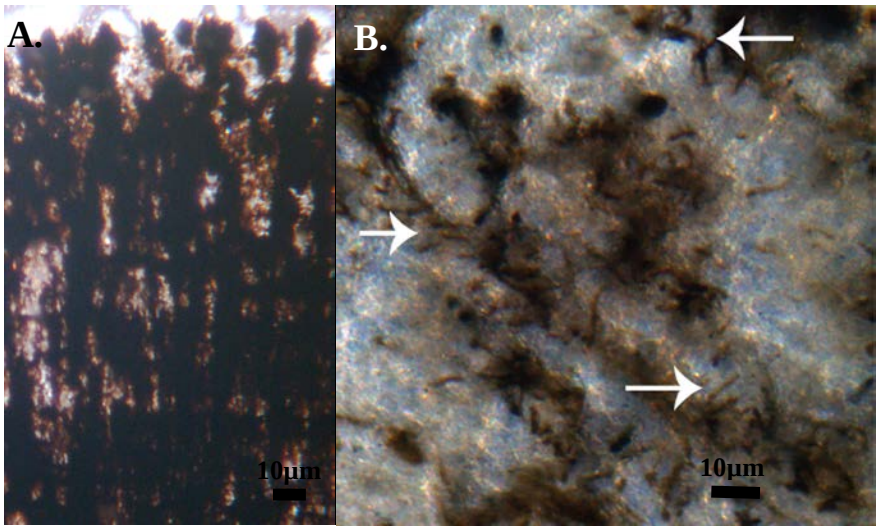

Figure 7. Petrographic thin-sections using optical light microscopy. Left: Spicules of Mn minerals formed by templating onto biofilms. Right: Mn encrusted filamentous microorganisms (arrows) embedded in a silica matrix.

Biofilm Formation

The formation of a conditioning film on glass slides, as early as 1 hour after deployment, was observed using phase contrast microscopy. Initial biofilm colonization was by cocci (0.3 µm), presumably due to their small size and high surface area experience less fluid shear due to their small size attach to substrates first. As the biofilm matured the morphology of the microorganisms comprising the biofilm changed to rods (2 µm) within 3 hrs of deployment, followed by filaments (> 10 µm) after 24 hrs (Fig. 8)

Fluid shear on biofilm communities within the hot-spring appears to be the driving force controlling the rate of biofilm formation. We found that environments that experience high fluid shear, Vent 1 and OF 1, develop more extensive biofilm communities, produce more EPS and have high particle counts due to entrapment of microorganisms, mineral grains and other materials in hot-spring fluids. Microscopy revealed biofilms from Vent 1 and OF 1 were also comprised of microorganisms with more diverse morphotypes; cocci, rods and filaments suggesting a more mature biofilm community. Substrates deployed in low fluid shear environments, Vent 3 and OF 3, had lower particle counts and a biofilm comprised of rod and cocci morphotypes and noticeably less EPS on substrate surfaces.

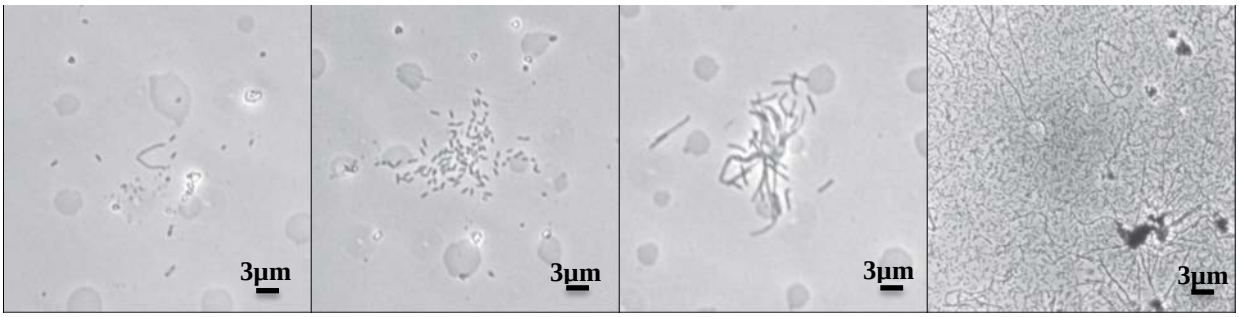

Figure 8. Phase contrast of colonizing biofilm forming on deployed glass slides showing progression of biofilm development. A) Attachment of individual cells after 1 hr in spring -fluids, monolayer biofilm. B) Formation of colony with slight expansion into surrounding environment after 3 hrs, monolayer biofilm. C) Expansion of colony with incorporation of Mn-oxides in biofilm after 4 hrs, monolayer biofilm. D) Colonization of entire slide surfaces with increase in mineral incorporation after 48 hrs, biofilm no longer monolayer.

Particle Counts and Manganese Incorporation

Particle count analyses of biofilms attached to glass slides demonstrated an increase in material, both organic and inorganic, as a function of time. However, not all sample sites demonstrated the same degree of biofilm maturation. Vent 1 was the only site that demonstrated a consistent increase in biomass; biofilms from Vent 1 and 3 demonstrated the highest particle counts after 24 hrs (Fig. 9).

LBB assays of biofilms from all-time intervals allows us to identify the time it takes biofilms to begin incorporating Mn oxides through Mn entrapment by EPS or due to Mn oxidizing microorganisms along the temperature gradient. All sites were LBB negative during the first two time points, 1 & 2 hrs. Vent 1 was slightly LBB positive after 4 hrs, while Vent 3 was slightly LBB positive after 3 hrs and a strongly positive after 24 hrs. The biofilm from Vent

3 began having Mn-oxides associated with the biofilm after 3 hrs; after 24 hrs significantly more Mn-oxides were associated with the biofilm as evidenced by a strong positive LBB reaction. OF 1 was consistently LBB negative at all-time points (Table 4).

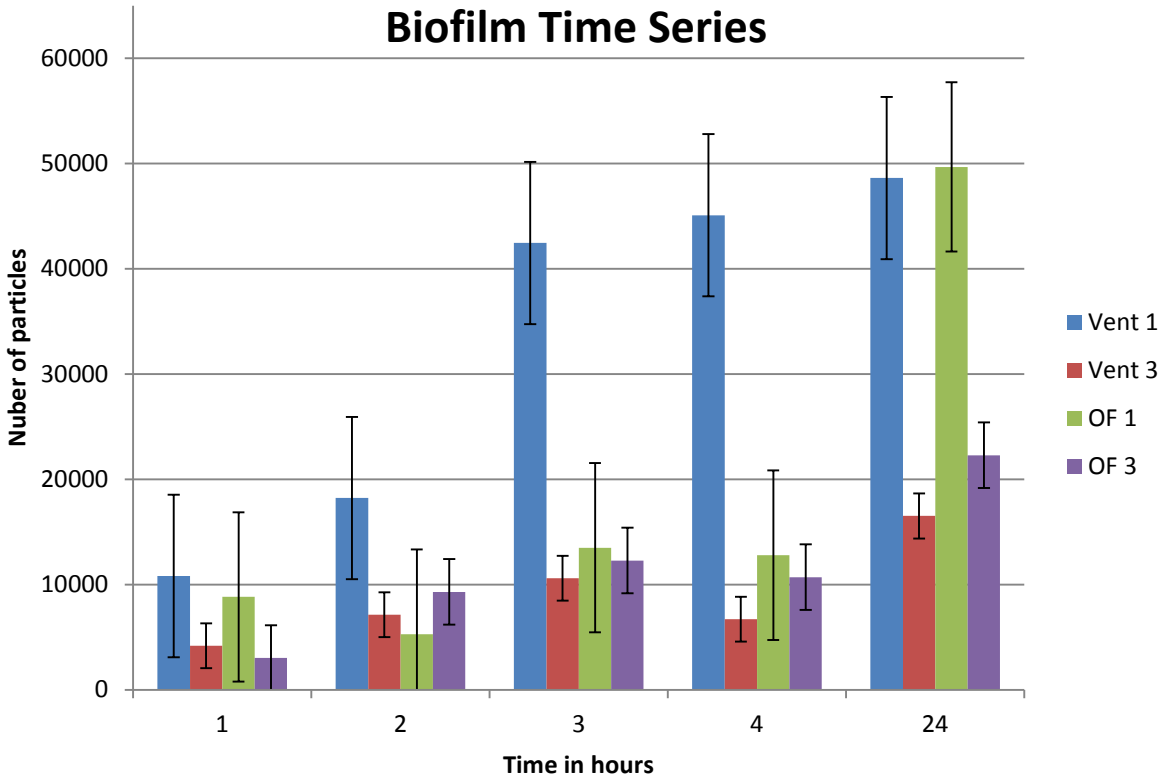

Figure 9. Graphical representation of biofilm particle density as a function of time. Vent 1 (blue) has a rapid and significant increase in particles, with Vent 3 showing a slow incorporation of particles within the biofilm. The outflow sites OF 1 and OF 3 have low particle counts, which may be a result of the increase in stream flow at these two sites flushing microbes and minerals off the glass slides.

Cationic Staining of EPS

Biofilms affixed to glass slides and stained with cationic stains allowed us to observe the rate of biofilm formation and production of EPS associated with the microorganisms and minerals entrained within the biofilm. Stains reacted with the biofilm matrix were bound by reactive side chains within the biofilm allowing for visualization of the invisible EPS matrix. The conditioning film that formed the first hour after deployment strongly bound the ruthenium red stain, whereas biofilms collected after one hour, 2 - 48 hrs, strongly bound the alcian blue stain, suggesting that a chemical modification in the composition of the EPS occurred with the

maturation of the biofilm (Fig. 10A). These findings suggest that the conditioning film was composed primarily of EPS possessing O-specific polysaccharides, which are a variable component of the large lipopolysaccharide (LPS) of gram-negative bacteria (Kenyon et al., 2011). The presence or absence of O-side chains strongly influences the surface texture (rough or smooth) of the LPS (Rittig et al., 2003). EPS of maturing biofilms strongly bound the alcian blue stain suggesting the presence of sulfated and/or carboxylated reactive side chains (Fig. 10 B-C).

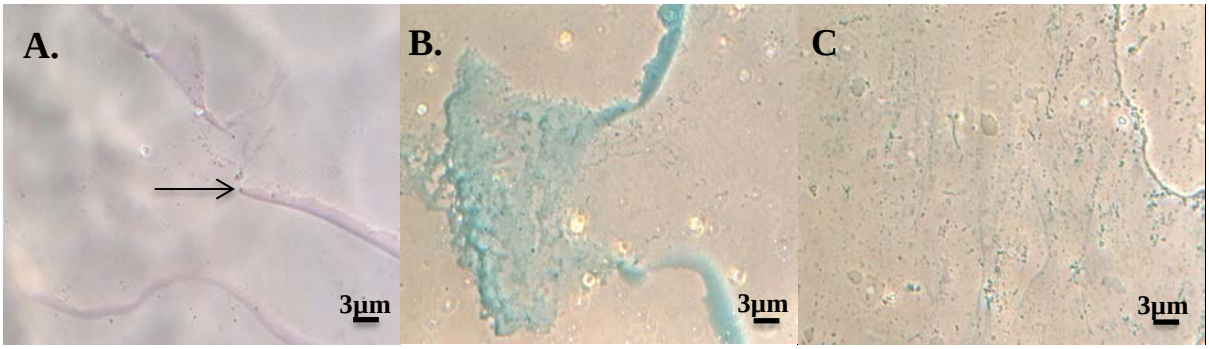

Figure 10. Optical light microscopy using phase contrast, 10x, of cationic stained biofilms attached to glass slides through time. A. After 1 hr there is a conditioning film forms a monolayer of organic material on the slide that preferentially binds ruthenium red stain. Notice the biofilm rolling up off of the slide (arrow) B. After 3 hrs the biofilm has continued to develop in specific regions with the colonization if microorganisms, the EPS preferentially bind alcian blue stain. B. After 24 hrs the biofilm has a spread across the entire surface of the slide and the biofilm continues to bind alcian blue stain.

Table 4. LBB biofilm reaction as a function of time illustrates the incorporation of Mn into the developing biofilm.

Site	1 Hour	2 Hour	3 Hour	4 Hour	24 Hour	48 Hour
Vent 1	-	-	-	+	+	+
Vent 3	-	-	+	+	+++	+++
Outflow 1	-	-	-	-	-	+
Outflow 3	-	-	-	+	+	+

Characterization of Natural Biofilms and Biofacies

Visualization of microorganisms associated with rock surfaces using TEM microscopy was exceedingly difficult due to the abundance and density of Mn and silica minerals; however, there were a few instances where Mn encrusted microorganisms were visualized. SEM of rocks demonstrated significant differences in the texture biofilm and mineral precipitates from each of the four sample sites. Sample sites experience differences in temperature and fluid flow rate. Mn

oxide deposits along the temperature gradient illustrated distinct textures, with increasing silica content as temperature decreased. Biofacies from Vent 1 were comprised of a porous layered Mn oxide deposit with little silica incorporated into the rock matrix.

Vent 1

Rock and biofilm samples collected from the high temperature region of Vent 1 were comprised of columnar spicules. Temporal growth of spicules was evident from SEM analysis, with the extension of spicules by an average of 350 µm per growth season (Fig. 11). SEM of biofilms collected from rock surfaces forming within the hot-spring at Vent 1 showed that the rock is composed of silica minerals 0.5 µm in diameter and encased in nano-particulate Mn-oxides. SEM observation of the columns showed microorganisms intertwined with EPS and minerals, strongly suggesting that the spicule formation was driven by microorganisms (Fig. 12, Top). Biofilms were comprised of cocci, rods, and filaments, all of which were encased in an EPS that had smooth sheet morphology (Fig. 12, Top). TEM analysis of ultra-thin sections showed Mn oxides that formed thin wispy plates that taper averaging 150 nm in length; the presence of Mn oxides was confirmed using EELS analysis (Fig. 12, Bottom). Petrographic thin-sections of spicules show that the structures are porous with little infilling by mineral precipitates.

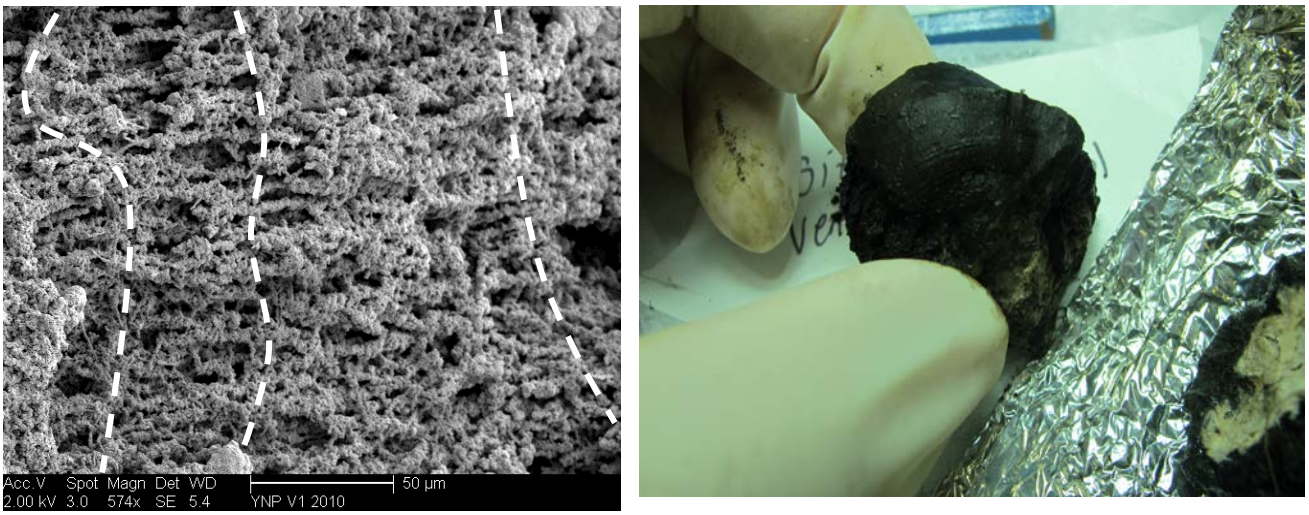

Figure 11. (Left) SEM micrograph of biofacies cross-section from Vent 1 shows subtle banding patters caused by seasonal growth of microorganisms (dashed lines). (Right) Sample showing the thick Mn deposit overlying a silica base.

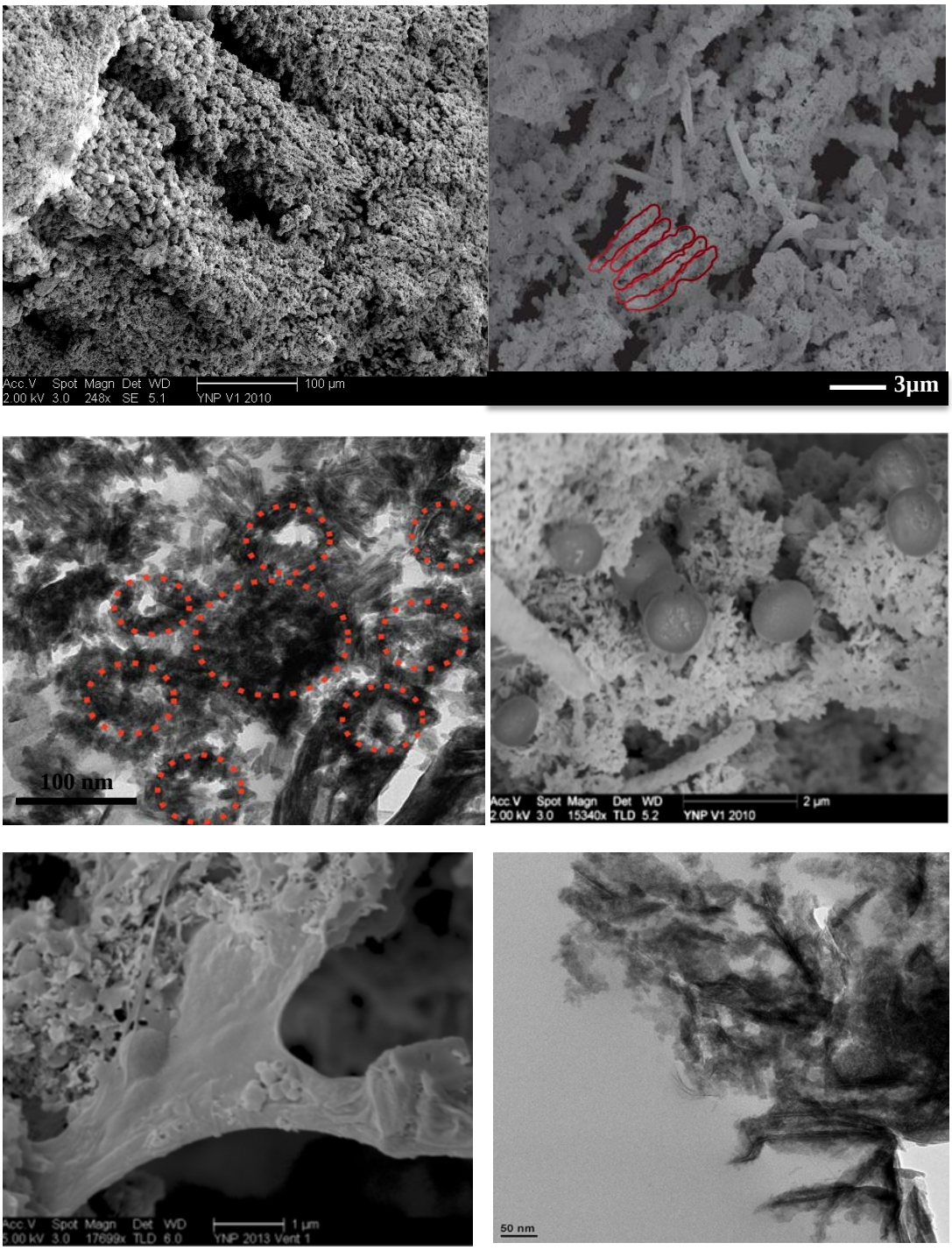

Figure 12. EM micrographs illustrating microbe mineral associations and EPS of the biofilm from the high temperature Vent 1 sample site. (Top Left) SEM of spicules (Top Right) microorganisms intertwined around spicules. Notice that each column is comprised of serval smaller columns clustered together (circled in red) (Center Left) TEM showing a cross-section of spicules showing a dense core of column comprised of Mn oxides surrounded by smaller columns of Mn encrusted microorganisms associated with spicules (circled in red). (Center Right) SEM showing morphological diversity of microorganisms in biofilms. (Bottom Left) SEM showing the smooth sheet texture of EPS of the biofilm (Bottom Right) SEM showing texture of EPS from Vent 1 biofilms. (Bottom Left) TEM of wispy Mn oxides.

Vent 3

SEM observations of biofilms collected from the moderate temperature Vent 3 exhibited a compact texture possessing extensive thick sheets of EPS and filamentous microorganisms encrusted with Mn-oxides; silica colloids were present on all cell, EPS and Mn-oxide surfaces (Fig. 13). Petrographic thin-sections revealed microbial filaments encrusted in nano-particulate Mn oxides and silica. Samples from the outflow channel were composed of a thin Mn crust overlying extensive silica deposits. EM of biofilms revealed filamentous microorganisms encrusted with Mn oxides. EELS analysis and false color mapping confirmed the presence and location of Mn oxides biofacies from Vent 3 were comprised of a more dense Mn oxide mineral layer 23 mm thick, overlaying a thin silica base (Fig. 14, Bottom). The biofilm on the surface was heavily encrusted with fine silica colloids and wispy Mn oxides. Fossilized cell walls were observed by TEM (Fig. 15).

Figure 13. (Left) SEM illustrating the filamentous morphology of the biofilm and the associated Mn oxides encrusting filament surfaces. (Right) Rock showing Mn deposition overlying a silica base. Notice the intergrowth of silica (white) in the Mn oxide deposit.

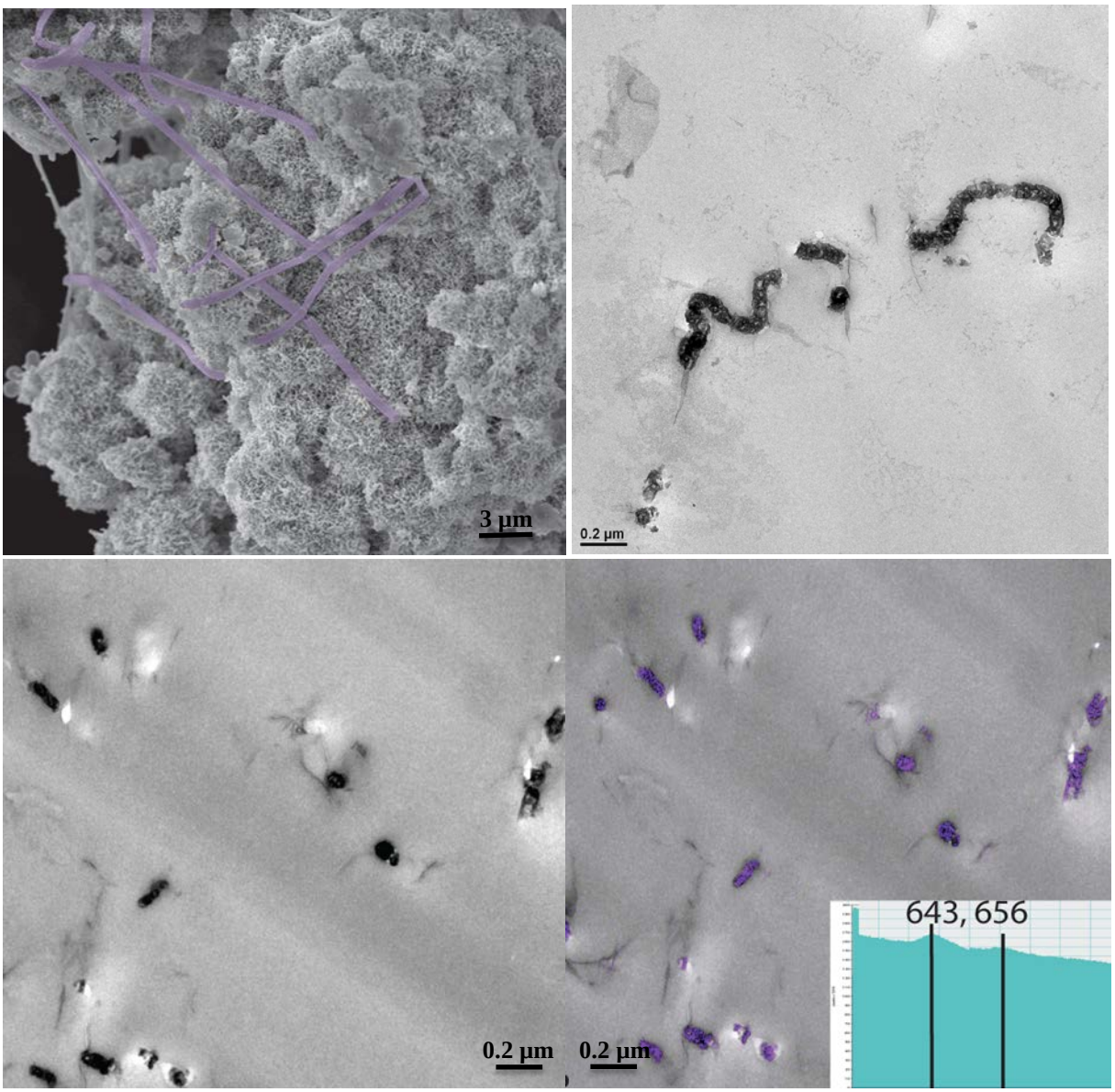

Figure 14. (Top Left) SEM of filamentous biofilm (false color purple) colonizing the surface of Mn oxides. (Top Right) TEM of a Mn encrusted filament from the biofilm colonizing Mn oxide deposits from Vent 1. (Bottom Left) TEM of Mn encrusted microorganisms (Bottom Right) same image using EELS to map the location of Mn within the sample (Mn is colored purple) inset of spectra showing characteristic peaks for Mn (IV) at 643 and 656kv.

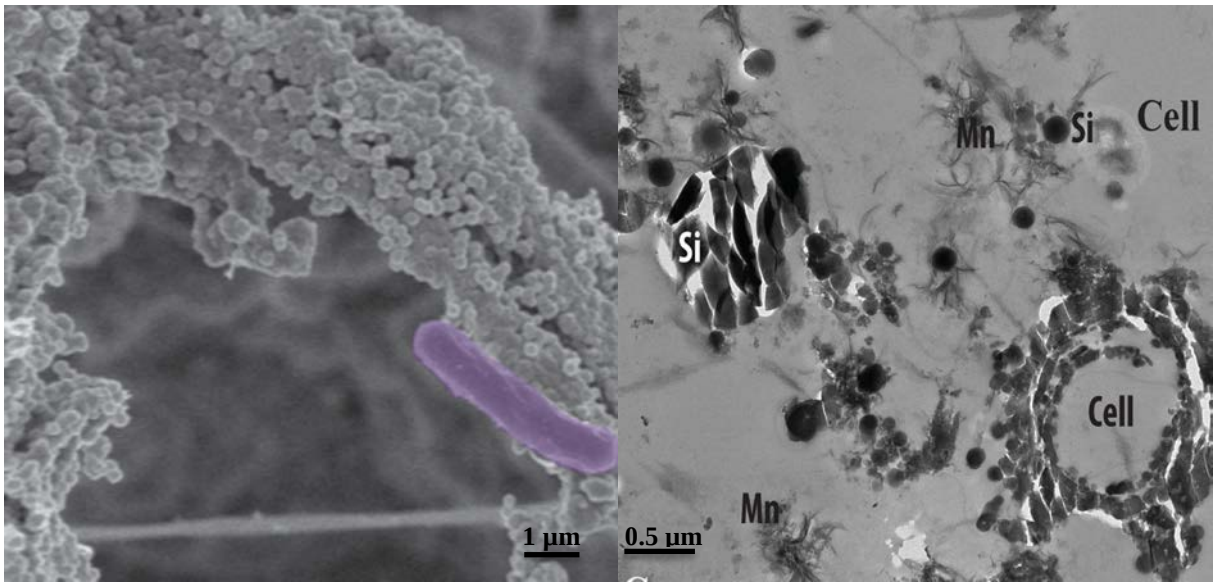

Figure 15. (Left) SEM of biofilm encrusted with silica colloids, microbial cell is colored purple. (Center) TEM showing fossilized cell wall, wispy Mn plates, and silica colloids.

Outflow 1 and 3

Biofilms collected from OF 1 and OF 3 were more mineralized than samples collected from the vent sites. This may be due to the shallow flow and decreased temperatures, which allowed rapid nucleation and precipitation of minerals. Spicules were only a few millimeters in height and compact caused by infilling of pore spaces by mineral precipitates due to the shallow environment in which they formed. Mn oxides from OF 1 formed rosettes averaging 1.5 µm in diameter, composed of Mn plates as observed from petrographic microscopy (Fig. 16), and overlain by a thin biofilm community and a thin sheet of EPS. SEM of the biofilm shows the rosettes were composed of short Mn oxide plates averaging 50 nm in length and Mn oxide encrusted filaments (Fig. 17). Biofacies from the two outflow channels were comprised of a very thin 1 mm dense Mn oxide layer. The deposit had very few pore spaces due to infilling by silica.

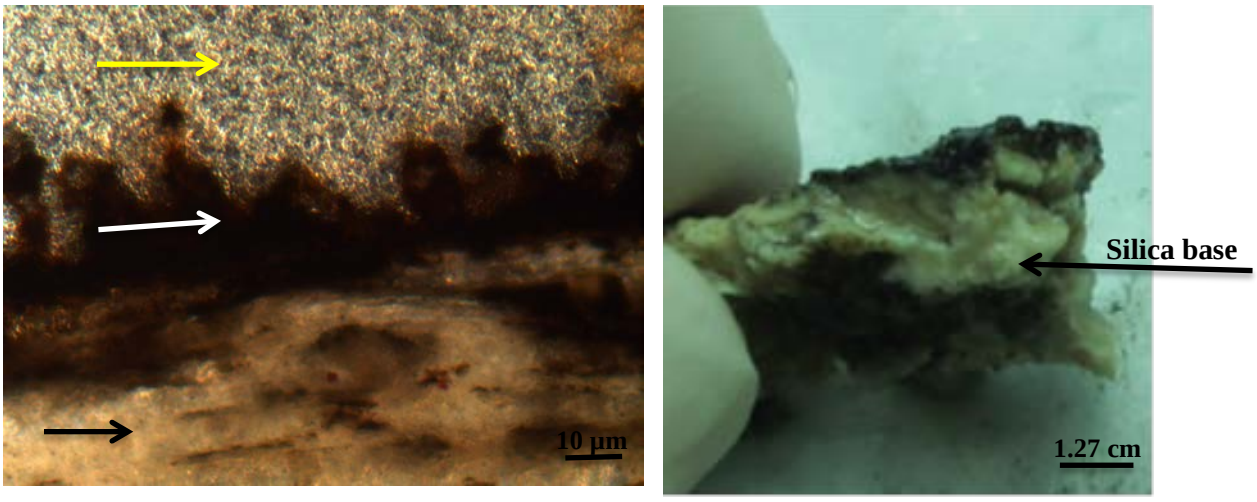

Figure 16. (Left) Petrographic thin-section illustrating the thin layer of Mn oxide spicules (white arrow) overlying a silica base (black arrow). Note the density of the Mn oxide layer as compared to spicules from Vent 1, this is due to the shallow environment resulting in rapid infilling of Mn and silica minerals. The yellow arrow indicates the embedding matrix affixing the sample to a glass slide and is not part of the sample. (Right) Specimen from OF 1 the Mn oxide layer is thin black layer overlying a massive silica base (arrow).

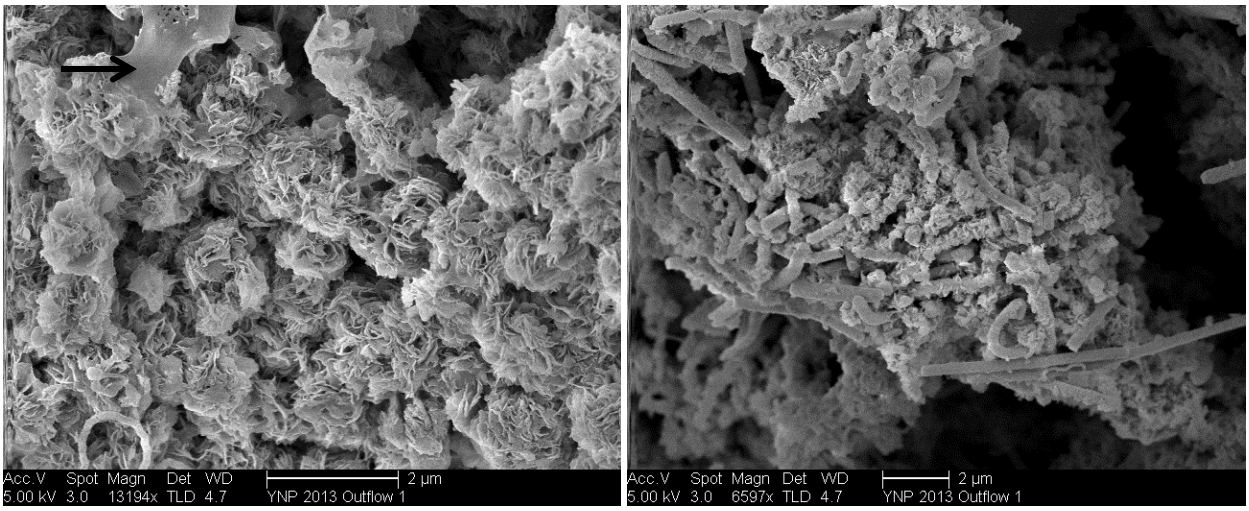

Figure 17. (Left) SEM micrographs of Mn oxide rosettes and EPS (arrow) from OF 1. (Right) Spicule from OF 1 showing the density of the structure as well as the density of the Mn encrusted biofilm forming the structure.

Microbial Diversity: T-RFLP

Environmental microbial communities are exposed to rapid and extreme physical, temperature and chemical gradients across the extent of their spatial confines within the biofilms. These communities are intrinsically linked to the physical and chemical properties of their environment and may be varied and ephemeral. T-RFLP analysis indicated that there was strong

spatial variability along the temperature gradient from the high temperature pool, 92.2°C, to the lower temperature outflow, 77.3°C. Variability in the outflow channel appeared to be driven by flow shear. OF 1 is located in a high flow region and OF 3 is located in a low flow region of the channel. Cluster analysis showed a bifurcation occurred separating the clusters from the main pool/vents and the outflow channel (Fig. 18). Vent 1 and 2 are in close proximity to one another in the high temperate main pool in which both experience the same environmental conditions (i.e. flow rate, temperature, pH, spring fluids) resulting in biofilms with similar community richness as reflected in the dendrogram with nearly 100% similarity. Biofilm mat sampled from the main pool clustered with Vents 1 and 2 with 72% community similarity. Vent 3 is located at the mouth of the outflow and experiences lower temperatures, 78.3°C, with 51% similarity to biofilms from the high temperature main pool. Biofilms collected in the shallow low temperature outflow channels OF 1 and OF 3 have 61% similarity to microbial communities experiencing fluid shear and 43% similarity to the high temperature region of the pool.

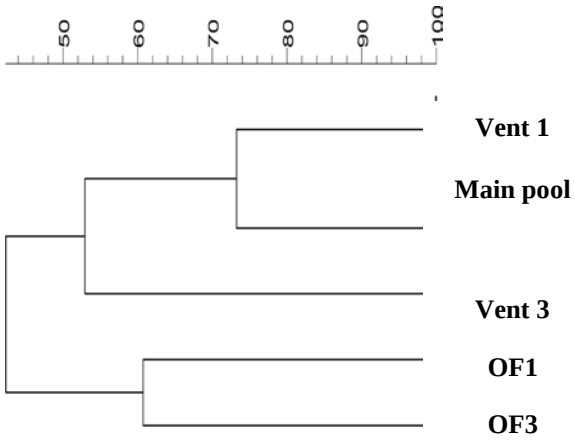

Figure18. UPGMA dendrogram of biofilms from Purple Pool collected in 2010. The dendrogram illustrates the variability with regard to community composition along the temperature gradient. T-RFLP results indicate that there is a bifurcation between the high temperature vents and outflow channel microbial communities using cluster analysis.

Clone Library

Characterization of the hot-spring microbial communities using a small clone library of small subunit ribosomal RNA gene (rDNA) sequences (96 clones) from OF 1 revealed that a majority of the sequences (54%) represented novel deeply rooted bacteria (Fig. 19).

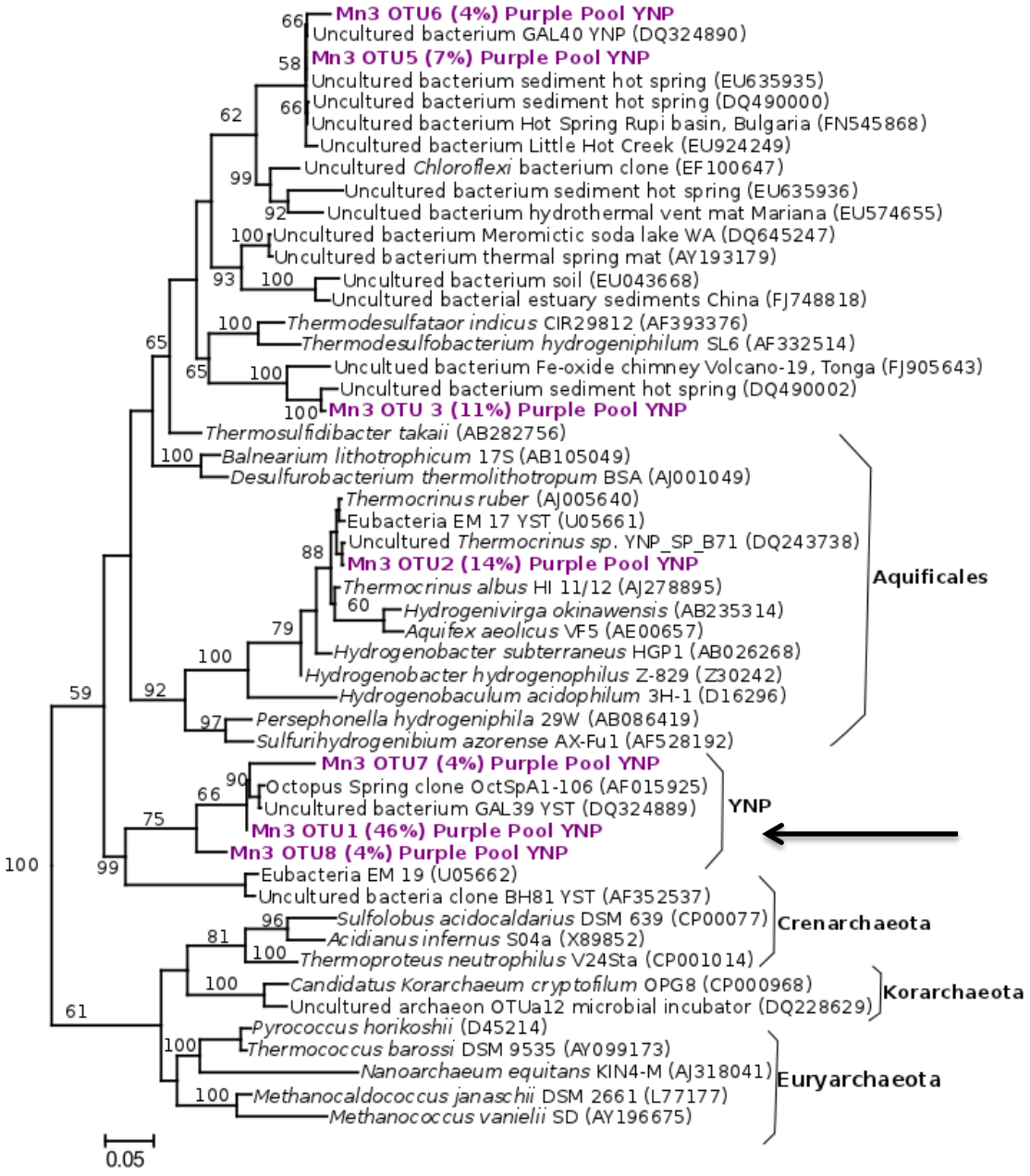

Figure 19. Phylogenetic tree illustrating OTUs from clone library from outflow channel, 75.5°C. Over 50% of the clones are unidentified deeply rooted bacteria often found at other hot springs (arrow).

Quantitative PCR

QPCR of small subunit rRNA genes was conducted to examine the contribution of bacteria and archaea to the biofilm communities found along the temperature gradient. Bacteria comprised 50-70% or more of the biofilm while archaea comprised 30-50% (Fig. 20).

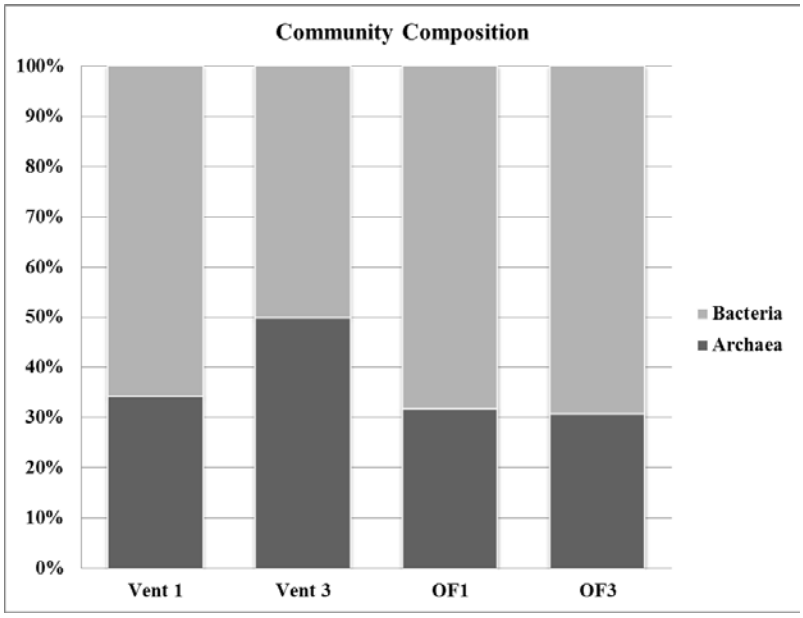

Figure 20. Graph illustrating QPCR results showing the percentage of bacteria to archaea along the temperature gradient at Purple Pool.

EMIRGE Analysis

Microorganisms from this hot-spring ecosystem were found to belong to several known bacterial taxa, including the *Aquificales* phylum, *Proteobacteria* phylum, and the *Deinococcus-Thermus* (phylum) with the most abundant archaeal division being from the *Crenaraechota* (phylum) (Fig. 21). Some of the microorganisms from biofilms were unrelated to any known cultured divisions. Results indicated that *Aquificae* was the dominant bacterial community member and *Crenarchaeota* were the dominant archaeal community members. As expected the composition of biofilm communities across the temperature gradient, 92°C to 72°C, had a relatively low diversity with only one type of archaea present and various other bacterial microorganism present in low abundance at most sites. Analysis of SSU rRNA genes from EMIRGE analysis showed that about a third of the microbial populations were archaea, with the exception of Vent 3, from which a smaller metagenomic library was generated. Examination of biofilm diversity from each site showed that the archaea community was composed primarily of

Thermoprotei and the bacterial population was composed of *Aquifex*, with the exception of the high temperature Vent 1 which was dominated by *Beta-proteobacteria*.

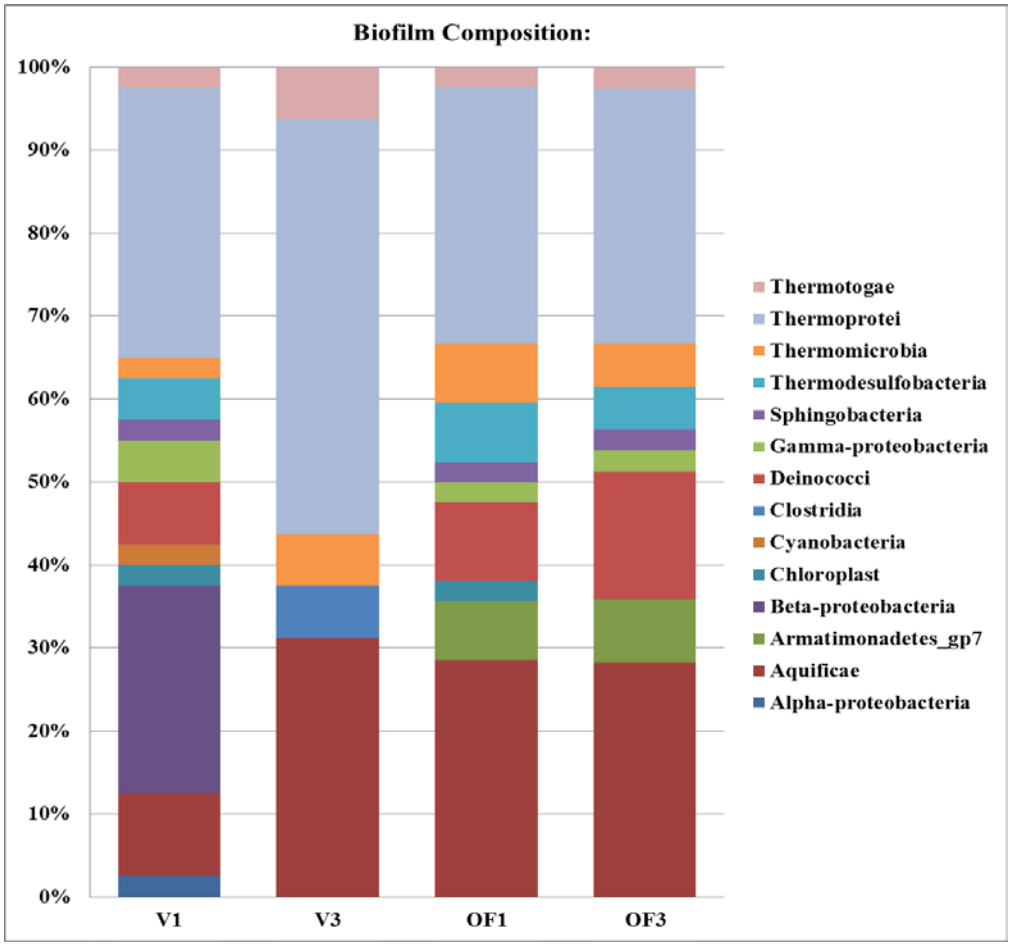

Figure 21. Percent distribution of biofilm community along temperature gradient.

Chemoautotrophy

Chemoautotrophic microorganisms derive all of their organic carbon from inorganic carbon sources such as carbon monoxide, carbon dioxide or methane. This fixation of C-1 compounds requires a significant amount of energy. In the case of carbon monoxide and methane, energy can be obtained through their oxidation, which can be used to fix C-1 compounds for biosynthesis. Microorganisms that obtain their energy using reduced inorganic chemicals, such as HS^-,Fe(II) or NH_4^+, and fix CO_2 in the process are referred to as chemolithoautotrophs.

Of the six known pathways for CO_2 fixation, two were identified from the metagenome of the biofilms collected at Purple Pool hot-spring by examining the KEGG pathways represented using the MEGAN metagenomic analysis software package (Algorithms in Bioinformatics, Germany). The two prevalent pathways were the Calvin-Benson-Bassham (CBB) cycle and the rTCA cycle (rTCA). The CBB cycle is the most thoroughly studied pathway (Tabita, 1999) and is thought to be responsible for most of carbon fixation on Earth today. The CBB cycle is the pathway most commonly used by chemoautotrophic and photoautotrophic microorganisms and utilization of this pathway is energetically expensive. Many chemoautotrophic and photoautotrophic bacteria, including the *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Firmicutes*, and *Thermus*, all of which were identified in natural biofilms forming at Purple Pool, use the CBB cycle.

In contrast to the CBB cycle, the rTCA cycle shares many of the intermediates and enzymes as the tricarboxylic acid (TCA) cycle, however the rTCA cycle operates in reverse order with the subsequent formation of acetyl-CoA. The rTCA cycle is commonly limited to microaerophilic or anaerobic environments due to the oxygen sensitivity of the key enzyme, 2-oxoglutarate synthase (Xiang and Martin, 2006). Other key enzymes unique to the rTCA cycle are ATP citrate lyase; 2-oxoglutarate: ferredoxin oxidoreductase and pyruvate: ferredoxin oxidoreductase (Boundless, 2015). The rTCA pathway is thought to be a prime candidate for early Earth conditions, and a key pathway in the evolution of life. This pathway is also energetically expensive as it fixes carbon dioxide, utilizing hydrogen, sulfide, thiosulfate and some minerals as electron donors. This pathway appears to be tolerant to high temperatures, as many hyperthermophilic *Aquificales* use the rTCA cycle (Hügler et al., 2007). *Aquificales* were found at all sample sites along the temperature gradient, from 92.2°C to 73.2°C. The archaea *Thermoprotei* are chemoautotrophs that use sulfur reduction of thiosulfate for energy and fix carbon via the rTCA cycle to reduce carbon dioxide (Ramos-Vera et al, 2010).

Discussion

Characterization of the process of manganese biomineralization in extant hot- spring environments allows us to begin to build a knowledge base to identify Mn biominerals from ancient geologic deposits on Earth and on other planetary bodies. In addition, it allows us to infer the environmental conditions in which these deposits formed and to better hypothesize as to the role microorganisms played on early Earth and shaping the world we live in today. This

knowledge base that we are building through the characterization of biogenic Mn oxides, identifying potential thermophilic Mn oxidizing microorganisms, and the role EPS plays in mineral nucleation and biofacies formation, will allow us to confidently determine the origin, biotic or abiotic, of Mn oxides in geologic deposits based on telltale characteristics, such as mineral morphology and detection of biosignatures within Mn minerals (i.e. microfossils or chemofossils) in the same way as is done for other biogenic minerals, such as iron and silicates.

Our findings indicate that there may also be temporal variability playing a role in community structure and diversity due to factors such as the extreme fluctuation in seasonal water runoff and variability of sunlight. Glass slides were deployed to allow for biofilm formation and observation as a function of time. These in situ experiments revealed microbial colonization and the rate of formation, and development of biofilms along the temperature gradient and in different flow regimes and the time point at which Mn oxides become incorporated into the biofilm (Fig. 6, Table 3). This allows us to better understand the rate of biofilm formation and biomineralization along the ecosystem as a function of both time and the many environmental conditions (i.e., flow rate, temperature, pH, Mn concentration, oxygen concentration) in which these biofilm and biominerals form.

Visualization of EPS is difficult using traditional microscopic techniques due to the instability of the three-dimensional structure, the lack of contrast in the EPS and the high water content. We used polycationic stains which bind to functional groups associated with the EPS to enhance visualization of different phenotypic structures in the biofilm EPS, particularly structures responsible for cellular attachment to substrata by (Bober, 2005; Erlandsen et al., 2004). Cationic stains allowed for visualization of not only the microorganisms within biofilms but also the EPS providing a better understanding of the three-dimensional biofilm architecture. Characterization of EPS within biofilms in Purple Pool using cationic stains and microscopy illustrated a chemical modification of the EPS after the formation of the conditioning film at 1 hr; this is likely an active microbially driven process altering the reactive side chains in the EPS. We found that biofilms demonstrated a positive reaction to LBB solution after 4 hours, indicating that Mn oxides, biogenic or abiotic, became part of the biofilm matrix within 4 hours of development. EPS likely plays an important role in Mn oxidation within biofilms, serving as sites for mineral nucleation, templating, or sorption sites for Mn oxide minerals or providing a microenvironment suitable for Mn oxidation to occur.

Petrographic microscopy of Mn(III/IV) deposits collected at Purple Pool suggest that biofilm communities have a profound influence on the formation of the Mn-deposits as well as the morphology of mineral deposits (Fig. 4 and 9) due to templating of minerals on to cell and EPS surfaces. Mn oxides formed spicules suggesting a biological origin of the Mn oxides within Purple Pool. SEM analysis revealed the formation of Mn oxides on the surface of microorganisms within biofilms as well as Mn oxides formation closely associated with EPS. TEM further supported the templating of Mn oxides on biological substrates, cell walls and EPS, through the examination of spicule formation.. Further analyses of metagenomic data will allow us identify both the mechanisms of Mn oxidation occurring in a high temperature hot-spring and the microorganisms involved.

Metagenomic analysis provides insight into the microbial diversity and possible identity of novel thermophilic Mn-oxidizers, specifically identifying novel chemotrophs, and the identification of novel enzymes and metabolic processes. The metagenomes were compared to identify those sequences that are most commonly found in the samples. Formation of biofilms on Mn deposits within the hot-spring and the overall morphology of biofilms along the temperature gradient showed microbial communities exhibiting low diversity relative to biofilms formed in other low temperature groundwater springs. The microbial community in biofilms from each of the four study sites were dominated by a single taxon each of bacteria and archaea, with the *Crenaraechota clade* as the only archaeal division detected and the *Aquificales* clade as the most abundant within the bacterial division except at Vent 1 which also had an abundance of *Beta-proteobacteria* represented. There are several microorganisms from these study sites that, to date, have not been identified nor characterized. Examination of biofilm diversity using T-RFLP indicates that temperature plays a significant role in community composition; this is evident from the clustering of high temperature biofilms from the vents and main pool, 78.2-92.2°C, and the lower temperature biofilms from the outflow channels, 72.3°C.

Microscopic examination of Mn oxide deposits from Purple Pool suggests a strong relationship between biofilm formation and Mn oxide deposition due to the columnar morphology of deposits and abundance of Mn oxide encrusted microorganisms. We propose that Mn oxidation is mediated by thermophilic Mn oxidizing microorganisms as the concentration of dissolved Mn(II) within the hot-spring is low with little possibility of abiotic Mn oxidation and Mn oxides are always associated with microorganisms/biofilms. Microscopic analysis

demonstrated that spicule formation is strongly driven by microbial biofilms and Mn oxides associated with these biofilms. We found through EM observations that Mn oxides nucleate onto biological surfaces forming microfossils within the rock matrix around the hot-spring.

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Chapter 6

Comparison of Two Iron and Manganese Depositing Groundwater Ecosystems

Introduction

The research presented in this dissertation was conducted in an effort to characterize the morphology of biogenic Fe- and Mn oxides, to identify the microorganisms responsible for the biogeochemical cycling of Fe- and Mn in groundwater, the mechanisms by which oxidation occurs, and to better understand the preservation of biosignatures in rock deposits. Knowledge gained from studying modern analog environments allows us to better identify and interpret biosignatures, biominerals and unique oxidation products from ancient geologic deposits such as BIFs and cherts formed on early Earth and possibly other planetary bodies.

Here I compare and contrast the geochemistry, microbial diversity and geomicrobiology of two unique Fe- and Mn-depositing groundwater ecosystems. The first ecosystem is comprised of several Fe-depositing cold-seeps located at Soda Bay, in Southeast Alaska. Soda Bay is a lotic to marine ecosystem. Fluids from the seeps have an average temperature of 10°C. The second environment is a Mn-depositing hot-spring, Purple Pool, from YNP; the temperature of spring fluids range from 92.2°C to 72.2°C.

Geology

The geology of both study sites directly influences the dissolved chemical species in the groundwaters, which eventually erupt from cold-seeps and hot-springs. At Soda Bay the groundwater dissolution of carbonate (limestone), Fe-enriched metamorphic green schist bedrock, and Mn-enriched dikes have led to the formation this environment. In contrast, Purple Pool is a circumneutral hot-spring located in the Lower Geyser Basin of YNP. Purple Pool is located in a volcanic caldera where surface rock is comprised of silica rich rhyolite overlying a shallow magna plume. Surface waters percolate downward through fissures and pore spaces becoming superheated by a shallow magma plume underlying the region. Subsequent water-rock interactions lead to the enrichment of hot-spring fluids with silica from the rhyolite bedrock and Mn from Mn-enriched dikes in the region of the park.

Geochemistry

The geochemistry of Soda Bay is a dynamic groundwater system with rapid and extreme temporal fluctuations in geochemical composition. The geochemical variability we are observing at Soda Bay may be due to the small dataset with which we are making observations, with only one year of data collected. Continued sampling and analysis of the geochemistry will be necessary if we want to understand the geochemical cycling of Soda Bay and the biogeochemical cycling of carbon and metals by collecting baseline data. Our observations strongly suggest that climatic forcing directly affects the geochemistry of the cold-seeps at Soda Bay. We found that there was a significant increase in precipitation in April and May 2014 as compared to the average precipitation rates for Southeast Alaska, leading to an increase in percolation of surface water into the karst aquifer and subsequent mobilization and discharge of dissolved chemical species of varying composition from cold-seeps (Fig. 1). Longer term observations will be important to understand the effects interannual variability and climatic change have on the system.

In contrast, the geochemistry of hot-springs in YNP has remained relatively stable for decades (Inskeep and McDermott, 2005). Even so, volcanic activity in the region has resulted in a change in the groundwater conduits and the geochemistry of Purple Pool as is evident in examination of rock deposits in and around the hot-spring. Rock specimens collected from the rim of Purple Pool hot-spring show a massive underlying sinter deposits overlain by a thin (mm) Mn crust, indicating a change in the geochemical composition of this hot-spring within the last decade (Fig. 2).

Both groundwater systems are thought to have low oxygen concentrations due to very different causes. Soda Bay cold-seep fluids are oxygen deplete due to rapid drawdown by microbial respiration as organic rich muskeg fluids percolate through fissures moving toward the main karst aquifer (Murphy and Schramke, 1998). In contrast, Purple Pool fluids are oxygen deplete due the degassing of fluids as they become superheated as they percolate through fissures to the groundwater aquifer at depth.

Geochemical analyses of fluids were done to measure concentrations of Mn(II/III) and Fe(II) from both Soda Bay and Purple Pool using ICP-MS. It is evident that there is

significantly more dissolved Mn(II/III) from the cold-seeps at Soda Bay, average 23 µM, than Purple Pool, average 1.14 µM; Purple Pool had more Mn deposition encrusting the surfaces of the spring (Fig. 3).

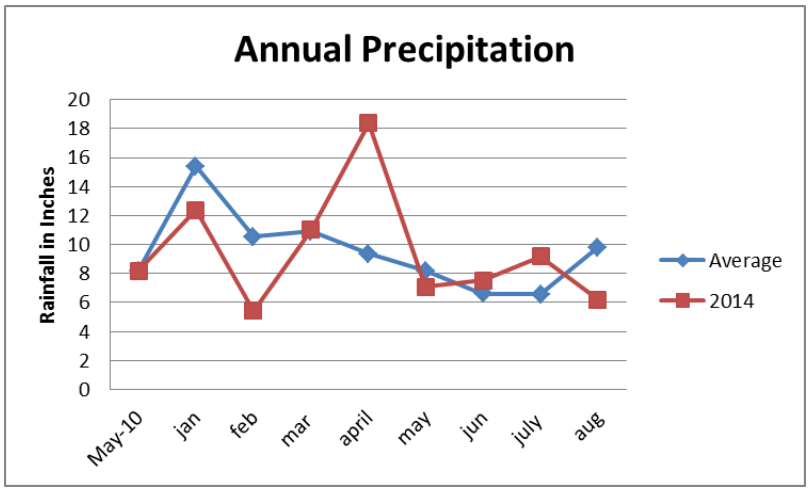

Figure 1. Graphical illustration of average annual precipitation for Soda Bay in Southeast Alaska and the increased precipitation for 2014 (NOAA , National Weather Service, http://www.arh.noaa.gov/public).

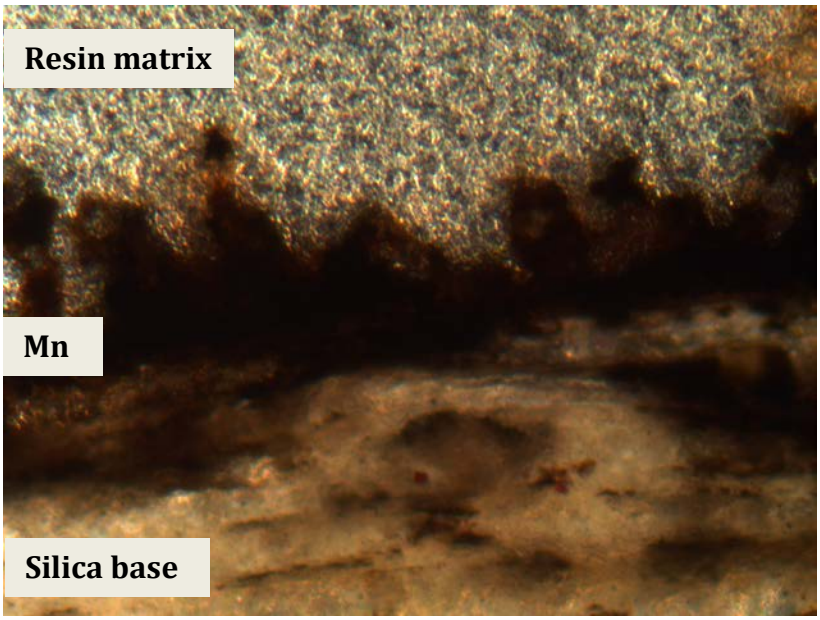

Figure 2. Bright field image of a petrographic thin-section of rock from Purple Pool hot-spring. The silica base was formed when the hot-spring deposited primarily siliceous sinter. The Mn (black) layer on the surface is from present day deposition of Mn oxides.

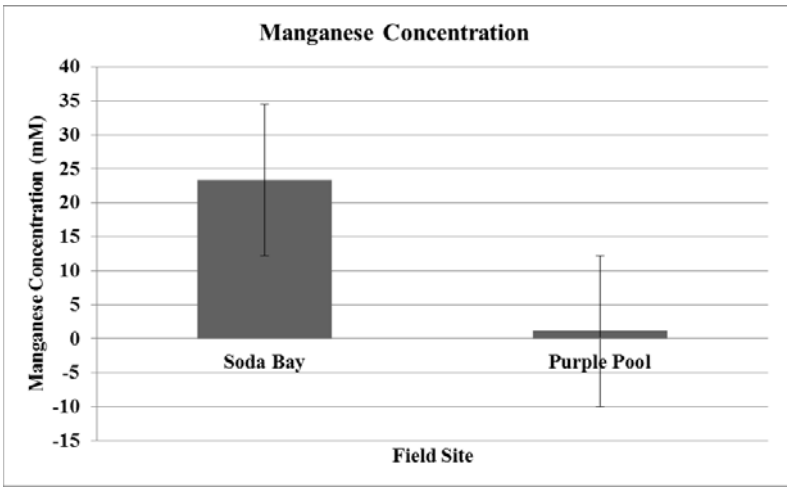

Figure 3. Graph of ICP-MS data illustrating the vast differences in the concentration of dissolved Mn(II/III) from Soda Bay and Purple Pool.

Measurements of dissolved Fe(II) from both field sites demonstrates why Soda Bay was studied for Fe deposition and the presence of Fe-oxidizing microorganisms. Dissolved Fe(II) from Soda Bay cold-seeps ranged from 0.73 µM to as high as 1,175 µM; at Purple Pool dissolved Fe was below detection limit (Fig. 4).

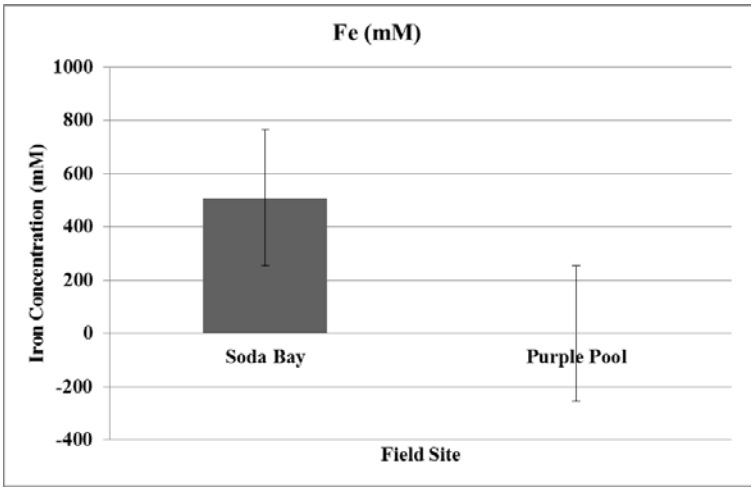

Figure 4. Graph of ICP-MS data illustrating elevated concentrations of dissolved Fe(II) at Soda Bay.

Silicic acid concentrations showed that geothermal fluids in YNP were enriched with silicic acid from dissolution of the rhyolitic rock, with an average concentration of 1,784 µM; in contrast, concentrations at Soda Bay averaged 161 µM (Fig. 5).

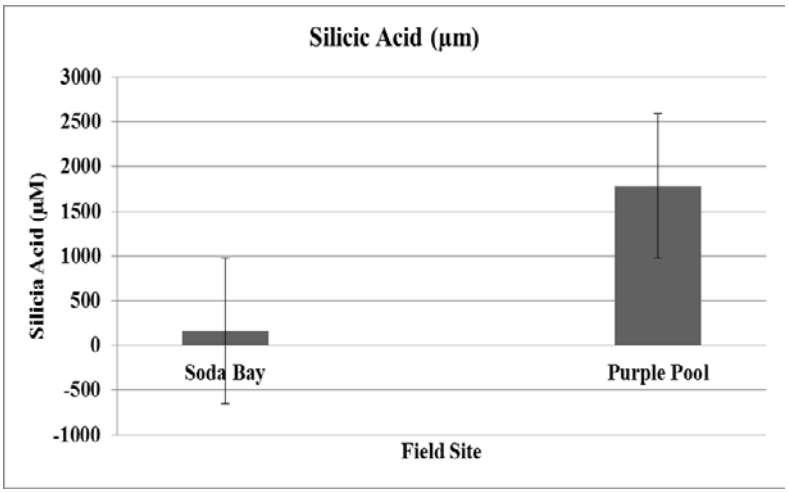

Figure 5. Graph illustrating the concentrations of silicic acid from Soda Bay and Purple Pool.

Microbial Diversity

Analysis of SSU rRNA genes from the metagenomic sequences showed that the microbial communities from the cold-seeps at Soda Bay were more complex and diverse than those from the Purple Pool hot-spring. From examination of the diversity based on archaeal and bacterial 16S SSU ribosomal genes it was evident that there was significantly more bacterial diversity at Soda Bay (Fig. 6). The compositions of the microbial communities from Purple Pool were less diverse as we would expect from a hot-spring ecosystem with a nearly equal abundance of archaea and bacteria.

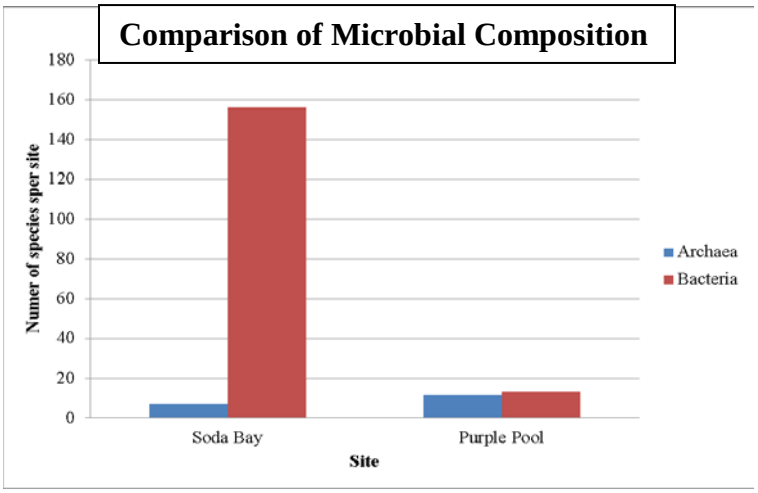

Figure 6. Graph illustrating microbial diversity examining bacterial and archaeal genes from metagenomic data using EMIRGE. The composition of the microbial community from Soda Bay have more bacterial species represented than the bacterial communities from Purple Pool.

Discussion

My research conducted at both Soda Bay Fe depositing cold-seeps and Purple Pool Mn depositing hot-springs allowed for observations and comparisons to be made of two unique metal depositing groundwater ecosystems. These sites were studied as modern analogs to the extreme environments present on early Earth, such as shallow marine environments where BIFs formed or from ancient hot-springs. The extreme environmental conditions of both of these study sites are reflected in the groundwater geochemistry and microbial diversity.

Biofacies from Purple Pool produced distinctive mineralized columnar structures overlain with an easily identifiable microbial biofilm. In contrast, biofacies at Soda Bay were composed of amorphous Fe oxides and carbonate minerals associated with a microbial-EPS precipitate. The supersaturated cold-seep fluids precipitate rapidly upon exposure to the oxidizing atmosphere forming amorphous minerals and encrusting microorganisms making identification of individual cells difficult.

Deposition of metals from both study sites is influenced by biological processes as evidenced by the identification of putative Fe- and Mn-oxidizing and reducing from metagenomic data. However, at Soda Bay mineral deposition is heavily influenced by abiotic processes as the dissolved Fe- and Mn saturated fluids erupt from cold-seeps and experience changes in pH and oxygen concentration as well as from mixing with seawater. Knowledge about the microbial communities will allow us to better understand the role microorganisms continue to play in shaping life on Earth though biogeochemical cycling of elements.

Future Work

There is still much work to be done at both Soda Bay and Purple Pool. Continued geochemical sampling and data analysis of cold-seeps fluids from Soda Bay will allow us to better understand biogeochemical processes occurring along this lotic to marine ecosystem. First a focused investigation of nitrogen and carbon cycling at Soda Bay using QPCR of target genes and metagenomic analysis to further elucidate biogeochemical processes in these dynamic cold-seeps will provide a better understanding of the range of processes that are important in this system. Continued and in-depth analysis of the

metagenomes from both Soda Bay and Purple Pool to identify novel Fe- and Mn- cycling microorganisms and their mechanisms of involvement will expand our knowledge of microbial and metabolic diversity and allow us to not only better understand biogeochemical cycling of elements but also provide insight into the evolutionary history of microorganisms, their role in biogeochemical cycling and ultimately their impact on the planet.

Enrichment studies will allow us to identify the presence of chemoautotrophs and Mn oxidizing microorganisms at Soda Bay and Purple Pool. Enrichment studies can be conducted using geochemical data to design media that would allow the growth of chemoautotrophs and Mn oxidizers. DNA from enrichments or eventual isolates can be compared to the metagenome of biofilms from the environment, which may allow us to identify novel microorganisms and possibility identify their role in biogeochemical cycling of elements in these environments.

Our ability to decipher patterns of mineral deposition from modern spring/seep environments provides insight into environmental conditions that were present when ancient geologic deposits such as BIFs formed. Climatic forcing has a direct and profound influence on the geochemistry and composition of the microbial communities in these environments. For example an increase in precipitation results in mobilization of pools of dissolved chemical species with an initial increase in nutrients followed by the subsequent dilution and depletion of nutrients resulting in a decrease in biomass and biogeochemical cycling of elements.

Acknowledgments

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Chapter 7

Geoscience Education

Introduction

Geoscience Education in Alaska Native Communities

American Indian and Alaska Native K-12 students face numerous challenges both socially and academically. Schools typically employ non-native teachers who lack a fundamental understanding of the community in which they are teaching. This lack of understanding thereby prevents teachers from being effective, as without an understanding of the culture it is exceedingly difficult to teach Native students STEM disciplines. Native students also suffer from a lack of Native mentors in their communities, schools and in STEM fields in general, due to a lack of these mentors, the path to careers in STEM fields is obscured. However, there is a revitalization occurring in Indian education, in which traditional knowledge is being coupled with western science to teach culturally relevant STEM disciplines. Indian country is poised to nurture a new generation of scientists and natural resource managers who can guide their communities to a sustainable future.

Questions to be addressed are:

- Does a multi-year place-based geoscience education program, which incorporates Traditional Ecological Knowledge (TEK) and culture increase content knowledge of marine systems in an indigenous 4-12 peer group?

- What effects do a multi-year place-based geoscience education program, which incorporates TEK knowledge and culture, have on developing a local community workforce which is able to monitor and protect natural resources on lands under tribal control?

- How does TEK in STEM education influence Native students' perceptions of STEM disciplines?

- Can an improvement be made in science literacy of 4-12 grade students from Alaska Native communities through the incorporation of TEK?

Student progress will be evaluated using various assessment techniques with collaborators The University of Washington Office of Educational Assessment (OEA) Evaluations will dovetail with existing, ongoing CMOP assessment work, and proposed data collection will inform evaluation questions for this project, as well as broader questions tied to the Center's education and diversity goals. Pre- and post-surveys for students involved in field studies: Instruments will include some technical knowledge questions (i.e., a pre- and post-test) developed in

collaboration with co-investigators, as well as questions about students' knowledge about and interest in STEM pathways. Data from the evaluation activities will not only document progress towards program goals, but also to inform ongoing program improvement.

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It Takes a Community to Raise a Scientist: A Case for Community-Inspired Research and Science Education in an Alaskan Native Community

By Nievita Bueno Watts and Wendy F. Smythe

The quote, "It takes a village to raise a child," is attributed to *African tradition and carries over to Alaskan Native communities as well (Hall, 2000). Without the support of their community and outside resources, Alaska Native children have a difficult time entering the world of science. Yet increasing the awareness of science, as a tool to help a tribal community monitor and maintain the health of their environment, introduces conflicts and misconceptions in context of traditional cultural practices. Rural communities depend upon traditional food harvested from the environment such as fish, wild game, roots, and berries. In many Native Alaskan villages the health of the environment equals the health of the people (Garza, 2001). Integrating science with culture in precollege education is a challenge that requires sensitivity and persistence.*

The Center for Coastal Margin Observation and Prediction (CMOP) is a multi-institutional, National Science Foundation (NSF) Science and Technology Center that takes an interdisciplinary approach to studying the region where the Columbia River empties into the Pacific Ocean. Two of CMOP's focus areas are biogeochemical changes affecting the health of the coastal margin ecosystem, and socio-economic changes that might affect the lives of people who harvest and consume fish and shellfish.

The Columbia River waters touch the lives and livelihoods of many people, among them a large number of Pacific Northwest Indian tribes. These people depend on the natural and economic resources provided by the Columbia River. Native peoples from California through Alaska also depend on resources from their local rivers, and, currently, many tribes are developing a workforce trained with scientific skills to manage their own natural resources in a way that is consistent with their traditional way of life. The relationship between Traditional Knowledge (TK) and practices, which are informed by centuries of observation, experimentation and carefully preserved oral records, and Western Science, which is deeply rooted in the philosophies and institutions of Europe, is often an uneasy one.

National progress is being made to open pathways for individuals from Native communities to Western Science higher education programs and back to the communities, where tribal members are empowered to evaluate and monitor the health of their environment. CMOP is part of this national movement. CMOP science is developing tools and techniques to observe and predict changes in the river to ocean system. CMOP education, an essential element of CMOP, supports American Indian/Alaska Native students in pursuing academic and career pathways focusing on coastal margin sciences (Green et al., 2013). One of CMOP's initiatives is the CMOP-School Collaboratories (CSC) program.

CMOP-SCHOOL COLLABORATORIES

The CMOP-School Collaboratories (CSC) program is based on the idea that Science, Technology, Engineering, and Mathematics (STEM) pathway development requires an intensive and sustained effort to build relationships among science educators, students, school personnel, and the tribal community. The over-arching goal is to broaden participation in STEM disciplines. CMOP educators developed the CSC model that includes integration strategies for a community, development of appropriate lessons and field experiences and student action projects that connect local and traditional knowledge with science. Educational experiences are place-based, multi-disciplinary and culturally relevant. The objective is to open students' minds to the reality of the need for scientists with many different world views and skill sets working together to address our planet's pressing problems in a holistic manner. CMOP seeks to encourage these students to be part of that solution using both Traditional Knowledge and STEM disciplines.

The program encourages STEM education and promotes college preparatory awareness. This CSC program has three unique characteristics: it introduces coastal margin science as a relevant and viable field of employment; it integrates STEM learning with Traditional Knowledge; and, it invites family and community members to share science experiences. The example presented in this article describes a four-year program implemented in a small village in Southeast Alaska, 200 miles from the capital city of Juneau.

Figure 1: Students, scientists, a cultural expert, and a teacher with scientific equipment used to collect data from the river.

ALASKA NATIVE VILLAGE CASE STUDY

Wendy Smythe, a CMOP doctoral candidate and principal investigator for an NSF Enhancing Diversity in the Geosciences (OEDG) award, is an Alaska Native Haida. As she advanced in her own education, she wanted to share what she had learned with the youth of her tribal community, striving to do so with the blessing of the tribal Elders, and in a way that respected the Traditional Knowledge of the Elders. Dr. Bueno Watts is a mentor and expert on broadening participation. She acts in an advisory capacity on this project.

The village school consists of 15 staff members and 50 K–12 students, with the school experiencing high administration turnover rates. In the first two years of the program we recruited non-native graduate students to participate in the CSC program. This effort provided them experience working in Native communities. In the last two years we recruited Native American undergraduate interns to teach lessons, assist with field activities and provide students with the opportunity to become familiar with Native scientists [Figure 1]. Interns formed part of the science team.

STEPS TO GAIN ENTRÉE TO A VILLAGE

The community must support the concept to integrate science education with traditional practices. Even for this Alaska Native (Smythe), the process of building consensus from the tribe and gaining approval from the Elders and school district for the program was a lengthy one. The first step required letters of support from school district and tribal leaders. The difference in geographical locations proved difficult until Smythe was able to secure an advocate in the tribe who spoke for her at tribal meetings. Face-to-face communications were more successful than distance communications. Persistence proved to be the key to achieving success at getting the consensus of community leaders and school officials' support. This was the top lesson of 10 learned from this project (Table 1).

Traveling to the school to set up the program is no small feat and requires extensive coordination of transportation and supplies. A typical trip requires a day-long plane ride, overnight stay in a nearby town to prepare and gather supplies, a three-hour ferry ride, acquisition of a rental truck and a one-hour drive. Accommodations must be made to board with community members.

The development of appropriate lessons for the curriculum engaged discussions with tribal Elders and community leaders on an individual basis. Elders agreed to provide videoed interviews and were given honoraria as a thank you for their participation. Smythe asked the Elders what scientists could do to help the community, what stories can be used, where students and educators could work in the community to avoid intruding on sacred sites, and what information should not be made public. Once Elders agreed to provide interviews and share stories, other community members began to speak about their lives and concerns. This included influence of boarding schools, life as it was in the past, and changes they would like to see within the community. This was a significant breakthrough.

1. Persistence is key.
2. Face-to-face communication is vital and takes time.
3. A community advocate with influence and respect in the community is critical.
4. Consult with the Elders first. They have their finger on the pulse of the community and are the center of the communication network. Nothing happens without their approval. Find out what it is okay to talk about and where your boundaries are and abide by them. Include funds for honorariums in your proposal. Elders' time and knowledge is valuable and they should be compensated as experts.
5. Partner with individuals or groups, such as the Department of Natural Resources.
6. Find a relevant topic. Be flexible with your curriculum choice. It must reflect the needs and interests of the community and the abilities of the teacher you are working with.
7. Be prepared, bring supplies with you. Ship items in advance if going to a remote location.
8. Have the ability to provide individual instruction for students who need it to prepare projects and practice giving presentations.
9. Involve the community. Hold events in a community center to encourage everyone to attend.
10. View your involvement as a long-term investment in a committed community relationship.

Table 1. Lessons Learned: ten things to consider when developing a science program with Native communities

In addition to the Elders, support was needed from a natural resources representative who functioned as a liaison between our group and the community members. This person's role is found in most villages and could be the head of the Department of Natural Resources or a similar tribal agency that oversees fish, wildlife, and natural resources. This person provides a critical link between the natural environment and the community. The next step is to go in the field with the natural resources representative, science teachers, Elders, and interested students to identify a meaningful focus for the community. Initially we focused the project with a scientist's view of teaching microbiology and geology of mineral deposition in a river ecosystem. However, the team found community interest low and no enthusiasm for this project.

Upon our return to the village, the team and CMOP educators found the focus, almost by accident. We were intrigued by "boil water" notices posted both at the home in which we were staying and on the drinking fountains at the school. The students were all talking about water, as were the Elders. It was clear that the community cared about their water quality. The resulting community-inspired research educational plan was based on using aquatic invertebrate bioindicators as predictors of water quality (Adams, Vaughan & Hoffman Black, 2003). This student project combined science with community needs (Bueno Watts, 2011).

CURRICULUM LESSONS

The first classroom lessons addressed water cycle and watershed concepts (Wolftree, 2004), which were followed by a field lesson on aquatic invertebrates. Students sampled different locations in an effort to determine biodiversity and quantity of macroinvertebrates. While students were sitting at the river's edge, the site was described in the students' Alaska Native tongue by a cultural expert, and then an English translation was provided. This introduced the combination of culture and language into the science lesson.

Figure 2: Students use data loggers to collect data on temperature, pH, and location.

The village water supply comes from a river that runs through the heart of the community. Thus, this river was our primary field site from which students collected water for chemical sampling and aquatic invertebrates using D-loop nets. Physical and chemical parameters of the river were collected using Vernier LabQuest hand-held data loggers. Students recorded data on turbidity, flow rate, temperature, pH, and pinpointed locations using GPS coordinates [Figure 2].

Aquatic invertebrate samples were sorted, classified, counted, recorded, and examined through stereoscopes back in the classroom. Water chemistry was determined by kits that measured concentrations of alkalinity, dissolved oxygen, iron, nitrate/nitrite, dissolved carbon dioxide, and phosphate.

Microbiology assessments were conducted in an effort to detect fecal coliform (using m_FC Agar plates). Students tested water from an estuary, river, drinking fountain, and toilet. Results from estuarine waters showed a high number of fecal coliform, indicating that a more thorough investigation was warranted. While fecal coliform are non-disease causing microorganisms, they originate in the intestinal tract, the same place as disease causing bacteria, and so their presence is a bioindicator of the presence of human or animal wastes [Figure 3 and Figure 4].

Students learned that the "dirty water" they observed in the river was actually the result of a natural process of acidic muskeg fluids dissolving iron minerals in the bedrock, no health danger. The real health threat was in the estuarine shellfish waters. Students shared all of their results with their families, after which community members began to approach the CMOP science team with questions about the quality of their drinking water. The community was relieved to find that the combined results of aquatic invertebrate counts and water chemistry indicated that the water flowing through their town was healthy. However they were concerned about the potential contamination as indicated by fecal coliform counts in the local estuary where shellfish were traditionally harvested.

In the second year, a curriculum on oceanography developed by another STC, the Center for Microbial Oceanography: Research and Education (C-MORE) was introduced (Bruno, Wiener, Kimura & Kimura, 2011). Oceanography lessons focused on water density as a function of salinity and temperature, ocean currents, phytoplankton, and ocean acidification, all areas of research at CMOP. Additional lessons used local shipworms, a burrowing mollusk known to the community, as a marine bioindicator (CMOP Education, 2013). Students continued to conduct bioassessments of local rivers and coastal marine waters.

Figure 3: Students sort and count aquatic invertebrates as a bioindicator of river health.

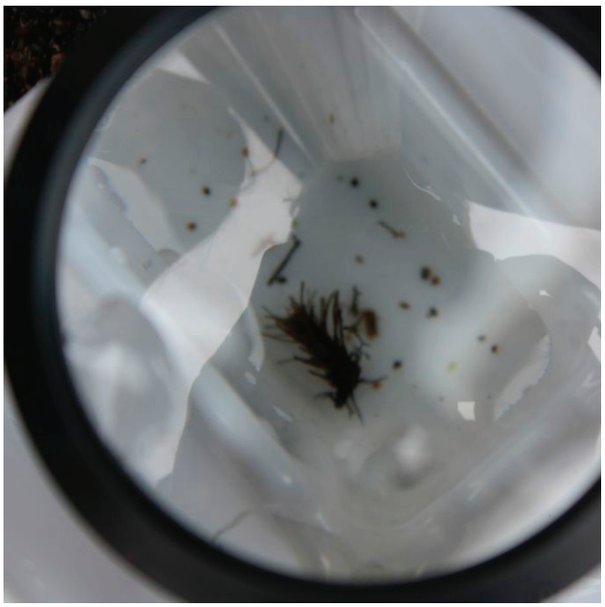

Figure 4: Caddis fly larvae are highly sensitive to pollution. Their presence indicates a river is healthy.

Students used teleconferencing technology to participate in scanning electron microscope (SEM) session with a scientist in Oregon who had their samples of aquatic invertebrates. Students showcased their experiments during parent day. Five students (10%) had parents and/or siblings who attended the event.

SHARING KNOWLEDGE

As a reward for participation in the science program, two students were chosen to attend the American Indian Science and Engineering Society (AISES) 2009 conference in Oregon. Travel expenses were shared between the school, CSC program, and the tribe. In the following three years an additional ten students attended the AISES conference and presented seven science research posters in New Mexico, Minnesota and Alaska. In 2012, one student won 3rd place for her shipworm poster presentation [Figure 5]. These conference presentations enabled some students to take their first trip out of Alaska.

In May 2011 the first Science Symposium for grades K–12 allowed students to share their science projects with parents, Elders, and tribal community members. Both students and teachers were prepared on how to do a science fair project. Work with students had to be accomplished on a one-on-one basis, and members of the team were paired with students to assist with completing projects and polishing presentations. Students were not accustomed to speaking publicly, so this practice was a critical step.

The event was held at the local community center, which encouraged Elders and other community members to attend. Elders requested a public education opportunity to teach the community about watersheds and the effects of logging. Our team incorporated this request into the science symposium. Students led this project by constructing a 3D model of the watershed for display. People could simulate rainfall, see

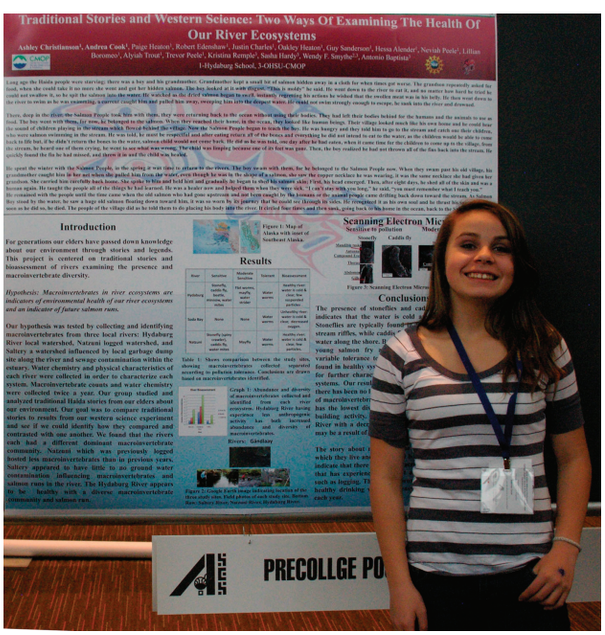

Figure 5: Science fair participant presents her work at the national AISES conference.

how land use affects runoff, and make runoff to river estuary connections. Scientists conducted hands-on demonstrations related to shipworms, local geology, ocean acidification and deepsea research. Language and culture booths were also included. During the symposium, a video of one of the interviews we had conducted with an Elder was shown as a memorial to his passing. The symposium was considered a huge success and was attended by 35 students and 50 community members.

COMMUNITY RESPONSE

The CSC program garnered results that could not have been predicted at the outset. For example, the tribe requested our input when deciding which students should attend a tribal leadership conference and summer camp. Three student interns participated in a collaborative project with the tribe to conduct bio-assessment studies of local rivers and a key sockeye breeding lake. Interns operated a remotely operated underwater vehicle (ROV) for data collection, resulting in video documentation of the salmon habitat. In addition to the bio-assessment, the interns conducted interviews with Elders about the rivers in the monitoring project. The results of this study were used to stop logging around sockeye spawning habitat and to ban the harvest of shellfish from contaminated parts of the estuary. Now the tribe is monitoring rivers on its own. In the near future CMOP plans to install a sensor that can be monitored remotely, and to train people to read and interpret the data.

CONCLUSION

Community-inspired research often produces a ripple effect of unforeseen results. In this case, inclusion of Elders in the design and implementation of the project produced large scale buy-in from community members at all age levels. Consequently, in a village where traditionally students did not think about education beyond high school, we have had two students attend college, two students attend trade school, five students receive scholarships, and eight Native interns conducting science or science education in the community. And, given the low numbers of Alaska Natives pursuing careers in science, we find those numbers to be remarkable.

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ADDITIONAL RESOURCES

The educational resources of CMOP are available on their website: URL http://www.stccmop.org/education/k12

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Lessons Learned from a Geoscience Education Program in an Alaska Native Community

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Abstract: Alaska Native communities depend on customary and traditional use of natural resources for physical, emotional and cultural sustenance, and community members are concerned about threats to local ecosystems posed by logging, mining, overharvesting, invasive species, fresh and marine water pollution, and climate change. To support one community's efforts to address these concerns, we have developed an experiential geoscience education program for grades 5 – 12 that draws upon both western science and traditional knowledge. In this program we have found that students are best served by a pedagogy that is founded upon community partnerships, focuses on community needs, reinforces cultural traditions, and presents western science and traditional knowledge as equal and complementary bodies of knowledge. This program has contributed to an increased number of high school graduates pursuing college degrees and has been welcomed by the community as an integral component of cultural revitalization.

Keywords: native pedagogy; geoscience education; traditional knowledge; cross-cultural education

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Introduction

The Center for Coastal Margin Observation and Prediction (CMOP) is a multi-institution, NSF-funded Science and Technology Center that uses an interdisciplinary approach to study coastal margins, with primary geographic focus on the Columbia River and its interaction with the Eastern North Pacific Ocean. With place-based research as a catalyst, CMOP is working with Native American communities in the Pacific Northwest (PNW) and Alaska across multiple dimensions of the center's mission, including education and knowledge transfer. Workforce development is a high priority issue for PNW tribes, who are actively seeking Native scientists able to incorporate scientific skills within "traditional knowledge" in order to manage natural resources in a way that is congruent with tribal values and traditional way of life (Bueno Watts & Smythe, in review).

The incorporation of traditional knowledge and practices with western science is often challenging (Mazzocchi, 2006). Even the definition of traditional knowledge is not without ambiguity. We adopt in this paper the definition offered by the American Association for the Advancement of Science:

> Traditional Knowledge (TK) is the information that people in a given community, based on experience and adaptation to a local culture and environment, have developed over time, and continue to develop. This knowledge is used to sustain the community and its culture and to maintain the genetic resources necessary for the continued survival of the community (Hansen & Vanfleet, 2003, p. 3).

A potential solution to this challenge is to create open, supportive, and well-defined pathways from native communities to western science higher education programs and back to the communities, where tribal members will ultimately be empowered to evaluate and monitor the health of their environment. With this framework in mind, CMOP seeks to support American Indian/Alaska Native students in pursuing academic and career pathways in coastal margin sciences, through programs such as our CMOP-School Collaboratories program (Bueno Watts & Smythe, in review).

This paper illustrates one such collaboratory – which in fact incorporates an entire community – created through a geoscience education program for grades 5-12 in the Haida community of Hydaburg, Alaska. The objective of this program was to incorporate traditional knowledge into geoscience education, thereby improving geoscience literacy and attitudes towards both traditional knowledge and western science by Alaska Native students and community members. To accomplish this objective we employed language, tools and skills from both disciplines to offer an engaging and culturally relevant geoscience curriculum. This effort was led by a consortium of scientists, educators, elders, and tribal groups focused on empowering Hydaburg youth to one day assume leadership roles in the management of natural resources that are key to sustaining their community and their way of life.

In this effort we presented western science not as the primary method to understand local ecosystems or to drive resource decision – after all, Alaska Natives have successfully studied, utilized, and managed lotic and coastal ecosystems for thousands of years. Rather, we presented western science as a tool to understand how local ecosystems are changing in response to rapid anthropogenic inputs, to augment traditional resource management techniques, and to reclaim

traditional practices gone underutilized as a result of the historical repression of Native cultures.

Our pedagogical approach emphasized a respect for local traditions, knowledge, and culture, and was explicitly driven by community-identified needs. Each individual science topic was chosen due to a specific interest expressed by teachers, students, or the tribal community. For each topic our group learned the traditional perspective and language terms by consulting with one or more community members with traditional expertise.

Our program provided a variety of student-centered, inquiry-based, and culturally relevant activities that utilized both western science and traditional knowledge. These activities included both field and laboratory components and were enriched by the use of remote scientific instruments, including a scanning electron microscope (SEM), computed tomography and a remotely operated vehicle. Topics included watershed, lake and ocean studies, bio-assessments, water chemistry experiments, microbiology studies, mollusk ecology and anatomy, and dendrochronology. Cultural activities included video and audio recordings of elder interviews, translation of scientific terms into the Haida language, use of traditional stories and songs, and summer culture camp support. We also supported the local chapter of the American Indian Society of Engineering and Science (AISES) by subsidizing student trips to AISES and Geoscience Alliance conferences.

Teaching Science in Native American and Alaska Native communities – a Review of the Literature

The challenges faced by native schools and communities are well-documented by both native and nonnative authors. This review focuses on studies conducted by or in close association with Native Americans and Native Alaskans, in an attempt to better understand issues and community dynamics from a native perspective (Swisher, 1996).

Many studies (Brave Heart & DeBruyn, 1998; Brave Heart, 2003; Whitbeck, Adams, Hoyt, & Chen, 2004) point to a historical trauma on native communities that is transmitted across generations. This trauma is identified as deriving from a history of genocide, displacement, abusive boarding schools, prejudice, and forced acculturation–and as being compounded by ongoing poverty, discrimination, depression and anxiety, substance abuse, suicide, and violence. These issues are often reflected in the schools.

Although education reform in the 1970s and later replaced federal and state schools, which focused on acculturation, with locally controlled schools (Deyhle & Swisher, 1997; Dlugokinski & Kramer, 1974; Lomawaima, 1995), native schools still employ an overwhelmingly non-native teacher base with an exceedingly high turnover rate (Barnhardt & Kawagley, 2004). In 2003, the U.S. Commission on Civil Rights found that native students commonly experienced "deteriorating school facilities, underpaid teachers, weak curricula, discriminatory treatment, and outdated learning tools" (Berry et al., 2003).

Despite these challenges, native communities and their schools have begun to revitalize (Brave Heart, 1999; Brave Heart & DeBruyn, 1998), reclaiming traditions, culture, and language. Effective science education efforts can both support and capitalize on this movement by adopting culturally relevant and student-centered pedagogies and by educating non-native teachers about the specific cultures of the native communities in which they live or work (Hatcher et al., 2009). Cajete (1999) notes that pedagogies must recognize the current state of Native American culture, both accommodating the ways in which Native Americans have traditionally learned and recognizing how native cultures have changed since the 1900s.

Although native cultures are far more diverse than often recognized by most non-natives, a number of core values, beliefs and practices are shared by many native communities (Cajete, 1999). Traditional knowledge has been acquired over multiple generations through firsthand interaction with local ecosystems (Cajete, 1999; Deyhle & Swisher, 1997; Kawagley & Barnhardt, 1999; Mack et al., 2012; Nelson-Barber & Estrin, 1995; Simpson, 2002; Zandvliet & Brown, 2006). Competency in natural resource science and management was essential for–and defined by–survival (Barnhardt & Kawagley, 2005; Simpson, 2002). Thus, traditional knowledge systems contain abundant and time-tested information despite the fact that the confirmation mechanism is different than that of western science.

Unlike the compartmentalized knowledge systems of western culture, in which science is a highly specialized practice, traditional knowledge systems are woven into a single body of knowledge (Barnhardt & Kawagley, 2005). Both traditional knowledge and western science are founded on empirical observations, but traditional knowledge incorporates a holistic approach that perceives natural phenomena in non-deterministic, cyclical, contextual terms (Haig-Brown, 1995). Though this view differs from the more deterministic and axiomatic science generally taught in mainstream schools, Riggs (2003) has noted that it may present natives with an advantage understanding such concepts as complex natural systems, geologic time, and long-term environmental cycles. Riggs also notes that because these integrative concepts are fundamental in the geosciences, an earth systems pedagogy has advantages for native students.

Despite many challenges over the past few centuries, much traditional knowledge has been preserved through both continued practice and the telling of oral traditions, which typically interweave allegorical lessons and cultural wisdom with empirical observations (Brighthurst, 2000). The ability of cultures with no written languages to maintain knowledge across multiple generations is non-intuitive to a western scientific culture highly dependent on written records. However, the amount of accurate detail in oral traditions describing, for example, the eruption of Mt. Mazama in Oregon seven thousand years ago (Heusser & Grabher, 2002) or the timing of catastrophic tsunamis along the pre-colonial Oregon coast (Ludwin et al., 2005) demonstrate the effectiveness of native oral histories. Increasing recognition of the added value of traditional knowledge has led to its incorporation into a number of scientific studies (Barnhardt & Kawagley, 2004, 2005; Davidson-Hunt & O'Flaherty, 2007; Nelson-Barber & Estrin, 1995).

The cultural differences between traditional and western knowledge systems have created challenges in equitably educating native students. A first challenge is posed by the common belief that traditional knowledge systems are inferior and lacking in real or meaningful data (Deyhle, 2010; Deyhle & Swisher, 1997; Haig-Brown, 1995; Kawagley & Barnhardt, 1999a). This belief stimulates teachers to "correct" the ethnoscientific knowledge with which many native students are endowed (Kawagley & Barnhardt, 1999b; Deyhle & Swisher, 1997). Native students are forced to choose between the "correctness" of one knowledge system over the other; many choose the system in which they were raised, withdrawing from active participation in school and sometimes leaving school altogether. Although choosing their native cultures leads to "failure" as defined by their teachers and mainstream society, students making this choice view their actions as a successful rebellion against an unjust system (Deyhle, 2010; Deyhle & Swisher, 1997).

A second challenge is a set of teaching strategies that conflict with the learning methods of many native students (Barnhardt & Kawagley, 2005; Cajete, 1999; Kawagley & Barnhardt, 1999b; Nelson-Barber & Estrin, 1995). The teaching of global axioms in mainstream schools is in opposition to the situational ecological relationships taught in native cultures (Haig-Brown,

1995). Common classroom practices such as rapid-fire question and answer sessions may pose difficulties for native students, who tend to prefer contextualized lessons in which they observe before practicing – i.e. "watch-then-do" (Cajete, 1999). Although native cultures vary, Cajete (1999) describes some common attributes of native learners, including quietness and patience, a cooperative social orientation, a holistic and spiritualistic view of nature, a non-linear sense of time, an open work ethic (i.e., work is valuable when it serves a valuable purpose) and a cautious approach to new situations.

The shortcomings of educational systems based on forced acculturation have been documented in other cultural contexts (Cobern, 1996; Kirkness & Barnhardt, 1991; Phelan, Davidson, & Cao, 1991). Conversely, studies have shown that, depending on the degree of cultural difference between students and mainstream society, students whose worldviews are explicitly respected and affirmed may be more competent in both their native culture and in the mainstream culture of the schools (Aikenhead, 2001; Aikenhead & Jegede, 1999; Barnhardt & Kawagley, 2004; Brown, Collins, & Duguid, 1989; Costa, 1995; Deyhle & Swisher, 1997; Phelan et al., 1991). Further, studies have shown that native students taught in their own language fare better on standardized tests and exhibit higher self-esteem (Deyhle & Swisher, 1997; Wright, Taylor, & Macarthur, 2000).

Nelson-Barber and Estrin (1995) note that pedagogies well suited for native communities align closely with constructivist best practices in modern science education – i.e., new knowledge is built upon prior knowledge through direct interaction in an appropriate, understandable context (Bransford, Brown, & Cocking, 2000). In particular, there are clear parallels between traditional knowledge and the constructivist notions that an individual's body of knowledge is constructed from direct experiences with the world; that knowledge exists within the contexts of culture and personal perspective; and that "truth" varies from person to person (Colburn, 2000).

However, Nelson-Barber and Estrin (1995) also note that mainstream constructivist pedagogies must be augmented with specific cultural practices to significantly improve Science, Technology, Engineering and Math (STEM) education for native students. Mack et al. (2012), through a series of interviews with informal educators in native communities, identified a comprehensive set of attributes necessary for success in informal native science education program. Subsets of these attributes have been identified by others (Barnhardt & Kawagley, 2004, 2005; Burhansstipanov, Christopher, & Schumacher, 2005; Cajete, 1999; Davidson-Hunt & O'Flaherty, 2007; Deyhle & Swisher, 1997; Haig-Brown, 1995; Hatcher et al., 2009; Inglebret et al., 2008; Kawagley & Barnhardt, 1999b; Kirkness & Barnhardt, 1991; Nelson-Barber & Estrin, 1995; Riggs, 2005; Simpson, 2002).

From these standpoints, a well-designed program for native students includes the following elements:

- place-based, experiential lessons focused on traditional homelands,
- curriculum created in collaboration with native community members, especially elders, with native cultural and ethnoscientific expertise,
- the use of native language in instruction,
- the use of culture as a foundation for the program,
- learning outcomes matched to the specific values of the community,
- the use of locally appropriate traditional pedagogies,
- collaborations with regional, local and tribal entities to share resources,
- respect for sacred aspects of native knowledge,

- respect for all of the knowledge and experience brought to bear by students and other participants,
- the application of research on both native ways of knowing and western science.

Teaching Geoscience in the Community of Hydaburg, Alaska

Southeast Alaska is the main territory of the American Haida population. The community of Hydaburg, located in a temperate coastal rainforest on Prince of Wales Island, is richly endowed with a wide variety of natural resources and has retained much of its knowledge and skills pertaining to customary and traditional use of local resources. The community is centered on the Hydaburg River, which empties into the narrow Sukkwan Strait and ultimately in the Eastern North Pacific Ocean, presenting a multitude of freshwater and marine opportunities for fishing, hunting, and gathering. Hydaburg River and other nearby rivers support generally healthy salmon runs, though according to tribal elders and fishermen the numbers of salmon have declined in certain watersheds due to logging activity (Glenn Douglas and Claude Morrison, personal communication, 2010). Local old-growth, second-growth and recently logged watersheds provide wild game and berries, tea and medicinal plants, and wood products for firewood, building materials and art products. Sukkwan Strait and the surrounding environment supply an abundance of fishery resources such as anadromous fish, halibut, herring, crab, shrimp, bivalve mollusks, salicornia and other sea greens.

The American population of Haida originally settled on Prince of Wales Island in the 16th century, having emigrated from Haida Gwaii, located off of what is now the coast of British Columbia. As with many other tribes, the past two centuries have provided daunting challenges to the Haida people and culture. The introduction of Euro-American diseases occurred in the late 18th century and by the late 19th century; smallpox and other diseases had reduced the Haida population by 90% (Brighthurst, 2000). Soon after, state and federal boarding schools began separating young children from their families, systematically disciplining them for speaking their native tongues or practicing native traditions. This practice continued into the mid-20th century and many of today's Haida elders experienced the abuses of boarding school (Georgianna Douglas and Glenn Douglas, personal communication, 2011). Although native students at boarding schools employed a number of strategies to hold on to their culture (Brave Heart, 1999), by the end of the 20th century, the Haida language was spoken fluently by an estimated total of only 45 people – 15 in America and 30 in Canada (Lewis, 2009).

However, as in other Alaska Native/Native American communities, Hydaburg tribal members are forging a cultural revival. Haida linguists are teaching Haida language classes and archiving traditional stories of Haida history from tribal elders. Though school instruction is in English, elementary school students learn Haida-language songs and games as well as oral traditions translated into both English and Haida. The community has a number of master and apprentice carvers, weavers, and artists who use traditional tools to create stunning works of art founded on traditional designs. Hydaburg holds an annual culture camp that emphasizes teachings in Haida language, art, and traditional practices.

Although the youth in Hydaburg have maintained a strong Haida identity and community elders are revered by the younger generations, few youth openly practice the traditional culture of their grandparents and great-grandparents. Instead, the youth with whom we worked were oriented to mainstream popular music, movies and technology. The dominant social and physical

youth activity is basketball. The school system struggles with low student achievement, and the youth in Hydaburg exhibited many of the symptoms of historical trauma described by Brave Heart (2003), specifically withdrawal from school participation and a lack of self-confidence in academic disciplines.

Our Teaching Strategies

In traditional Haida culture, parents support and nurture their children while skills, knowledge, and discipline are taught by extended family members such as aunts and uncles (Glenn Douglas, personal communication, 2010). Children with particular abilities are mentored by specialists in the appropriate aspect of traditional knowledge. According to Haida elders, many aspects of traditional knowledge are considered sacred or privileged and are taught only when students are considered to be sufficiently mature and competent (Georgianna Douglas, personal communication, 2011).

Although we could not replicate this teaching model, our goal was to offer a culturally relevant, engaging geoscience curriculum that supported the community's and students' needs. This program did significantly deviate from the best practices identified by Mack (2012) in that we did not take an explicit "culture first" approach. This was for two reasons: (a) because only two tribal members - the town's environmental planner and our team leader - had Western science expertise, a significant portion of the program was led by non-natives who were not culturally fluent; and (b) because no students spoke fluent Haida, instruction was primarily in English with Haida terms and phrases incorporated as a second-language supplement. Thus, the program's *de facto* approach was to place western science on an equal footing with traditional knowledge. We felt that because students were heavily oriented to modern culture, our approach would be more effective than attempting to ground our lessons entirely in traditional values and culture.

Instructional activities

In this program we provided a wide variety of STEM enrichment activities for students in grades 5-12. Lessons were provided using a student-centered strategy, probing students for their knowledge, experiences and interests before deciding what material we would teach and at what level we would teach. The material taught was placed in practical rather than traditional context, emphasizing how watershed ecology and oceanography connected to the community's water supplies for consumption, recreation, and fishery resources. When the students would share their traditional knowledge, we would use this opportunity to actively reinforce the validity of that knowledge and to point out the ways in which western science complemented it. Whenever possible we incorporated stories told by community members in Haida and translated into English, and introduced Haida terms to the students (Appendix I).

STEM enrichment activities were delivered to students in three discrete weeks of each school year and one week each summer of the program. Each week-long visit had a different purpose and emphasis. The first visit, at the start of each school year, focused on field and laboratory exercises related to the nearby coastal watersheds and served to align our instructional activities on practical and traditional community needs. Mid-year visits focused on maintaining relationships with students and teachers, helping teachers make progress with student projects, and establishing projects for the spring science symposium. End of year visits focused on the

science symposium, with team members and teachers helping students complete and exhibit their science projects. The summer trip's purpose was to support culture camp activities with family science experiences and to support tribal assessments of key fishery and watershed resources conducted with the help of high school and undergraduate interns. Each visit to Hydaburg was followed by a team debriefing in which we identified what students learned and what questions were raised by the activity.

The fall field science activities included macroinvertebrate-based bioassessments (Figure 1) and chemistry-based water quality studies of local rivers, deployment or collection of marine shipworm traps at the local marina, dendrochronology studies of newly sawn totem poles to document climate change, microbiology studies of various surfaces and water sources, and oceanography science. We loosely followed the KWL (What do you *Know*, What do you *Want* to know, What did you *Learn*) format (Carr & Ogle, 1987) for introductory and follow-up discussions of each activity. Field trips and experiments were preceded by a discussion of students' prior knowledge and an introduction of science concepts using diagrams, maps, group discussion, and traditional stories relevant to the topic.

Figure 1 Elementary school students identifying macroinvertebrates.

During the winter trip, one member (Smythe) traveled to Hydaburg to reiterate material taught at the beginning of the school year and to introduce new material for the upcoming spring visit. In meetings with teachers we discussed any activities that had been introduced as a result of our fall trip; although we encouraged teachers to build on the fall field studies, for logistical reasons (primarily sports schedules and poor weather conditions) this follow-through was often minimal, especially at the high school level. Finally, in classroom discussions we helped students plan science fair activities that they would pursue during the winter and spring.

An engaging activity that allowed us to extend the fall field studies into the winter was the remote examination, via a SEM physically located at Portland State University, of macroinvertebrate specimens gathered by students in the fall. These specimens were preserved in

50% ethanol and brought to Portland by project team members. Unlike many advanced scientific instruments, the fundamental data produced (secondary electron images) by an SEM can be interpreted intuitively and without special training (Beane, 2004), although a detailed understanding of SEM contrast mechanisms requires more study. Further, modern SEM instruments are controlled by computer interfaces and therefore can be remotely operated via an internet interface. Figure 2 displays a sample SEM image that was acquired remotely by Hydaburg students.

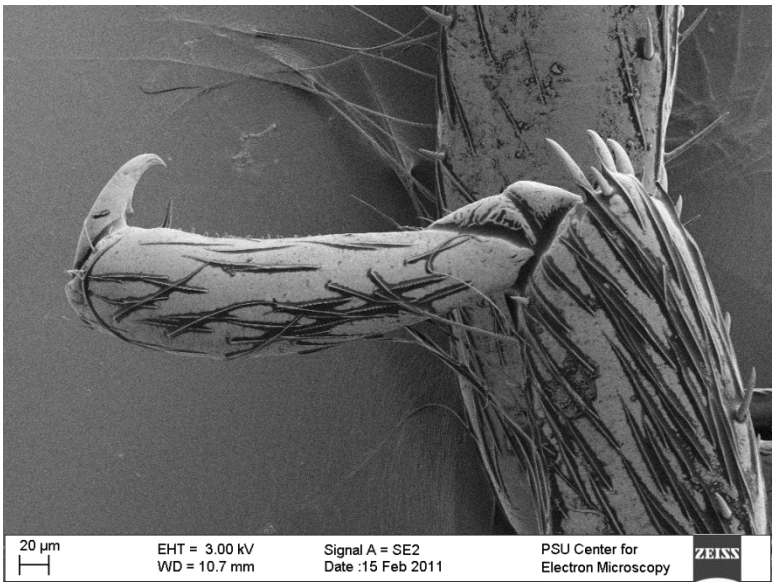

Figure 2. SEM image of a Golden Stonefly claw

The spring trip was focused on science experiments for the year-end, non-competitive science symposium. However, our team of 6-9 scientists also led throughout the week a variety of fun, hands-on experiments and demonstrations, such as Mentos™ geysers, crystal growth experiments, luminous bacteria streak plates, and science-themed skits. For most of the week, project team members helped individual students or small groups complete a wide variety of science experiments. Over the course of the two and a half-year enrichment program, students in grades 5-6 performed consumer research (e.g. "which bubble gum blows the biggest bubbles?") and electricity experiments. Students in grades 7-12 pursued small-group projects appropriate for their earth science, biology, and chemistry classes or participated with a larger group that built a three-dimensional, spatially accurate coastal watershed styrofoam-and-clay model based on a topographic map of Hydaburg (Figure 3). The multi-grade special education class performed remarkably sophisticated, social peer-pressure experiments in which survey subjects were pressured into choosing a particular brand as the tastiest peanut butter (younger group), or identifying the larger of two polygons based on a previous student's identification.

Figure 3. 3-D model of Hydaburg built with styrofoam sheets and modeling clay. Vertical relief is exaggerated. The model is waterproof so students can simulate surface water flow by pouring "rainwater" on the surface.

Summer activities included CMOP- and tribal government-sponsored watershed assessment internships and and enrichment activities that added value to the tribe's annual culture camp (Figure 4). During culture camp, family groups were taught to perform water quality assessments using macroinvertebrate surveys and tide pool ecology. A strong emphasis was put on how these types of assessments could help protect the community's natural resources and complement traditional resource management practices. The inclusion of parents in these activities was intended to build broad community support for our science enrichments.

Figure 4. Totem pole raising, performed using traditional engineering methods, at the summer culture camp. CMOP-sponsored watershed assessment activities during culture camp emphasize

the complementary nature of traditional knowledge and western science.

In every visit to Hydaburg we supported Haida cultural reclamation efforts by funding the language preservation activities of a local linguist and by holding frequent meetings with community leaders and elders. This support led to the creation of audio and videographic records of Haida-language stories and recollections by elders and the translation of western scientific terms into the Haida language. The translation process resulted in the creation of new Haida phrases for scientific terms. For example, prior to this project there were no Haida terms for the words "computer," "calculator" or "camera." These and other words were added to the Haida vocabulary through a consensus effort by the elders. Appendix I gives a listing of relevant Haida terms.

Assessment Methods

Our assessments were aligned with our student- and community-centered program objectives and were guided by the basic question of how to best serve this specific community's needs. We employed a mixed-models methodology (Johnson & Onwuegbuzie, 2004) which emphasized qualitative assessments to both measure the impacts of the program on students and community members and to provide feedback for program adjustments. Quantitative surveys, which focused on each year's science fair, provided an independent measurement of program effectiveness and student attitudes toward science. We note that though the surveys were summative in nature and were focused on each year's culminating event, our explicit purpose for the survey was to provide ongoing feedback for a program and partnership that will continue beyond the life of an NSF-funded project. Unlike studies that intend to produce generalizable results, our focus was on specific long-term benefits to the communities of Hydaburg and Prince of Wales Island.

Our qualitative assessments included interviews of students, teachers, and community members, structured and informal observations of student performance and attitudes, and written assessments of student learning gains. Semi-structured interviews and video recordings of tribal elders provided the foundation for the relevant cultural content and Haida language support. Community needs were assessed through informal interviews with tribal leaders and school parents. Teacher interviews allowed the project team to respond to specific curricular needs and align project activities with learning objectives. High school graduation and college/trade school attainment rates were retrieved via informal interviews with students and teachers.

Student participation, attitudes, and learning gains were measured during the KWL exercises described above, with one researcher leading the students in the exercise and another researcher recording student responses and behavior. Learning gains were also measured using "draw-a-watershed" activities in which students were asked to draw and label important components of either the Hydaburg watershed or the marine environment at the mouth of the river, depending on the subject of the learning activity.

Quantitative evaluation surveys were independently designed and administered by the University of Washington's Office of Educational Assessment. Surveys were given to students at two time points: once in Fall 2010 and again in Fall 2011. These survey served two evaluative purposes: (a) to assess the impact of the instructional activities on student's self-reported knowledge, their interest in science, and their future plans; and (b) to gather feedback about the activities themselves, including students' satisfaction and suggestions for improvement. In 2010,

the survey was administered on paper by the Hydaburg school teachers after the research team had departed. In 2011, the survey was implemented using an online survey tool (University of Washington's Catalyst Tool); students completed the survey on in-class computers with assistance from their teachers. The survey questions are given in Appendix II. Comparisons of students' responses to identical questions across the two time-points provide some indication of impact of the program. The school population in this community is quite small (30 students from 5^{th}-12^{th} grade), and although response rate was higher than 50% on any one survey, there was inadequate statistical power to yield significance in parametric tests (i.e., matched-sample t-tests) of longitudinal changes in numerical ratings. Survey results were therefore interpreted qualitatively, relying on open-ended responses, descriptive statistics, and non-parametric tests (i.e., effect size) of longitudinal changes.

Results

Because of limitations noted above, our quantitative survey results are most valuable when used to verify and extend our qualitative results. Therefore we present our findings as a series of qualitative lessons learned which we hope will be useful for other researchers and educators working in similar communities.

Non-traditional native students benefit from a creative, flexible, and contextualized approach.

Although students in this community generally revered and respected their tribal elders, there was sometimes a cultural disconnect. Unlike other tribes that have much or most of their indigenous language intact, this community's language and traditional knowledge base has been fragmented by historical traumas. The youth in this community have a strong emotional attachment to a Haida identity but speak little Haida and practice their traditional culture sporadically. Thus we could not emphasize Haida language and culture as a way to make science relevant, as recommended by Mack (2012) and others. In fact, the approach that proved effective was the opposite: we used science as a way of affirming traditional Haida knowledge and culture.

The effectiveness of our approach was best demonstrated by informally observable improvements in student engagement over time, particularly among students who participated as summer bioassessment interns. Student interest in science grew significantly in year two of the project, with students designing their own science projects, taking part in fund raising activities to attend national or regional science conferences and actively participating in field investigations. To date, 16 students have participated in team poster presentations at national conferences, with two students winning conference awards in 2012.

Survey data showed that students were most interested in topics that were both locally relevant and related to traditional Haida culture. In Figure 5, survey topics which addressed questions explicitly related to customary and traditional use of local resources (i.e. those at the upper end of the chart) were generally of more interest to students than the abstract, generalized science topics at the bottom of the figure.

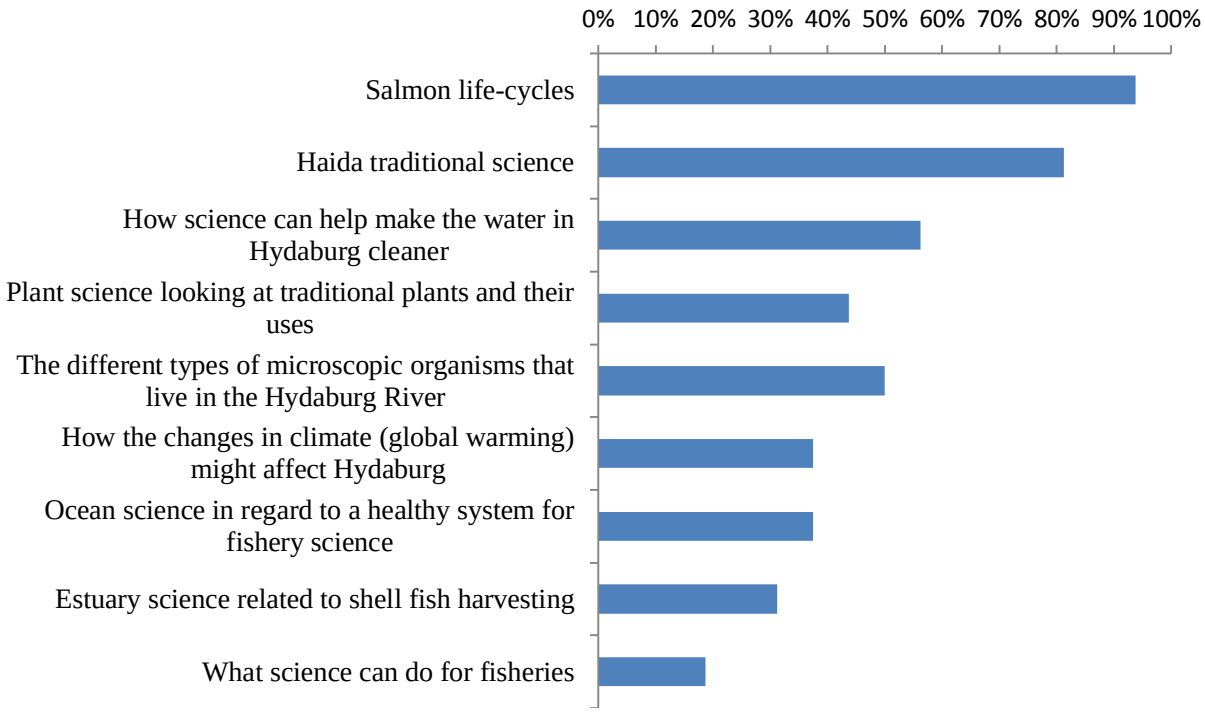

Figure 5. Percentage of 2011 survey respondents (N = 16) indicating they would be interested in studying various topics during future project team visits.

Other survey data of student attitudes reinforced the need for contextualization. Participants' ratings of their interest in general science fields and topics, as well as their confidence with specific skills or knowledge (e.g. how to sample water, or how climate change might affect the Hydaburg river) remained statistically unchanged or, in some cases, decreased between the 2010 and 2011 surveys.

Table 1. Descriptive statistics and effect sizes for students' ratings of their own interests in different fields, from 1 - "Not at all interested" to 4 – "Extremely interested," across time points.

	2010		***2011***			
Field	***M***	***SD***	***Mean***	**SD**	***n***	***Δ***
Biology	2.55	1.13	1.91	0.94	11	-0.62
Chemistry	2.11	0.93	2.22	1.09	9	0.11
Oceanography	2.67	1.41	1.89	1.05	9	-0.63

Field	2010		2011			
	M	*SD*	*Mean*	SD	*n*	*Δ*
Geology	1.78	0.97	2.00	1.12	9	0.21
Microbiology	2.00	1.16	1.86	1.22	7	-0.12
Marine biology	2.00	1.16	2.00	1.00	7	0.18
Science in general	2.44	1.01	2.11	0.93	9	-0.34

* Participants who responded "I do not know what this is" or did not respond to the item on either the pre- or post-survey were not included in mean calculation or effect size.

Students and community members were extremely positive about team visits.

Many students and community members informally reported looking forward to team visits and were enthusiastic when the team arrived. Parents and teachers often thanked project personnel for their efforts. Students often had favorite team members and would ask about any team members missing from subsequent visits. Open-ended survey data indicated that students found our team's week-long visits to be enjoyable, with the examination of macroinvertebrates (or, as students reported, "little bugs" in the water) via microscope and the "crystal gardens" activity standing out as most engaging and/or "cool."

Project leadership by a community member was critical

In this effort a Haida community member (Smythe) was the explicit project leader, while others were present to provide resources and assistance. Philosophically, this structure was a critical component of our effort as it demonstrated to the tribe that the project team was ready to assist the community in its own agenda. Without this structure we would simply have been a group of outsiders dictating outside solutions to problems which we independently identified. From a practical standpoint, community leadership in the project was necessary to facilitate discussions with parents and other community members. In these discussions, our team leader's understanding of tribal politics and cultural mores was critical. Navigating this complex social structure requires the proper mix of assertiveness and deference, and it is essential to understand what constitutes appropriate gifts, visits, and social acknowledgments for elders and other community leaders. These social acknowledgments need to be handled with an astute understanding of the social and power relationships between modern and traditional governmental entities, clans, and families.

Face time was critical

Throughout our program, efforts by teachers, administrators and students were much higher during personal visits by the team. For reasons both within and outside of their control,

follow-through by teachers and administrators was rather weak when we were away. A major impediment for this follow-through by teachers was the lack of reliable telecommunications due to both technical and personal issues.

Most students had worked with our team leader one-on-one during the winter visit to develop their science fair projects. Survey data indicated that students were overwhelmingly positive about this learning experience. Most students stated that they "learned a lot" and would not have performed a science project without this assistance. See Figure 6.

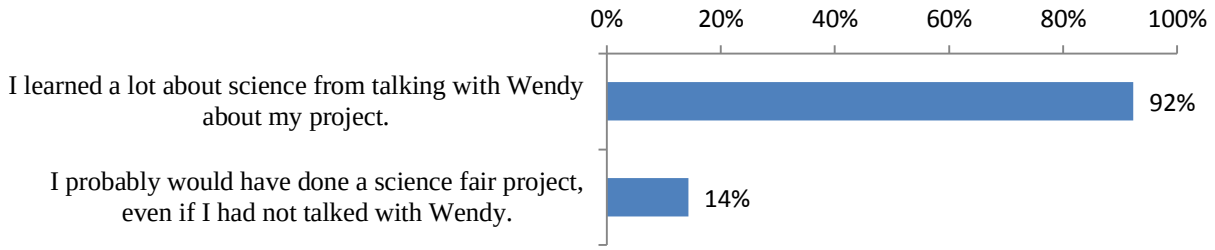

Figure 6. Percentage of students (N = 14) who agreed or strongly agreed with statements about the importance of individual attention from the project team leader.

A culminating event – the Science Fair - piqued student interest and stimulated community involvement

Most of the students in grades 5-10 presented an exhibit at the science fair at the end of each year of the project. The event was well attended by proud family members and tribal elders. Other community partners such as the tribal government and a regional health care provider prepared exhibits devoted to environmental and health issues. Student and family participants were enthusiastic and supportive of each other's work.

Survey data showed that students were generally positive about the experience, although a number of students in grades 10-12 did not participate in the science fair in spite of completing their projects. See Figure 7. Open-ended responses to the survey indicated that for many students, the science projects were themselves the most important part of the experience, e.g., "The best part about the science fair was the experiment part." Other responses indicated that most students were proud of their work, e.g., "The best part was finishing, learning and feeling good about mine and my partners project."

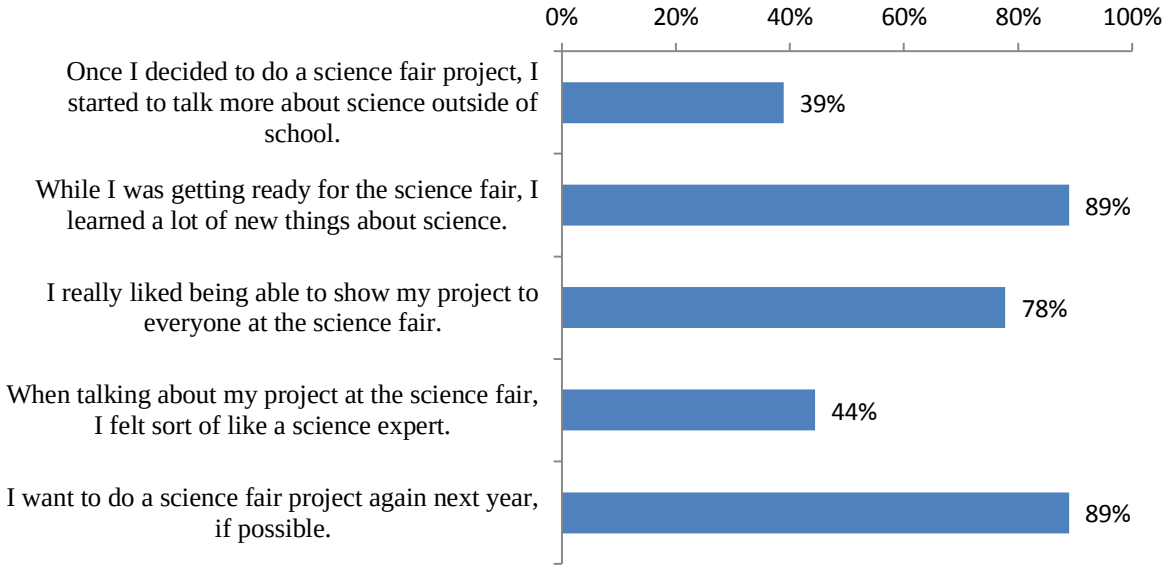

Figure 7. Percentage of 2011 survey respondents (N = 18) who agreed or strongly agreed with positive statements about the science fair

Establishing credibility in the community takes time

The community of Hydaburg has multiple entities - tribal elders, city government, tribal government, the regional Native Corporation, the school district, the school board - with formal or informal authority over specific aspects of life. Sincere attempts to acknowledge and respect a variety of individual, cultural and governmental perspectives were critical for a successful collaboration. As in most rural communities, citizens have witnessed many short-term programs delivered by outsiders that had little long-term impact, and residents were naturally skeptical of outside participants taking part in the education of students. Long-term persistence by the project team was required before the community became fully vested in the effort. This was true for both community members and school teachers. School teachers in general resist making major curricular changes to accommodate temporary interventions by outside interests, and even after long-term trust has been gained, teachers must be allowed time to alter their curriculum to incorporate new concepts and plan for adequate field time.

Community partnerships are necessary for program sustainability

Within the school district, teacher and administrator turnover limits both long-term cooperation and cultural fluency of the staff. This is a problem for many rural school districts and cannot be solved by our team. Rather, we have built an expectation of high personnel turnover into our long-term plan and have built partnerships with community entities that can demand and support high-quality school programs from the school district while the district staff changes over time. We have continued to actively seek partnerships with regional organizations such as business development and environmental agencies and nearby colleges. Progress is sometimes slow, and this program must have a long-term commitment in order to help students secure their footing in both the traditional and modern worlds.

Community-centered science education efforts benefited the whole community

Though results are not encapsulated in our formal surveys, discussion with elders and

other community members has revealed that our program has had a dramatic effect on the entire community. Students have privately expressed their excitement about the activities we provide. Parents have communicated their excitement about the opportunities that this program brings to their children and to their community. Many of the positive effects are related to joint efforts with our community partners, and others have resulted from activities that we initiated and were later sponsored by other partners. Some concrete effects of this broad effort included:

- An increase in college/trade school attainment from 5% in 2000-2009 to 62% in 2010-2012
- 71% of 2010-2012 college entrants received scholarships
- After summer bioassessment internships, 8 summer interns have worked for the tribal government performing water quality assessments.
- The tribal government has used water chemistry and bioassessment data from student projects and summer internships to write major grant proposals that were awarded for remediation of contaminated river/estuaries
- The tribe has made watershed science a permanent component of the annual culture camp activities.
- Tribal organizations across Prince of Wales Island have taken notice and are developing an island-wide watershed science consortium

Discussion

In our geosciences education program we have developed a bottom-up, community-based approach that strives to keep native students grounded in their native culture by incorporating cultural traditions into geoscience education and by maintaining positive relationships with the tribal community. In this endeavor we seek to develop not just scientists, but native scientists who will support culturally appropriate management and utilization of natural resources in the modern world. Our approach can be thought of as a pyramid that supports student success. At the foundation of this pyramid lie elders and tribal leaders. This essential foundation supports families and the community at large. This community in turn supports teachers and school administrators, who ultimately prepare students to succeed in a variety of career pathways. Every element of this structure is necessary for student success, and the effective approach is always bottom-up.

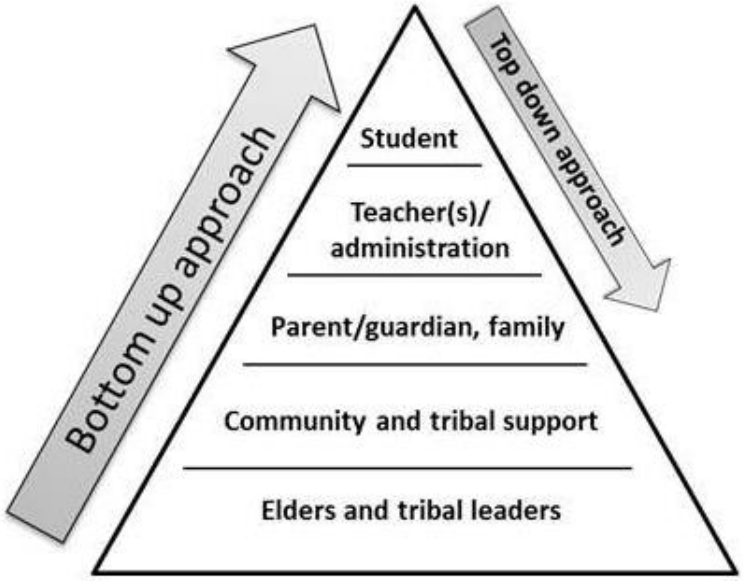

Figure 8. Community based education model. Elders are always the foundation in a tribal community.

By building collaborations with tribal leaders, working directly with community members to identify community needs, and coupling western science with traditional knowledge, we have demonstrated the value and utility of geoscience to the community. This approach establishes a positive feedback mechanism of support and encouragement for students interested in science careers, and will ultimately help create a scientifically trained workforce that becomes an integral part of the tribal community (Smythe and Bueno Watts, in review). Based on our experience in southeast Alaska, we posit that education programs using this community-based approach will increase college attainment, degree completion, career success, and positive community contributions by native students.

Conclusions

In our geoscience education program, we have established and maintained a positive working relationship with a tribal community that has a multi-generational legacy of distrust in the outside world. Through positive interactions with working scientists, sponsored trips to national conferences, and summer internships, we have exposed a group of students with no prior college aspirations to the possibility of a career in science. After a successful short-tem experience, we are shifting our attention towards the long-term sustainability of the program, which might over time require its progressive institutionalization within the traditional tribal structures.

Our support in recording Haida stories and adapting the Haida language has contributed to the preservation and revitalization of the community's language and culture. Further, by incorporating traditional knowledge into science lessons, we encouraged students to think of

Science as a pursuit that is compatible with Haida culture. We have taught students and their parents that their watershed and marine environments are complex systems with both abiotic and biotic components, and that their water quality and resource base depends on a properly functioning watershed. We have demonstrated the importance and utility of geoscience to a community with few scientist role models. Thanks to relevant geoscience experiences for students, community meetings, summer culture camp programs and informal discussions, students and community members understand how geoscience and western science in general can be used to protect and maintain their quality of life.

Acknowledgments

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Appendix I - Glossary of selected geoscience terms in English and Haida

The following words were used in Haida instruction during our geoscience education program. The list includes both pre-existing words and new words created to describe modern concepts and objects. New words and phrases were formulated by tribal linguist Benjamin Young with instruction and advice from tribal elders.

- Adult (adult salmon): Chúinaay íwaandgan
- Binoculars: Tlág Kéeng waay
- Camera: Níijaang
- Calculator: Gudii gin hl kihlaas, Híisdluu gids díig gin súudgans aa
- Carbon dioxide: Gin akyáag tl'áng gagánjuugans aa (what we breathe out)
- Coho: táay
- Compass: Tlíitsaan dángg gin súulgans aa
- Computer: Gin 'wáadluwaan stlá K'áalangs an únsadsaa
- Dissolve: wáa sgáawgang (to dissolve, melt) Aájii xílaay
- Dog salmon: skág
- East: sáawtlagáas
- Fresh water: Ga'nd (water)
- Gas: táw, tawáay
- Glacier: Kálgaasii jíinaagang (The ice is lasting a long time)
- Hatchling/fingerling/fry: Máaluud
- Humpy Salmon: ts'atáan
- Image/picture: Níijaang, Dáng tl' hiijaangaan aa Ritliiuaa (Put your picture on the wall)
- Ice age: Awáahl gagwíi kálgaa jíingaangiinii (A long time ago the ice lasted a long time)
- Insect Larvae: Káanuu (maggot)
- Juvenile (young salmon): táayee ts'úudalgan, chíiaay ts'úudalgan (s aa)
- King salmon: Táa'un
- Liquid: tantl'dáagaagang (liquid, damp)
- Map: Tlíitsáan dáng is Kugíinaay dángg súutgans aa
- Mountains: tlatáawaay
- Migrate: ts'áagaa (migrate, get up, move)
- North: xáagw
- North pole: xáagw, Damaan uu xáagw tadáang
- Oxygen: Gin tl'áng gagánjuugans aa (what we inhale)
    - Aajii táw tláatsgaa jahlu'gang
- Population: xáadgaay
    - Asgáay t'iits xáat'aagang. (These ones are part Haida)
- Rain: gwáaw (gang) daláay
- River: Gándlaay
- Rock: Kwa'áay
    - One type of rock: Kwáa
- Saltwater: Cháan tángaa (salty water in the sea)
- Sockeye: sgwáagaan
- Solid: K'ats'áang (to be hard) K'ayee k'ats'aang.

- South: xíwg
- Soil: Cháan (kind of soil you can plant)
    - Cháanaa (kind of soil that is dirty & muddy)
- Spawn: xáydang, Káawiigang.
    - Máaluud gántlaay aa kwáan awy (There are lots of minnows in the creek)
- Steel head: tayáng
- Temperature: Sangáay
- Trout: táatl'aad
- Turbidity/Water Cloudiness: Gándlaay sk'íileelga
    - Súuwaa káamuutlang t'ás cháan eelgan. (I stepped in it and it became cloudy)
- West: Káágwaa

APPENDIX II: Survey instrument (2011)

Survey Introduction

The University of Washington's Office of Educational Assessment (OEA) has been asked to conduct an evaluation of the geoscience education sessions you have taken part in. We are asking you to help us with this evaluation by completing this online survey. Your answers are very important in helping make sure this program continues and gets better.

Your participation is voluntary: you do not have to complete the survey if you do not want to and you may skip any questions you do not want to answer.

Your responses will remain private and confidential. That means that only OEA will know who gave which answers. Wendy, Rick, and your teachers will not know that you were the one who gave your answers. Even though you are giving us your name below, only OEA will have access to the list of names.

OEA will put all the answers together and submit a report to the project leads (Wendy and Rick). Your name will not appear in that report.

If you have any questions about this study or the survey, you can email or call Bayta Maring at the University of Washington Office of Educational Assessment (baytam@uw.edu, 206-543-5190).

To begin, please enter your name below and click "Next."

Name: _____

Section 1: Information about You

1. Are you a . . .

- ○ Boy
- ○ Girl

2. What is your ethnicity? (select all that apply)

- ○ American Indian/Native American/Alaska Native
- ○ African American
- ○ Asian
- ○ Hawaiian/Pacific Islander
- ○ Hispanic/Latina/o
- ○ White/Caucasian
- ○ Other: _____

3. What grade are you in? [choose from 5^{th} through 12^{th}]

Section 2: Science Fair

4. Did you participate in the Science Fair?

- Yes, I presented a science fair project. [4a, then 4b]
- I went to the science fair, but I didn't make a poster. [4a, then 4h]
- I did not go to the science fair [skip to next section]

4a. Did anyone in your immediate family (parent, guardian, sister, or brother) go to the science fair?

- Yes
- No
- Not Sure

[***4b – 4g only seen by participants who did take part in the science fair]

4b. Describe your science fair project:

4c. In February, Wendy was available to talk with students one-on-one about their science fair projects. Did you talk with Wendy about your project?

- Yes [to 5d]
- No [to 5e]
- Not Sure [to 5e]

4d. Please rate how much your agree or disagree with the following statements about your conversation(s) with Wendy about your science project.

	Strongly Disagree (1)	**Disagree (2)**	**Neutral (3)**	**Agree (4)**	**Strongly Agree (5)**
I learned a lot about science from talking with Wendy about my project.	❒	❒	❒	❒	❒
I probably would have done a science fair project, even if I had not talked with Wendy.	❒	❒	❒	❒	❒

4e. What would say was the most helpful thing Wendy said or did when you were talking with her about your project?

4f. Is there anything else Wendy could have said or done that would have helped you more?

4g. Please indicate how much you agree or disagree with the following statements:

	Strongly Disagree (1)	Disagree (2)	Neutral (3)	Agree (4)	Strongly Agree (5)
Once I decided to do a science fair project, I started to talk more about science outside of school.	❒	❒	❒	❒	❒
While I was getting ready for the science fair, I learned a lot of new things about science.	❒	❒	❒	❒	❒
I really liked being able to show my project to everyone at the science fair.	❒	❒	❒	❒	❒
When talking about my project at the science fair, I felt sort of like a science expert.	❒	❒	❒	❒	❒
I want to do a science fair project again next year, if possible.	❒	❒	❒	❒	❒

[4h is for students who only attended the science fair, but did not do a project]

4h. Please indicate how much you agree or disagree with the following statements:

	Strongly Disagree (1)	Disagree (2)	Neutral (3)	Agree (4)	Strongly Agree (5)
I learned something new about science while I was at the science fair.	❒	❒	❒	❒	❒
I want to do a science fair project next year, if possible.	❒	❒	❒	❒	❒

[4i and 4j are for all students who went to the science fair]

4i. What would you say was the best thing about the science fair?

4j. Is there anything that would have made the science fair better? What are your suggestions?

Section 3: Other Activities

During one in-class activity with Wendy, the class was able to see what a scientist saw through a microscope in Oregon. Students gave directions to Rick, and he moved the microscope so you could see different things.

5. Did you take part in this activity?

- Yes [to 5a]
- No [to 6]
- Not Sure [to 6]

5a. What, if anything, was the most important thing you learned during this activity?

5b. How interested would you be in doing something similar (using a computer to steer a microscope in Oregon) in the future?

- Not at all interested (1)
- A little interested (2)
- Somewhat interested (3)
- Very interested (4)

As you may know, Wendy is involved in the Center for Coastal Margin Observation and Prediction (CMOP) in Oregon. Each summer, the Center has internships available for students like you who might want to study science and how it might play a role in Hydaburg.

6. Would you be interested in such an internship?

- No [to 8]
- Maybe [to 7a]
- Yes [to 7a]
- Yes, and I have already told Wendy that I am interested [to 8]

6a. Is it OK if OEA tells Wendy that you are interested in being part of an interest.

- No
- Yes

7. What other things, if any, have you learned from the activities you have done with Wendy and Rick?

8. Which of the following topics would you like to study when Wendy and Rick come again? (Select all that apply)

- Salmon life-cycles
- What science can do for fisheries
- How science can help make the water in Hydaburg cleaner
- The different types of microscopic organisms that live in the Hydaburg River
- How the changes in climate (global warming) might affect Hydaburg
- Haida traditional science
- Ocean science in regard to a healthy system for fishery science
- Estuary science related to shell fish harvesting
- Plant science looking at traditional plants and their uses
- Other:

Section 4: What you Know and Like (or Don't Like) about Science
Some of the questions in this section might be familiar to you, because OEA asked them in a survey we did at the beginning of the year. Go ahead and answer the questions based on what you think *now* at the end of the year.

9. Please tell us how much you know about the following topics

Do you know . . . . ?	**Definitely Not (1)**	**Only Sort of (2)**	**I have a pretty good idea (3)**	**Yes, Definitely (4)**
a. … what an estuary is	❒	❒	❒	❒
b. … how to sample water	❒	❒	❒	❒
c. … what to test for in water samples	❒	❒	❒	❒
d. … how ocean acidification affects ecosystems	❒	❒	❒	❒
e. … how to identify which microorganisms are in a water sample	❒	❒	❒	❒
f. … how oil spills affect ecosystems	❒	❒	❒	❒
g. … how climate change might affect the Hydaburg River	❒	❒	❒	❒
h. … why the ecosystem of Hydaburg is unique	❒	❒	❒	❒
i. … why the geology of Hydaburg is unique	❒	❒	❒	❒
j. … how to use the scientific method	❒	❒	❒	❒
k. … what it is like to be a scientist	❒	❒	❒	❒
l. … what scientists do every day	❒	❒	❒	❒
m. … what it means to do "scientific research"	❒	❒	❒	❒

10. Please tell us about your interest in the following fields.

	I don't know what this is	**Not at all interested (1)**	**(2)**	**(3)**	**Extremely interested (4)**
a. Biology	❒	❒	❒	❒	❒
b. Chemistry	❒	❒	❒	❒	❒
c. Oceanography	❒	❒	❒	❒	❒
d. Ecology	❒	❒	❒	❒	❒

e. Geology	❒	❒	❒	❒	❒
f. Microbiology	❒	❒	❒	❒	❒
g. Marine biology	❒	❒	❒	❒	❒
h. Science in general	❒	❒	❒	❒	❒

11. When you hear the word "scientist," what it the first thing that comes to mind?

12. Do you think that science is important for people in Hydaburg? Why or why not?

13. Do you know any Alaska Native scientists? Who?

14. Do you think you can be a scientist if you wanted to? Why or Why not?

Section 5: Looking Ahead

15. Please tell us about your future plans.

Do you plan on . . . . ?	**Definitely Not**	**Probably Not**	**Not Sure**	**Probably Yes**	**Definitely Yes**
a. … going to college	1	2	3	4	5
b. … taking science classes when/if you go to college	1	2	3	4	5
c. … majoring in a science field when/if you go to college	1	2	3	4	5
d. … eventually having a job that involves science in some way	1	2	3	4	5

16. Any additional questions or comments about the activities you have done with Wendy and Rick, or about science, or your future plans?

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7

Geoscience Alliance: Building Capacity to Use Science for Sovereignty in Native Communities

Nievita Bueno Watts[1], Wendy Smythe[2], Emily Geraghty Ward[3], Diana Dalbotten[4], Vanessa Green[5], Mervyn Tano[6], and Antony Berthelote[7]

7.1. THE GEOSCIENCE ALLIANCE

The Geoscience Alliance (GA) is a national alliance of individuals committed to broadening participation of Native Americans in the geosciences. Its members are faculty and staff from tribal colleges, universities, and research centers; Native elders and community members; industry and corporate representatives; students (K–12, undergraduate, and graduate); formal and informal educators; and other interested individuals.

Mission and Vision of the Geoscience Alliance

We envision a future in which Native Americans are no longer underrepresented in the geosciences. We look to a future where Native scientists take a leadership role in helping to steer our country towards a more sustainable and environmentally ethical relationship with the Earth. To appreciate and advance the geosciences while being respectful of indigenous cultures, we articulate the following values:

- We focus on supporting students, even as we recognize that we all are students.
- We respect both western and indigenous approaches to knowing about the Earth, while recognizing that indigenous approaches historically have been undervalued.
- We believe that there are many paths to being a scientist and many traditions to draw from. Therefore, there is no single best practice; instead, we offer a collection of effective strategies to draw from.
- We will create ways for students to become scientists while holding onto and even strengthening their traditional knowledge.
- We are inclusive: our focus on increasing Native Americans in the geosciences doesn't confine membership to either geoscientists or Native Americans.
- We will explore, make mistakes, forgive and learn together.

The authors of this chapter are all members of the GA and of the GA's "Sustainability" Committee, which is a small group of GA volunteers committed to providing day-to-day leadership of the GA between formal meetings and events. In this chapter, members of the alliance present information pertinent to building capacity in Native communities to use science as a means of securing sovereignty in resource-management decisions on tribal lands. It is our position that this capacity is necessary and can be achieved through the attainment of high school diplomas and higher education degrees in Earth and environmental science by tribal members. In the sections which follow, the authors respond to recommendations of the GA for broadening participation of Native Americans in Geosciences [*Dalbotten*, 2010]: Incorporate traditional knowledge into geoscience education for Native students; understand and address barriers to obtaining undergraduate and graduate degrees in the geosciences; and create culturally appropriate assessment and evaluation in Indian Country. This research is supported by findings from the first Geoscience Alliance National Conference held in Cloquet, Minnesota in September 2010. Since then, members of the GA have been involved in several projects specifically aimed to address the issues and

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barriers raised here, and do so with a focus on responding to issues raised in Native American communities under the stress of anthropogenic global changes.

7.2. UNDERREPRESENTATION OF NATIVE AMERICANS IN THE GEOSCIENCES

Native Americans are underrepresented in Earth, environmental, geographical, and spatial science and other higher education programs. Compared with other science and engineering fields, the geosciences produce the lowest percentage of minority scientists with bachelor and master's degrees. Underrepresented minorities currently comprise 30.5 percent of the US population, with 15 percent of the population being non-Hispanic Blacks, 14 percent Hispanics, and 1.5 percent American Indian and Alaska Natives. The percentage of geoscience bachelor's degrees conferred on minority students is much less than 30.5 percent. In 2004, for example, Hispanics received 3.3 percent of the bachelor of science degrees, Blacks 1.7 percent, and American Indians 0.8 percent. For master's degrees in geosciences, the percentages drop to 2.3 percent for Hispanics, 1.4 percent for Blacks, and 0.5 percent for American Indians. At the doctoral level in 2004, the rates stayed about the same as for master's degrees, with Hispanics garnering 2.3 percent, Blacks 1.7 percent, and American Indians 0.4 percent [*American Geological Institute (AGI)*, 2009]. This level of underrepresentation and the underlying issues that lead to it are the motivating factors that led to the creation of the GA.

7.3. GEOSCIENCE ALLIANCE GOALS

The goals of the GA are to create new collaborations in support of geoscience education for Native American students; establish a new research agenda aimed at closing gaps in our knowledge on barriers and best practices related to Native American participation in the geosciences; increase participation by Native Americans in setting the national research agenda on issues in the geosciences, particularly those that impact Native lands; provide a forum to communicate educational opportunities for Native American students in the geosciences; and to understand and respect indigenous traditional knowledge.

7.4. BACKGROUND OF THE GEOSCIENCE ALLIANCE

The GA was formed in 2007 through the efforts of the National Center for Earth-surface Dynamics (NCED). Key partners from Salish Kootenai College, Purdue University, the GLOBE Program, University Consortium for Atmospheric Research (UCAR), and others joined and helped to bring about the first GA meetings, which were held in conjunction with other conferences, such as the American Geophysical Union (AGU), the Society for the Advancement of Chicanos and Native Americans in Science (SACNAS), and the American Indian Science and Engineering Society (AISES) national meetings.

7.5. THE GEOSCIENCE ALLIANCE NATIONAL CONFERENCES

In 2010, the National Science Foundation sponsored the first GA conference through an "Opportunities for Enhancing Diversity in the Geosciences (OEDG)" Award (NSF GEO 0939753). This conference brought together more than 100 individuals from tribal and other institutions—with more than half students—to discuss barriers to broadening participation and ways to overcome them. In addition, students had the opportunity to meet with program directors and hear about research opportunities in the geosciences. This led to many productive outcomes, including a summary report on the conference discussions [*Dalbotten*, 2010]; students participating in research programs, new research, and education collaborations; and a dissertation published on the topic of broadening participation of Native Americans in the geosciences [*Bueno Watts*, 2011]. The conference report and dissertation are important for being the first studies to address underrepresentation of Native Americans specifically in the geosciences and to examine key barriers and potential solutions to this problem.

The second GA national conference was held in March 2012 with the topic of *Home Places, Local Landscapes, Traditional Knowledge, and Modern Technologies*, with support from the National Science Foundation and NASA. It was located at Salish Kootenai College (SKC), a tribal college in Pablo, Montana. Several sophisticated research techniques and data management and visualization tools were highlighted. Keeping in mind the circle of learning principles that guide the GA (i.e., everyone teaches and everyone learns), these workshops were structured as a dialogue with positive feedback loops; participants not only explored the technologies and their potential for use in Native communities but were also engaged in helping the institutions who provided these tools to better understand and meet the needs of Native communities. Climate change and its impacts on Native reservations was a crosscutting concern of the conference and appeared as a theme in several of the posters and presentations of the students. Clearly, Native American students are concerned about the effects of anthropogenic change on their homelands and cultural resources. This chapter discusses research presented at the 2012

GA conference that was motivated by discussions at the 2010 GA conference as well as highlights the progress that has been made through collaborations born of the alliance. These studies are discussed in detail.

7.6. INCORPORATING TRADITIONAL KNOWLEDGE IN GEOSCIENCE EDUCATION

Native American and Alaska Native K–12 students of all ages face numerous challenges both socially and academically. In 2003, the US Commission on Civil Rights found that Native students commonly experienced "deteriorating school facilities, underpaid teachers, weak curricula, discriminatory treatment, and outdated learning tools" [*Berry et al.*, 2003]. There is a push for revitalization occurring in Native education and for traditional knowledge to be taught alongside other current science concepts to provide culturally relevant curriculum for STEM disciplines [*Brave Heart and DeBruyn*, 1998; *Brave Heart*, 1999]. Indian Country is poised to nurture a new generation of Native scientists and natural resource managers who can guide their communities to a sustainable future in the face of anthropogenic change.

Traditional knowledge, also referred to as indigenous knowledge or traditional environmental/ecological knowledge, refers to longstanding traditions and practices, encompassing the knowledge, culture, and spirituality of Native Americans, Alaska Natives, Native Hawai'ians, and other indigenous peoples around the world. Historically traditional knowledge has been orally passed on for generations and expressed through stories, legends, rituals, and songs [*Berkes*, 1993]. Traditional knowledge is founded on empirical observations, applied practices, and a lifestyle in which competency in natural resource science and management is essential for and defined by survival [*Simpson*, 2002; *Barnhardt and Kawagley*, 2005]. Indigenous cultures have collected and maintained extraordinary amounts of comprehensive knowledge through many generations of living in a specific region and through their spiritual ties to the environment. Coupling traditional knowledge systems with other science concepts provides a greater depth of understanding as to how natural ecosystems are changing and can augment resource management practices as managers respond to global climate change.

Traditional knowledge systems are holistic and interweave science, ethics, religion, philosophy, medicine, psychology, and economics as all part of the same body of knowledge [*Barnhardt and Kawagley*, 2005]. This holistic approach used by traditional knowledge provides Native students with a foundation from which to understand scientific concepts such as geologic time, climate change, ecology, and evolution.

7.6.1. Obstacles in Geoscience Education for Native Americans at the K–12 Level and the Role Traditional Knowledge Can Play in Removing These Obstacles

The difficulty of school systems to effectively educate Native students is a result in large part of four systemic, interrelated obstacles resulting in a divide that Native students and STEM professionals find challenging to overcome. The first is an attitude that traditional knowledge holds little or no value [*Haig-Brown*, 1995; *Deyhle and Swisher*, 1997; *Kawagley and Barnhardt*, 1999a; *Deyhle*, 2010]. This attitude manifests itself when culturally disengaged teachers attempt to challenge ethnoscientific knowledge, leaving Native students feeling pressured to choose between the correctness of one knowledge system over the other. Naturally many students choose the knowledge system with which they are familiar and as a result many withdraw from active participation in school, whereas other students leave school altogether [*Deyhle and Swisher*, 1997; *Barnhardt and Kawagley*, 2004; *Deyhle*, 2010].

The second obstacle is that non-native teachers are typically viewed as outsiders, and Native communities and students may have an us-versus-them relationship with teachers and administrators. This mutually exclusive type of relationship is detrimental to both the school staff and more importantly the students. Rather than continue this negative cycle of exclusion, solutions need to be found that overcome this obstacle for the future of Native students. One solution would be for the tribal community to embrace educators and school administrators, simultaneously implementing a "who we are," "what we stand for," and "why it is important" framework to assist nonlocal educators in understanding community and culture. The divide between teachers and Native students can be bridged through the combined commitment of mentoring by culturally competent tribal members and the acceptance of teachers and school administrators into Native communities. Educators who are welcomed are better suited to interact positively with the students, parents, community, and tribal members. Without awareness of the local culture, it is virtually impossible for culturally relevant curriculum to be taught. Therefore, it is imperative that Native communities work with educators and administrators, both Native and non-native, to develop culturally relevant STEM curriculum to ensure the educational success of their students.

The third obstacle is teaching strategies that contradict the learning style of many Native students, thereby setting up a psychological power struggle between traditional ways of teaching and learning and western educational system requirements [*Nelson-Barber and Estrin*, 1995; *Cajete*, 1999; *Kawagley and Barnhardt*, 1999b; *Barnhardt and Kawagley*, 2005]. Historically, Native peoples have not had the power to define what constitutes education, much

less science education [*Lomawaima*, 2000]. Pedagogies commonly used in geoscience courses expect students to adopt and understand an unfamiliar and abstract approach, which is in direct opposition to the situational, concrete knowledge emphasized in Native cultures. Although Native cultures vary, *Cajete* [1999] describes some common attributes of Native learners, including quietness and patience, a cooperative social orientation, a holistic and spiritualistic view of nature, and a nonlinear sense of time. Native students tend to prefer concrete, contextualized lessons in which they observe before practicing. Pedagogical approaches can accomplish this through the use of STEM curriculum that couples traditional knowledge and language with current science curriculums that include both a field and laboratory component.

The fourth obstacle resides in the inaptitude of Native people to identify as scientist, teacher, or any other position that requires higher education, hierarchical, or noncommunal thinking. This inaptitude is a result in large part of the historical trauma inflicted on Native peoples by governments that sought to erase cultural identity [*Lomawaima*, 2000]. Reforms in the 1970s and thereafter replaced federal and state schools, which focused on assimilation of Native peoples into western society [*Dlugokinski and Kramer*, 1974; *Lomawaima*, 1995; *Deyhle and Swisher*, 1997], with locally controlled schools, schoolteachers, and administrators.

Tribal elders experienced this cultural assault firsthand and survived through self-determination and courage; however, successive generations have also suffered as a result of the long-term effects of this cultural assault. It is imperative that we not only educate Native youth using traditional knowledge but also demonstrate that Native people can be the scientists, engineers, teachers, and educated tribal members within their own communities. Native communities have only recently begun to revitalize through healing [*Brave Heart and DeBruyn*, 1998; *Brave Heart*, 1999] by reclaiming traditions, such as canoeing, hunting, and languages that have not been practiced for generations [*Cajete*, 1999; *Deyhle and Swisher*, 1997; *Simpson*, 2002; *Deyhle*, 2010]. Science education programs can use the momentum of this movement by adopting culturally relevant and student-centered pedagogies that recognize the current state of Native cultures, both accommodating the ways in which Native peoples have traditionally learned and recognizing how Native cultures have evolved since the 1900s [*Cajete*, 1999].

Overcoming obstacles in Native education is no easy task. However, these obstacles can be overcome through recognizing the value in traditional knowledge and incorporating it into novel pedagogical approaches. Such approaches are student centered; inquiry based; respect local traditions, knowledge, and culture; and meet community-identified needs [*Bueno Watts and Smythe*, 2013; *Hugo et al*., 2013].

7.7. REMOVING BARRIERS TO BROADENING PARTICIPATION IN UNDERGRADUATE AND GRADUATE EDUCATION

Herein, we summarize the results from Bueno Watts's dissertation research on "Broadening Participation of Native Americans in the Earth Sciences," which was both inspired by her participation in the GA and facilitated by interviews with several GA members. The dissertation reports the results of semi-structured interviews, which ranged from 30 to 90 minutes in length, designed to discover what factors were barriers to attaining a degree, and what factors helped with completion of a geoscience program of study. These interviews were conducted with fifteen Native Americans who had already achieved, or were in the process of attaining, a postsecondary degree in Earth or environmental science and 10 directors of programs designed to increase the number of Native Americans graduating from Earth and environmental science degree programs. The interviews were analyzed qualitatively following methods outlined in *Miles and Huberman* [1984].

7.7.1. Demographics of Study Sample

Twelve of the 15 Native participants interviewed (80 percent) were female and three (20 percent) male. Eleven (73 percent) of the participants were first-generation college students, and the rest (27 percent) reported that one or both of their parents had been the first in the family to attend college, making them second-generation college students. About half (53 percent) were nontraditional students, defined here as someone who found themselves in school 20 years after high school graduation. Each nontraditional student either took an extended period of time off from school to work between degrees (75 percent) or worked full-time while pursuing a degree (25 percent), thereby extending the time required for completion.

Seven out of 15 participants (47 percent) started their college education at a public four-year institution; an additional 3 (20 percent) began at a private, religious, four-year institution. Five out of 15 (33 percent) completed their bachelor's degree without transferring or taking an extended break. Three out of the 15 (20 percent) participants began college at a local community college, and 2 out of 15 (13 percent) began their studies at a tribal college. Seven out of 15 (47 percent) of participants did not complete their initial choice of programs. Only 3 of the 15 (20 percent) participants started their educational path in a geoscience or environmental Earth science field.

Combined, the 15 participants had completed 6 associate degrees, 12 bachelor degrees (3 participants were undergraduate students), 7 master's degrees (with 2 enrolled in master's programs), and 2 doctorate degrees (with 4 more enrolled in doctoral programs).

7.7.2. Barriers to Completing a Geoscience Degree

The interviews uncovered a number of barriers that could be grouped into general themes: barriers to completing a college degree, impediments to making a decision to study Earth Science, and factors that impede progress in Earth science programs.

Many of these barriers are certainly not unique experiences of Native American students, but as expressed, compounded into nearly unbearable burdens for many students. Several factors were barriers to completing a college degree. For example, without financial aid resources 80 percent of the participants would not have been able to afford a college degree. In addition, pressures from familial obligations often interrupted studies. Some familial obligations, such as needing to go home for funerals or other ceremonial obligations, posed problems for some members of this group. In other cases students had families that they needed to rush home for, precluding them from taking advantage of programs and opportunities incompatible with the life styles of students who are also working parents.

Many participants in the study also described experiencing debilitating physical and mental health issues. Some mental health issues that arose, like those associated with feelings that a student "wasn't good enough" to be a graduate student in the Earth science department, or the depression and feelings of helplessness that occurred when work piled up too high, are not strictly conditions of being Native American. They were reported by a third of the women interviewed and were exacerbated by departmental environments that were particularly hostile to women. A good advisor or other support person, however, helped these students deal with both physical and mental health issues.

A second set of factors were impediments to making a decision to study Earth science. The most commonly reported was unfamiliarity with geoscience as a field of study or career path—80 percent of the participants talked about geoscience not being known in tribal communities as either a field of study or a career choice. Even if geoscience was selected as a field of study, however, the curriculum offered was often seen as being irrelevant to the practical needs of the community, and course names unintelligible to non-geologists, and the degree program was therefore discounted as a way of solving the problems found in Native homelands. The importance of this barrier cannot be overemphasized because an overwhelming percentage (93 percent) of participants expressed a desire to work on environmental geology issues. Particularly for re-entry students who were non-traditional in age, desire to heal the land was a motivating factor in their decision to return to school. Unfortunately, even after enrolling in a geoscience program, students found that the career they thought they were preparing for, where they would be able to use the skills learned to solve environmental problems back home, did not line up with the focus or program of the department, and this was a major disappointment. Locations of institutions themselves often pose an additional barrier because many are inaccessible geographically, and large segments of the Native population do not have access to Earth science courses near their homes.

A third set of factors impeded academic progress through Earth science academic programs. The most often expressed academic barrier reported was that of inadequate preparation in mathematics (73 percent). But it was not because Native students could not "do" math—most eventually took Calculus I and Calculus II and passed—but the quality and availability of mathematics instruction was the institutional barrier in this case, at *all* levels of the educational system. The biggest barrier this problem poses is the spiraling effect that bad grades in calculus and chemistry (another frequently mentioned barrier course), have on a student's grade point average, subjecting them to academic probation and making them ineligible for funding (another institutional barrier).

Lack of academic information and counseling was also a barrier to progress for students. Nontraditional students reported that the failure of both Earth science departments and university counseling systems to provide appropriate guidance for older students was problematic. They reported having to seek out information on graduate school, programs, and other opportunities and were not approached by faculty with opportunities for internships or scholarships as often as their younger peers. Some even felt that student counseling programs in place at the university level were not designed to accommodate their needs. Having a departmental mentor to talk to became imperative for these students' success.

Intradepartmental relationships were another barrier to success within the day-to-day workings of departmental politics. Conflicts between subfields were especially detrimental to student success when the advisor of a student was in a new, nontraditional field that the other members of the department did not support (i.e., biogeochemistry).

None of the participants described feeling discrimination in Earth science departments by virtue of the fact that they were Native, yet many of the women reported harassment by faculty members. Harassment was sometimes so intense that it affected the student's relationships with other students and jeopardized their ability to complete their program of study. The situation became so

intolerable in at least two cases that the student moved either to another department or another university. It must be understood that many Native women are respected leaders within their community and have grown up in the outdoors, often performing the same tasks that men do in white rural societies. Some of the women interviewed expressed surprise to find themselves in a field that was considered to be non-traditional for women because they came into the program assuming they were capable of doing anything a man could. Harassment was more reported by nontraditional students than by those who were of traditional age, and several incidents occurred during the last decade (2000s), indicating that this inappropriate behavior is not a thing of the past.

Some students, having entered geoscience programs, reported that the prevalence of western scientific perspective to the exclusion of all others became cognitively problematic. There was no acknowledgement of the local peoples or their knowledge of the land, even in schools with a high concentration of Native students. Furthermore, 11 out of the 15 participants (73 percent) described themselves as thinking holistically when approaching problems, which is contrary to methodologies that tend to break knowledge into smaller pieces for examination.

One promising move toward increasing geoscience degrees earned by Native Americans is the introduction of two- and four-year geoscience degree programs at tribal colleges and universities. In this way, many of the barriers described previously are removed. SKC recently announced the first two- and four-year geoscience degree offered at any tribal college or university in the country, offering new opportunities for Native students to participate in the geosciences.

7.7.3. The Salish Kootenai College Hydrology Degree Program

SKC received approval in the fall of 2010 from the Northwest Commission on Colleges and Universities to offer both associate and bachelor of science in hydrology, the first such degree programs among the tribal colleges and universities in North America. The hydrology program is aligned with the SKC strategic plan goal to "become a center of science education with an emphasis on Native American worldview and application of science to indigenous issues." SKC's hydrology program offers interdisciplinary study of physical, chemical, and biological water resources and their management. These efforts will significantly increase the number of Native Americans receiving degrees in the geosciences.

Water is a key natural resource in today's world and is a resource that is increasingly coming under stress as a result of climate change. Climate factors are also expected to play a growing role in increasing vulnerability to natural hazards such as floods or hurricanes. Tribal resource managers with skills in hydrology are needed to assess vulnerability of tribal lands to climate change and develop a plan for management. The summer 2012 flooding, which took place across the Fond du Lac Band of Lake Superior Chippewa reservation in Northeastern Minnesota, is an example of the devastating effect natural hazards can have on a reservation, impacting roads, fishing, forestry, wild ricing, housing, and health. Thomas Howes, Natural Resources Manager for Fond du Lac reservation noted: "we are gravely concerned about the future of *manoomin* (wild rice) in the face of climate change. Given the sensitive nature of *manoomin* to hydrologic events and the drastic landscape changes in the region that *manoomin* grows, we need additional hydrologists and researchers to explore wetland and ditching alteration remedies" [*personal communication*, May 9, 2013].

There is an urgent need for improved teaching and learning at tribal colleges and universities related to geoscience issues to support the next generation of future Earth land and water resource managers for Native American communities and across the United States. Twenty-three of the 37 tribal colleges and universities offer degrees, certificates, or courses in natural resources or environmental sciences, but these programs, which largely result in certificates of completion or associates degrees after completion, are in a constant state of flux. Most do not lead to transfers into four-year geoscience programs and therefore are not translating into increased numbers of bachelor's degrees in the geosciences.

Nationally, according to the 2008–2009 Occupational Outlook Handbook of the Bureau of Labor Statistics, the employment forecast for US hydrologists will experience a 18 percent increase from 2010 to 2020. An evaluation of the geoscience workforce indicates that there is a growing deficiency between the developing and needed workforce [*AGI*, 2009]. The nation will need new appraisals of water availability in the next decade that link both water quality and quantity; track changing flow, use, and storage of water, as well as models and predictive tools to guide its management decisions [*USGS*, 2007]. This has important implications for Native American reservations in the face of increasing anthropogenic changes.

The SKC hydrology program has the potential to have impacts nationwide as students take up jobs within the state or return to their reservations. Developing broader use of technology in the hydrology curriculum at SKC will prepare Native American students to face the challenges found in modern water resources research and management. Native American students will be better prepared to effectively participate in any discipline or profession where advanced geospatial, water quality, and water quantification tools have become an important and necessary job qualification. Graduates equipped

with the skills to use current technology in research and management will greatly aid tribal and nontribal agencies for which they will work.

The long-term impact of SKC's hydrology programs will culminate in Native hydrologists who can provide the unique perspective of Native peoples on natural resources while taking advantage of the most current technologies. Particularly, students will continue into professions or graduate education with knowledge of water-related issues including water rights, agriculture, environmental hydrohealth, beliefs, and spirituality related to water, and sustainability of water resources while having the advantage of developed proficiencies in current technologies. The purpose of including Native perspectives goes beyond a simple appreciation of Native culture and beliefs; SKC objectives are both to empower Native communities through a sharing of knowledge and experience and to enhance cross-cultural understanding and respect for different approaches to water and water development. Both cultural perspectives and technological objectives have practical and tangible expressions in the realm of improved legal frameworks, agricultural practices, water quality, health, public relations, water rights, sovereignty and other global, international, and local management concerns. The SKC hydrology degree programs strives to engage Native peoples in expressing unique perspectives on water priorities and on what constitutes "improvements" and "progress" for their societies while taking advantage of technological offerings and real-time data sets.

7.8. CULTURALLY APPROPRIATE, NATIVE-FOCUSED ASSESSMENT AND EVALUATION FOR THE GEOSCIENCES

A clear call from the first GA conference was for culturally sensitive or "native-friendly" practices that work to incorporate Native cultural perspectives and practices when planning assessment or evaluation of programs. Researchers have expressed concern about the cultural validity of science assessments in particular, arguing that sociocultural context influences both the interpretation and solution of questions [*Solano-Flores and Nelson-Barber*, 2001; *Lee and Luykx*, 2007]. *Solano-Flores and Nelson-Barber* [2001, p. 555] define cultural validity as "the effectiveness with which science assessment addresses the sociocultural influences that shape student thinking and the ways in which students make sense of science items and respond to them." The sociocultural context for Native communities is place, particularly for the study of natural sciences. Place is defined here as both the sociocultural landscape shaped by people and the physical landscape formed by Earth's processes [*Relph*, 1976; *Tuan*, 1977; *Gould and White*, 1986; *Cajete*, 2000; *Deloria and Wildcat*, 2001; *Aikenhead and Michell*, 2011].

Researchers have proposed infusing context to improve the effectiveness of assessment and evaluation practices [*Solano-Flores and Nelson-Barber*, 2001; *Nichols and LaFrance*, 2006; *LaFrance and Nichols*, 2010]. This type of assessment and evaluation design and implementation becomes an inherently collaborative process. Both assessment and evaluation methodologies resulting from this process are community focused and dependent on the program goals and cultural values of the stakeholders involved. An exemplar for contextualizing assessment is presented here and discussed in terms of its applications to evaluation practice.

The NSF-funded Cultural Validity in Geoscience Assessment project collaborative (NSF GEO-1034909 and GEO-1034926), in an effort to design place-based, culturally informed science assessment, identified experts from the Blackfeet and Navajo (Diné) communities who were involved in science education or had expertise in Native culture and language. The selected experts were involved in the entire process of assessment design. The expert group, along with tribal college faculty and students, were surveyed to ask them what geoscience topics were important to their community to identify important and culturally relevant geoscience concepts to be used in assessment. Their responses focused the assessment on relevant, place-specific content. Topics such as water, glaciers, and mountains were particularly relevant to Blackfeet community participants. Some topics identified embodied an Earth system science perspective, highlighting the interconnectedness of Earth materials and processes [*Ward, et al.*, 2011; *Ward, et al.*, 2014].

The Blackfeet expert group convened to discuss the survey results and to author a suite of open-ended assessment questions that would incorporate the place-specific content identified from the surveys. The questions focused on the elements of Earth, wind, water, and fire and were aligned with relevant Earth science literacy principles [*Earth Science Literacy Initiative (ESLI)*, 2009] as well as the Blackfeet Education Standards developed by the tribal college [*Blackfeet Community College Rural Systemic Initiative*, 2005]. Four questions were selected for piloting with science students attending Blackfeet Community College to collect their responses. The responses to the open-ended questions provided data for the expert group to develop closed-response assessment questions. The expert group identified themes using content analysis of the student responses and grounded the new assessment questions in the language and content provided by students for the open-ended assessment questions.

The product was a suite of geoscience assessment questions aligned with Earth science literacy principles and Blackfeet education standards that address important

and culturally relevant geoscience content for Blackfeet students. The newly constructed assessment suite provides a culturally validated measure of conceptual understanding for students from this community as a result of the collaborative process. The assessment process outlined previously aligns with the evaluation framework designed by American Indian Higher Education Consortium [*LaFrance and Nichols,* 2009] in that it is collaborative, contextualized, and in alignment with the cultural values of the project stakeholders. Hence, these methods of assessment can be adapted for the evaluation of new and ongoing research and education projects on the reservation.

Identifying meaningful outcomes for projects comes easily for these cultural experts. These outcomes often encompass a broad range of variables from gathering information from cognitive, behavioral, and affective domains, to increasing access to STEM experiences and giving back to the community. External evaluation can select data gathering methods that align with community preferences when partnerships are formed with local evaluators [*LaFrance and Nichols*, 2009; *Kirkhart et al*., 2011]. For example, if the local evaluator indicates that participants prefer oral administration of a survey or to have their answers audio-recorded rather than handwritten, data collection methods are able to accommodate these preferences, facilitating participant feedback.

Another effective practice is to share the data interpretation with participants to ensure validity, to retain the original voice, and to gather any additional feedback before reporting project findings [*LaFrance and Nichols*, 2009]. Assessment and evaluation practices in Native communities require a mixed methods approach. Practices that are collaborative, community driven, and contextualized offer rich and culturally valid approach to assessment and evaluation.*

7.9. CONTINUING ISSUES

Native Americans continue to be underrepresented in the geosciences, despite ongoing efforts to provide pathways into geoscience careers. These students have a crucial role to play in the future management of our nation's land and water resources, both on the reservation and across the country. Vast resources have been poured into creating technologies that help us understand and respond to the challenges that face future Earth, but these technologies are underused, particularly by Native communities. Despite recent investments, there continues to be a technology gap in reservation communities. And even in cases where computers and Internet are present, there is still an information and applications gap—the potential for these sophisticated tools to solve local problems is not realized by the potential user community.

7.10. FUTURE OF THE GEOSCIENCE ALLIANCE

The GA has worked between conferences and meetings to bring in new members and create multiple avenues for continued participation in the alliance. We have a blog, a Facebook group, and a listserv, all focused on providing information about opportunities and news related to the geosciences. Many organizations, including NSF Research Experience for Undergraduates programs, the American Geophysical Union, federal groups such as NASA, NOAA, and the USGS, and others send information to the GA for distribution to our members. Members are also active on social networks, sharing stories of issues related to indigenous groups around the globe. Our goal for the near future is to continue to build our membership while we develop a plan to support sustainability of the GA in the future. We hope you will join us in our endeavors.

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Title: Incorporation Of Traditional Knowledge Into Geoscience Education: An Effective Method Of Native American Instruction

Short Title: Traditional Knowledge and Geoscience Education Curriculum

Article Type: Curriculum & Instruction

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Key words: Geoscience education, Native curriculum, Native education, Traditional Knowledge

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Abstract

The development of curriculum which incorporates Traditional Knowledge with science, technology, engineering, and mathematics (STEM) disciplines has led to the development of a culturally-relevant, high-impact geoscience education program within an Alaska Native community providing Native students with a powerful and holistic form of science education. The geoscience education program was collaborations between the local school district, tribal government, community members, and the National Science and Technology Center for Coastal Margin Observation and Prediction (CMOP). These partnerships allowed for the development of place-based, culturally-relevant curriculum incorporating local Traditional Knowledge and the Native language into STEM disciplines. Place-based education helps communities through engaging K-12 students in addressing community issues of interest, such as water quality, thereby promoting learning that is rooted in local history, culture and environment. Place-based education uses students' local knowledge base as a primary resource for learning, rather than nationally referenced (non-context based) curriculum.

Introduction

Traditional Knowledge encompasses an understanding of geological, biological, environmental, and spiritual elements as one holistic system occupied by interconnected ecological communities accounting for the health of an entire ecosystem (Semken, 2005). The curriculum developed in this program used a model developed during the planning and implementation of this program (Bueno Watts and Smythe, 2014). The model uses a bottom up approach to teaching STEM disciplines and is structured as a community inclusive program which incorporates tribal elders and leaders, community members, parents, teachers, and students in an inquiry-based place-based geoscience research project building on the traditions by which Alaska Native students understand and interact with the natural world. This approach is designed to respect and reinforce Alaska Native Traditional Knowledge while providing a scientific basis for that knowledge, and follows current thinking in place-based education, which requires us to view humans as one part of the natural world and human cultures as an outgrowth of interactions between species and particular places (Smith and Williams, 1999). Inquiry-based learning is in stark contrast to modern mainstream pedagogical systems that discourage the natural process of investigation and questioning. Students taught using this traditional pedagogical method are less likely to ask questions being encouraged instead to listen and repeat the expected answers and outcomes (EBC, 2004). Inquiry-based education implies that student involvement will lead to greater understanding. The earth systems approach of modern geoscience disciplines is well suited to complement and support Traditional Knowledge and cultural belief systems in conjunction with STEM disciplines.

For generations elders have preserved Traditional Knowledge about the environment through continued practice and oral traditions, which interweave metaphorical lessons and cultural wisdom with empirical observations. This project incorporates Traditional Knowledge into

STEM content in an effort to develop culturally-relevant curriculum for Alaska Native 5^{th}-12^{th} grade students. Alaska Natives have survived on cultural and traditional use of locally-derived food and material resources for thousands of years, through the observation and understanding of environmental ecosystems and processes. Communal knowledge of these natural systems can be used to engage Native youth in learning western science in a way that is culturally relevant.

Students participating in this geoscience education program acquire skills that ready them for careers in resource management of tribal resources. It is important to encourage Native scientists not only because of their vested interest in the preservation of native lands and resources, but also because of their relationship to the historic knowledge of the region. From a young age, knowledge of the environment, natural resources and history of the region have been instilled in native youth. This fosters an inherent understanding of how the specific ecosystem works and what role the Native community plays in shaping that ecosystem. By encouraging native youth to pursue careers in STEM disciplines, traditional knowledge is incorporated into the task of the native scientist. Additionally, it is retained within the community, with the youth educated as tribal resource managers, environmental planners, and school teachers, leading to application of traditional knowledge in community planning and communication with policy makers.

Statistically, Alaska Natives/Native Americans are the most underrepresented group in STEM despite the fact that Alaska Native/Native American groups integrate an understanding of the natural world as an important part of their cultural heritage (Barnhart, 2005; Baker, 2007). The Commission on Professionals in Science and Technology found that only 0.3% Alaska Native/Native Americans were employed in traditional science and engineering occupations (Babco, 2000). As such, the specific objectives of this program are to encourage Alaska Native

participation in STEM education while fostering a community-wide appreciation of the value of the STEM disciplines when coupled with Traditional Knowledge.

Importance of Community Involvement

Alaska Native/Native American community members are bound together by intimate social and cultural connections forming close-knit groups. Education programs often fail to recognize that community support is vital to the success of Native students', serving as a source of encouragement and cultural identity. Native students that receive community support remain grounded due to solid support and cultural foundation, the incorporation of tradition, language, and spirituality, and by maintaining interpersonal relationships with tribal members and within tribal communities (Bueno Watts and Smythe, 2014).

A model illustrating community support uses a bottom up approach, in which elders and tribal leadership are placed at the base, as a strong foundation critical for student success. The remaining portions of the model progressively narrow, focusing in on community members, parents, family, school administration, and placing individual students at the top of the pyramid (Figure 1). Education programs and institutions using this bottom up approach will effectively educate, retain and empower Native students to succeed. Forming co-operative collaborations within tribal communities is essential for Native student success, and can be done by working directly with community members to support students, thereby establishing a positive feedback cycle of support and encouragement.

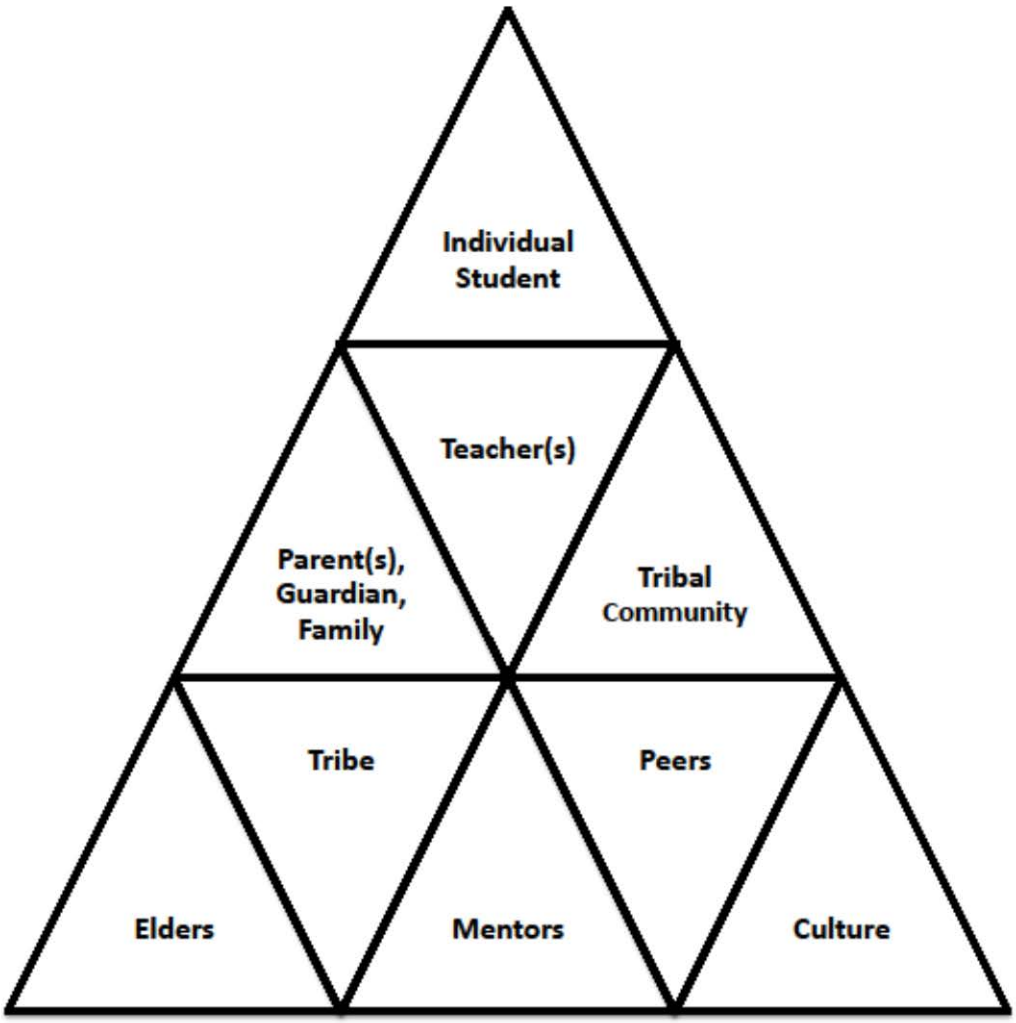

Figure 1: Model of community structure within tribal communities.

Research focused on rural communities, including Alaska Native/Native American communities demonstrates that this bottom up approach is a highly effective strategy for teaching STEM disciplines, particularly when science activities are tailored to the needs and interests of the community itself (Henderson & Royster, 2003; Boyer, 2006; Harmon). These studies have demonstrated that when indigenous youth were engaged in educational programs that used an outdoor inquiry-based science curriculum they achieved higher scores on standardized science tests (Zwick and Miller, 1996). In addition to improved test performance, these strategies also enhance indigenous youths' self-esteem (Cleary & Peacock, 1998), healthy identity formation

(Trujillo, Viri and Figueira, 2002), self-directedness (Garcia and Ahler, 1992), and most importantly, respect toward tribal elders (Agbo, 2004). All of these characteristics result in youth who provide a positive influence within tribal communities.

Curriculum Development

The pedagogical approaches that were used helped students develop critical science inquiry skills while applying a variety of math and science technologies. Initially low-tech, hands-on activities such as shipworm trap construction and monitoring (discussed below) were used to assess the environmental health of local marine ecosystems. These ecosystems are important to coastal tribes that practice cultural and traditional use strategies for survival. These initial environmental studies were then enriched by the incorporation of high-tech activities such as remote use of electron microscopy facilities and the use of computed tomography (CT) to visualize shipworms colonizing wood substrates in situ. These visualizations allowed students to see the number and location of live shipworms, which would have perished upon removal from their substrate.

Environmental Health Assessments Using Bioindicators: Shipworms

This geoscience education program focused on bioassessment of lotic and marine ecosystems to assess environmental health. Bioassessment or biological assessment refers to the use of organisms as indicators of environmental health, specifically looking at biodiversity, health, and abundance of organisms from particular locales. Bioassessment of lotic ecosystems focused on biodiversity and abundance of macroinvertebrates, while bioassessment of coastal marine ecosystems focused on the presence or absence of shipworms. The biological assessment of environmental health, in this case the water quality, is dependent on the observation of organisms susceptible to changes in certain environmental parameters or the introduction of allochthonous/exogenous toxins, which can come from human waste impacts. The study of

shipworm growth under variable environmental conditions is ongoing, though it is clear that recruitment to wood surfaces is highly dependent on the success of the broadcasted shipworm larvae (MacIntosh et al., 2012). Student observations of shipworm abundance and distribution was valuable to determine the susceptibility of the shipworms to changing environmental conditions.

Shipworms

Shipworms (Family *Teredinidae*) are not worms as the name would imply. Rather shipworms are wood boring marine bivalve mollusks that are related to soft shell clams (Family *Myidae*). These animals bore into wood substrates for shelter and nutrients using a ridged shell that is reduced in size and located at the anterior of the animal (Figures 2 and 3), and is adapted for the specialized function of grinding into wood as illustrated in the CT scan of a wood trap (Figure 4). There are very few organisms capable of utilizing wood as their primary food source due to the difficulty of digesting cellulose. Even so, these wood-boring bivalves are found in marine environments, and are widely distributed in shallow waters in tropical and temperate regions. Like most Mollusca shipworms are soft-bodied animals; unlike other mollusks they have an elongated body plan (Figure 5). While the presence of shipworms in an environment is dependent on many environmental variables that can be tested and observed using handheld portable instruments (such as YSI monitoring devices), they are tolerant to changes in temperature, salinity, and oxygen availability (Distel et. al, 2002; Betcher et. al, 2012). Shipworms are also well known in Coastal Native communities for contributing to the rapid degradation of wood structures such as boats and docks. The shipworm Bankia setacea is the most common species found along the west coast of the U.S. occupying the coastal regions from California to Alaska.

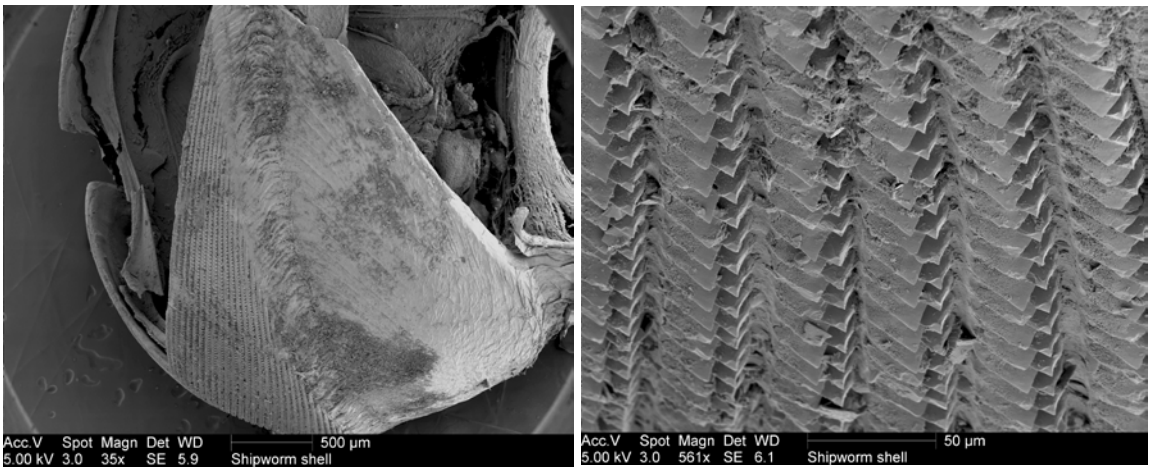

Figure 2: (Left) SEM micrograph of shipworm shell, magnification 36x. (Right) Close-up of shell surface, notice the toothed surface. The shell is used primarily for burrowing into wood substrates.

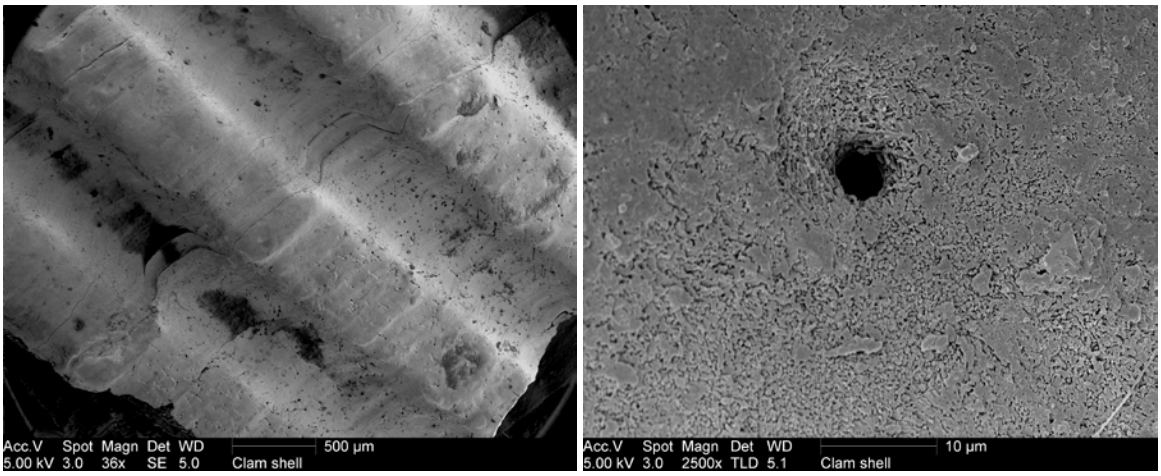

Figure 3: (Left) SEM micrograph of clam shell, magnification 36x. (Right) Close-up of clam shell surface, notice the smooth texture as compared to the shipworm shell.

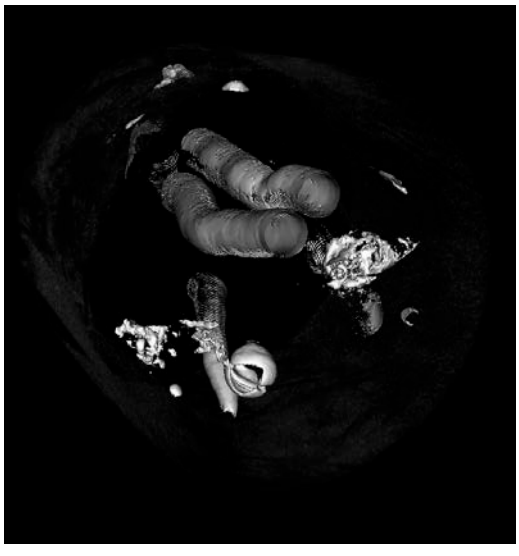

Figure 4: CT of alder wood colonized by shipworms. Here we can observe the calcareous lined tunnel, pallet and shell.

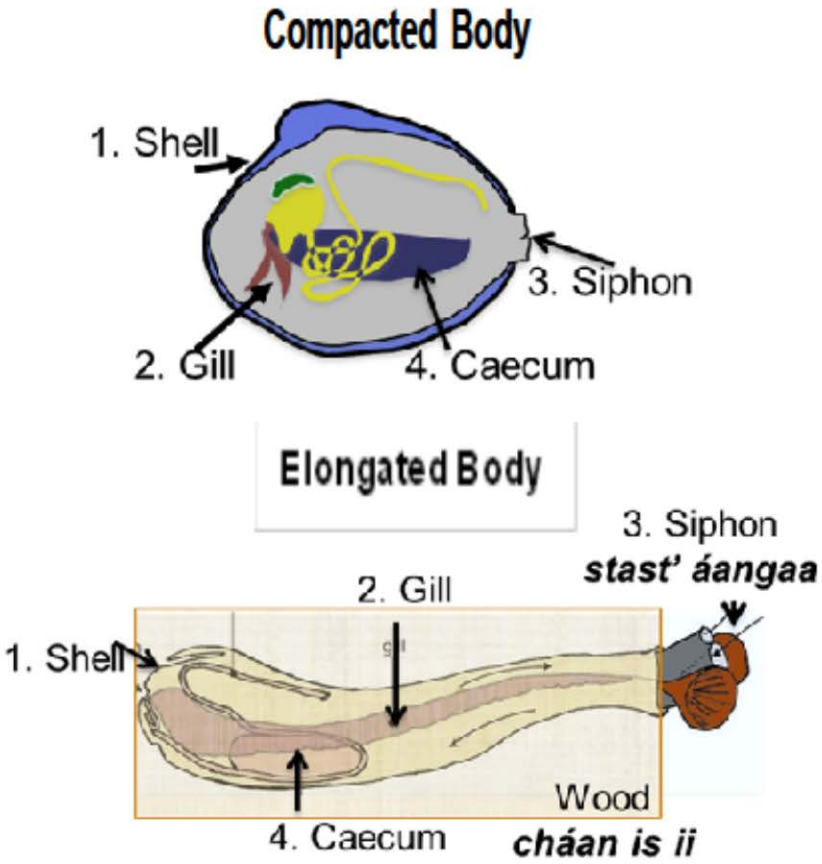

Figure 5: (Top) Cartoon illustrating the body plan of a clam (D. Distel) (Bottom) Illustration of the shipworm body plan (M. Betcher).

Curriculum was developed by first conducting interviews of tribal elders to record local Traditional Knowledge and to gain an understanding of what was known about shipworms. Traditional Knowledge was then coupled with western scientific knowledge about shipworms by collaborating with scientists that specialize in shipworm research. The coupling of these two knowledge systems resulted in the development of culturally relevant place-based and scientific inquiry-based curriculum. Once curriculum was developed, key vocabulary was identified to develop a glossary for each chapter. Glossaries were given to elders who are fluent in the Haida language and asked to translate into the Native language. This aspect of the project not only allowed for the incorporation of the Haida language into science education but also lead to the expansion of the Haida language as many of the glossary terms had no formal word in the Haida language. For these terms, elders used the definition of the term to formulate a new word. The English and Haida terms were taught to students, teachers, and scientists by a fluent Haida linguist, connecting western science, traditional knowledge, and Native language, resulting in a rekindled interest of the Haida language for many students.

National Science Standards

The following Next Generation Science Standards were identified as being met by the Shipworm curriculum described here (NGSS Lead States, 2013). MS – LS2 Ecosystems: Interactions, Energy and Dynamics

- MS- LS2-c: Construct and present arguments supported by empirical evidence and scientific reasoning for multiple explanations for how changes to physical or biological components of an ecosystem result in changes to the populations in the ecosystem.

- MS – LS2-g: Make an oral or written argument from evidence to support or refute the merits and constraints of different plans to solve a real world problem to restore a disrupted ecosystem.
- MS-LS2-i: Ask questions to clarify the scientific, economic, political, and social justifications used in making decisions about maintaining biodiversity in ecosystems.

MS – ETS1 Engineering, Technology, and Applications of Science

- MS-LS4-j: Use arguments supported scientific evidence and social and economic rationale to evaluate plans for maintaining biodiversity and ecosystem services.
- MS – ESS3-e: Design and communicate solutions that meet criteria and constraints for minimizing human impacts on environments and local landscapes by: 1) managing water resources, 2) reducing pollution, and 3) reducing the release of greenhouse gases.

Grade Level: Middle School (5-8th Grade)

Content or Subject Area: Life Science, Earth Science, and Ocean Science, Scientific Experimentation

Materials and Resources

- Anatomy, physiology and ecology of Marine Bi-valves (Mollusca)
- 2x4 foot long planks of different types of wood (soft and hard)
- Rope and weights to secure wood on docks
- Wood and dissecting tools for removal of shipworms following exposure

Instructional Practices: 5 E's

The following is a description of Shipworm curriculum developed by the author's written in an Instructional Practice (5E's) format. The 5 E's is an instructional model based on the constructivist approach to learning. This approach can be used with all age groups and states that learners build or construct new ideas and concepts along with preconceived ideas. Each of the 5 E's defines a phase of learning, with each phase beginning with the letter "E": This approach allows students and teachers to experience common activities, while building on prior knowledge and experiences to construct meaning, and to repeatedly assess their understanding of new concepts.

Engage: Introduce activity by discussing damage to boats, docks and wood structures as a mystery to be solved. Discuss and visually show pictures and examples from student's environments of areas with damage or susceptible to damage from marine life. Show several examples for evidence as clues to the mystery. Who might be responsible for this damage to wooden structures?

Explore: Introduce ecology, biology and physiology of Shipworms. Discuss modifications to Mollusca body plan by shipworms due to adaptation of their lifestyle. Discuss shipworm body plan and life stages. Show pictures, diagrams and dissected wood as example. If possible show video of Shipworms living in wood and filter feeding from environment. Show real dissection pictures of entire body along with dissected diagrams.

Explain: Shipworms as indicators of environmental health. Discuss why their presence or absence would be indicative of the health of the environment. What about their physiology allows them to only live in healthy environments? What impedes their existence in unhealthy environments? Do they exist in our marine community? How can we test for their presence

without disturbing our marine infrastructure? What kind of experiment can we carry out to test for the presence or absence of shipworms? Are there areas in particular in our community that we would want to test for their presence?

Elaborate: How will we test for the presence or absence of shipworms in our marine environment? Discuss possible variables in an experiment including incubation location, types of wood, depth in the water column (Independent Variables). What will we measure or collect data on regarding presence or absence of shipworms? Dependent variables might include: presence or absence of shipworms, numbers if present, colonization by other organisms, physical parameters (dissolved oxygen, nutrients, salinity, temperature, tidal influence, etc.) Our experiment will take place in our marine community and will be monitored by students throughout the year as colonization will take several months to a year. Discuss experimental set-up of wood pieces, type of wood, size, drilling holes for securing to rope, weight placement to keep wood hanging at depth, and discussion of depth for each piece of wood. Monitoring plan is set-up including data recording on a monthly, quarterly basis including plans for examining wood for presence of shipworms, extraction of shipworms from wood if discovered, removal of shipworms and preservation for later dissection.

Evaluate: Students will maintain records of experiment in a lab notebook including observations and collection of data from colonization of wood in the field. Categories for observations will be pre-discussed prior to notebook collection. Students will be divided up into teams with specific roles within each team for experimental management. Students will grade and evaluate themselves and their teammates for their perceived contributions to completion of the experiment. Finally, students will be responsible for putting together a presentation summarizing

their results from their experiment and will be graded on their team presentation. Rubrics are provided for each of the evaluation pieces as discussed above.

Engineering, Technology & Application of Science Extension

We will seek out avenues to share our information including possible collaborations with community councils, tribal governments, policy makers, and environmental advocates. We will work together in our teams to develop presentations, videos, posters along with developing our ideas through debates and discussions in class as to how we can apply our science to solve community problems or concerns.

Classroom Implementation

Curriculum incorporates both classroom lectures and field components allowing students to actively participate in experimental design, implementation, and data collection. The classroom component focuses on introducing the concepts of the scientific method for experimental design, environmental health, and monitoring techniques such as bioassessment of lotic and marine ecosystems. The concepts of dependent and independent variables are highlighted, with students choosing the independent variables (depth in water column and wood type) and measuring the dependent variables (shipworm presence/absence, abundance, and the degree of wood degradation). Students learn what indicator species are, how to choose an indicator species for different environments–e.g. shipworms for marine systems and macroinvertebrates for lotic systems–and how to collect organisms for bioassessment of ecosystems. Students worked together to construct shipworm traps and were responsible for selecting variables such as wood substrate, depth of wood deployment, and location of traps. Wood substrates were chosen from locally available wood products such as red cedar as a soft wood and alder and fir as hard woods.

The field component of the curriculum allows students to deploy traps and collect data from each deployment site, including GPS coordinates, temperature of surface water, and salinity. Students and teachers periodically monitored colonization of shipworms on deployed traps. Evidence of colonization by shipworms resulted in the harvesting of traps for observation in the classroom. Traps were photographed once removed from the water and cleaned of barnacles, sponges, and seaweed to allow for better visualization of shipworm tunnels (Figure 6). Collected traps were brought to the classroom for further analysis (Figure 7).

Traps were distributed to each science class and used for lessons covering a variety of topics from general biology to anatomy and physiology. Images taken of the traps prior to cleaning were used to teach oceanography with discussions on light penetration in the water column, the euphotic zone, and photosynthesis. The anatomy and physiology class dissected the shipworms examining the body plan and compared and contrasted them with traditional native foods such as clams and mussels.

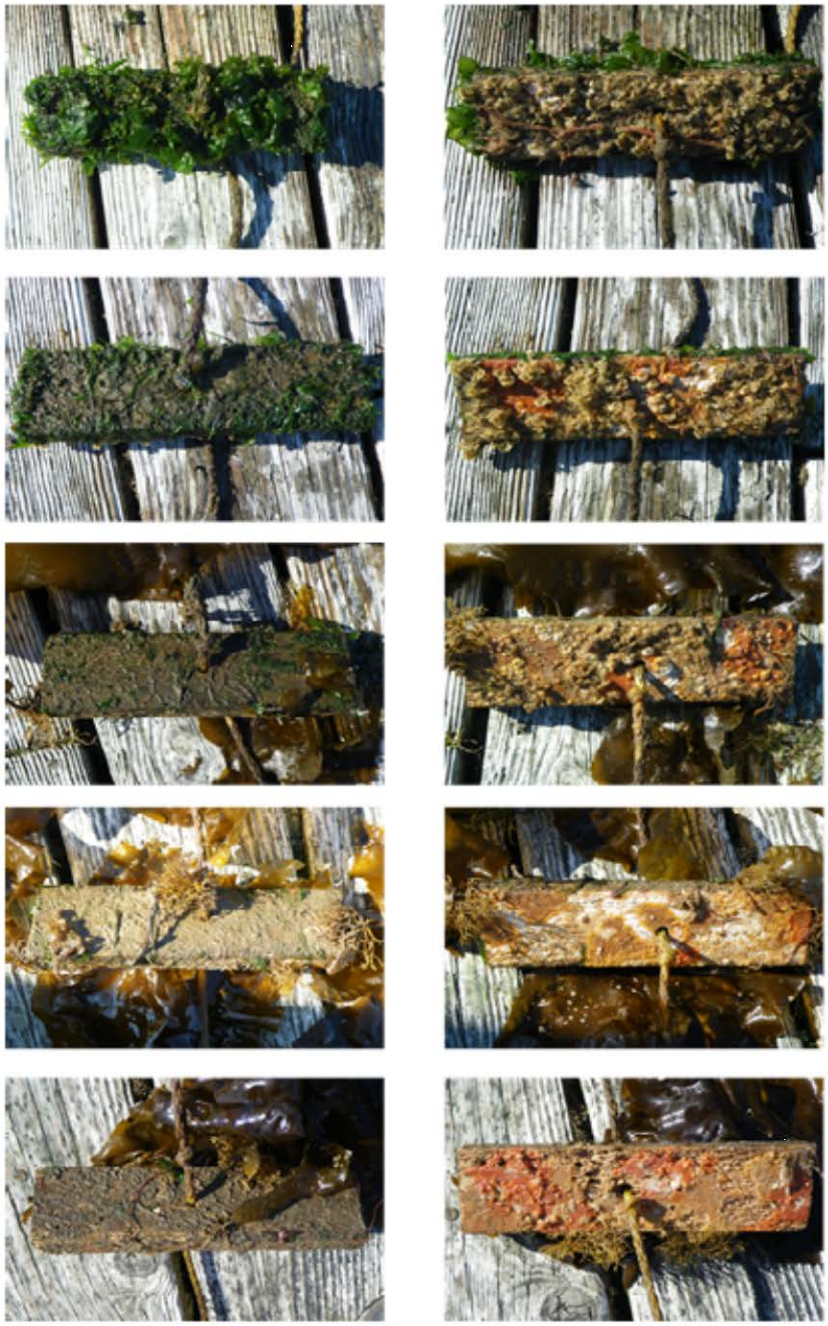

Figure 6: Colonized red cedar traps after 1 year deployment in the Hydaburg marina. (Left) Traps are place 1 meter apart for a total depth of 7 meters, the top traps is 2 meters below the water surface. Notice the sea weed growing on the top-side of the traps due to light penetration. (Right) Bottom side of traps; notice the absence of sea weed due to lack of light and the growth of sponges and barnacles.

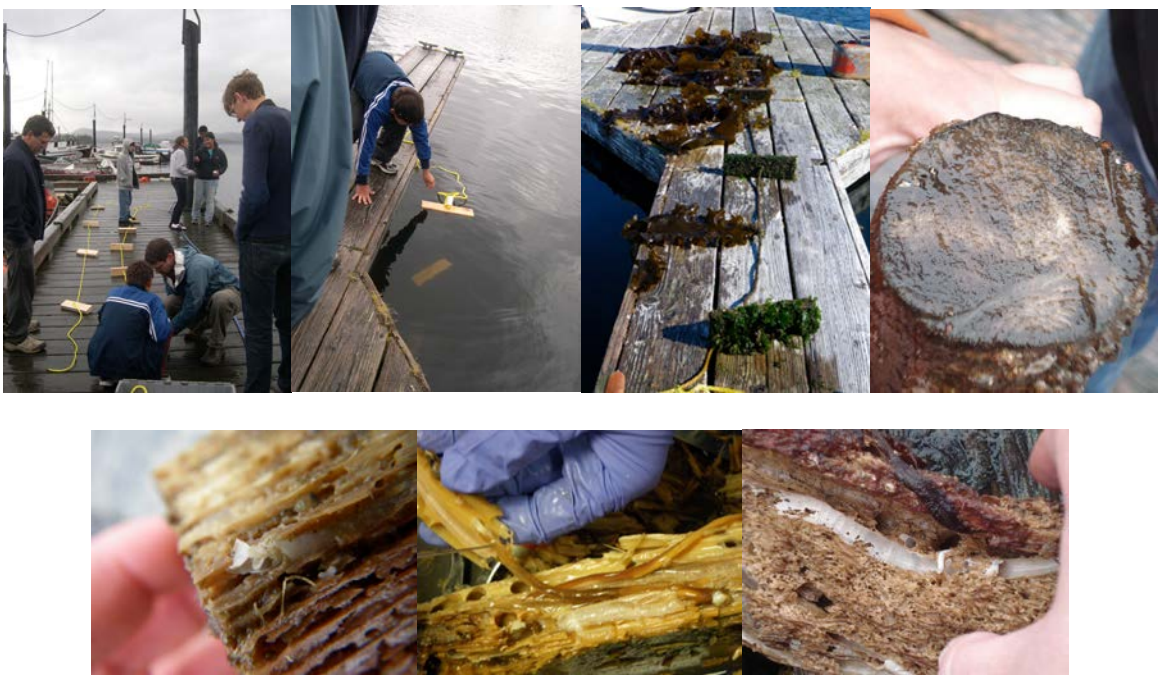

Figure 7: Images illustrating the deployment, collection and dissection of red cedar shipworm traps after a year in the Hydaburg marina.

Results of Shipworm Project

Colonization of shipworms in wood substrates typically occur independent of depth and type of substrate. Students found that shipworms colonized the softwood substrate more quickly than hardwood substrates. Students found that above 10 meters there was no colonization of traps, while traps below this depth were heavily colonized. The results indicated that there might be a pollutant in the surface waters of the marina inhibiting colonization.

The results and implications of this study were presented to the tribe who took a proactive approach working with the students to develop a remediation plan and to continue to monitor the health of the marina during the remediation process such as employing microbiology techniques to monitor water quality and extending efforts to clean up trash along the waterfront.

Discussion

Scientific skills gained from involvement in this geoscience program have led to tribal summer internships for students to continue to monitor both lotic and marine ecosystems and presentations by students at national and local science conferences. Additionally, the pathway for discussions pertaining to the anthropogenic influences on lotic and marine ecosystem has been paved and is commonly discussed with respect to traditional stories and future impacts. There has been an increase in student attendance at college and trade schools subsequent to their involvement in this culturally relevant geoscience education program that incorporates Traditional Knowledge systems, Native language, and western science. Students participating in this program have a positive perception of science and have begun to bridge the gap between generations by incorporating knowledge from the elders introduced through the curriculum. Interviews, both video and audio, with elders are archived for future generations.

We have addressed the National Science Standards (NGSS Lead States, 2013) that we believe that our curriculum meets for required classroom instruction. We recognize the importance of meeting standards in order to allow the lesson to be incorporated into a middle school science curriculum. The curriculum discussed here also has interwoven applications throughout courses in English, Native American Studies/Languages, History, etc. The most powerful piece of this curriculum, however, is the context in which it was developed. Its lessons are rooted in the community, in which it was taught, utilizing and recognizing the importance of traditional knowledge (through involvement of elders). The methods in which this curriculum was developed are as important as the lesson itself allowing other indigenous communities to model curriculum development for themselves around their own traditional knowledge and environmental issues and health.

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Appendix I

Medium recipes using for enrichments

Soda Bay Fe-oxidizing enrichments

1X Modified Wolfes Minimal Medium

For 1 liter stock medium
$CaCl_2 \cdot 2H_2O$ 0.1 g
NH4Cl 1 g
MgSO4 • 7H2O 0.2 g
K2HOP4 0.05 g
Distilled water to 1L volume.
Autoclave
Cool for 15 minutes then add
1 M $NaNO_3$
0.5M HEPES
1M $CaCO_3$
Adjust pH
*To make for heterotrophic growth add 6.5 μL of 2M sodium acetate.

1% Bottom Plug for Iron Gradient Tubes

Bake zero valent Fe to sterilize
Add agarose to MWMM to make a 1% solution
Autoclave
Add zero valent iron to a concentration of 223.88mM
Add 2ml of 1M HEPES
Dispense 2ml into the bottom of each tube with a glass pipette, careful not to get any on the sides of tubes.

Medium used for Mn-oxidizing cultures

K Medium

For 1L of seawater or artificial seawater
1 mL of NaNO3 (8.82 X 10-4 M)/ NH4Cl (5.00 X 10-5 M): use 1 mL of the solution containing both sources of N (NO3 and NH4) or 1 mL of each solution (1 mL of NaNO3 and 1 mL of NH4Cl) if they are separate solutions.
1 mL of Na2 beta-glycerophosphate 6H2O (1.00X10-5 M)
1 mL of H2SeO3 (1.00X10-8 M)
1 mL of Tris-base (1.00X10-3 M)
1 mL of K trace metal mix
0.5 mL of F/2 vitamins (kept at 4ºC or -20ºC)
Autoclave and cool to room temperature
Adjust for a final pH between 8.12 and 8.25.
Adjust pH by adding filtered sterilized (0.2 μm) 4M NaOH or 10% HCl directly into medium.

Trace Metal Solution for K Medium

Using18.2 MΩ water

$Na_2EDTA .2H_2O$	41.60 g
$FeCl_3 . 6H_2O$	3.15 g
$Na_2MoO_4 . 2H_2O$	6.3 g
$ZnSO_4 . 7H_2O$	22.0 g
$CoCl_2 . 6H_2O$	10.0 g
$MnCl_2 . 4H_2O$	180.0 g
$CuSO_4 . 5H_2O$	9.8 g

Make the EDTA first then add the Iron(III) chloride hexahydrate, followed by the remaining metals.
Autoclaved and store at room temperature or 4ºC in the dark.

Vitamin Solution for K Medium

Stock solution made in 18.2 MΩ water

Vitamin B_{12} (cyanocobalamin)	1g/L
Biotin	0.1 g/L
Thiamine –HCL	200 mg

Lept Medium

18.2 MΩ water	982 ml
1 M CaCl2	0.48 ml
1 M MgSO4	0.83 ml
Yeast extract	0.5 g
Agar	15.0 g
Autoclave	

Add pre-sterilized

1 M D-(+)-glucose	5.0 ml
Casamino acids	0.5 g
1 M HEPES buffer (pH 7.50)	10.0 ml
Lept trace element solution (filter sterilized)	1.0 ml
1 M MnCl2	0.1 ml
10 mM FeCl2 (made fresh and filter sterilized)	0.37 ml

Appendix II

Glossary of selected geoscience terms in English and Haida

The following words were used in Haida instruction during our geoscience education program. The list includes both pre-existing words and new words created to describe modern concepts and objects. New words and phrases were formulated by tribal linguist Benjamin Young with instruction and advice from tribal Elders.

Binoculars	Tlág Kéeng waay
Camera	Níijaang
Calculator	Gudii gin hl kihlaas, Híisdluu gids díig gin súudgans aa
Carbon dioxide	Gin akyáag tl'áng gagánjuugans aa (what we breathe out)
Coho	táay
Compass	Tlíitsaan dángg gin súulgans aa
Computer	Gin 'wáadluwaan stlá K'áalangs an únsadsaa
Dissolve	wáa sgáawgang (to dissolve, melt) Aájii xílaay
Dog salmon	skág
East	sáawtlagáas
Fresh water	Ga'nd (water)
Gas	táw, tawáay
Glacier	Kálgaasii jíinaagang (The ice is lasting a long time)
Hatchling/fingerling/fry	Máaluud
Humpy Salmon	ts'atáan
Image/picture	Níijaang, Dáng tl' hiijaangaan aa Ritliiuaa (Put picture on the wall)
Ice age	Awáahl gagwíi kálgaa jíingaangiinii (A long time ago the ice lasted a long time)
Insect Larvae	Káanuu (maggot)
Juvenile (young salmon)	táayee ts'úudalgan, chíiaay ts'úudalgan (s aa)
King salmon	Táa'un
Liquid	tantl'dáagaagang (liquid, damp)
Map	Tlíitsáan dáng is Kugíinaay dángg súutgans aa
Mountain	tlatáawaay
Migrate	ts'áagaa (migrate, get up, move)
North	xáagw
North pole	xáagw, Damaan uu xáagw tadáang
Oxygen	Gin tl'áng gagánjuugans aa (what we inhale) Aajii táw tláatsgaa jahlu'gang
Population	xáadgaay Asgáay t'iits xáat'aagang. (These ones are Haida)
Rain	gwáaw (gang) daláay
River	Gándlaay

Rock	Kwa’áay
Saltwater	Cháan tángaa (salty water in the sea)
Sockeye	sgwáagaan
Solid	K’ats’áang (to be hard) K’ayee k’ats’aang
South	xíwg
Soil	Cháan (kind of soil you can plant)
Spawn	xáydang, Káawiigang
Steel head	tayáng
Temperature	Sangáay
Turbidity/Water Cloudiness	Gándlaay sk’íileelga
West	Káágwaa