Microbial Biosignatures in High-Iron Thermal Springs

Mary Nichole Parenteau

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MICROBIAL BIOSIGNATURES IN HIGH-IRON THERMAL SPRINGS

by

MARY NICHOLE PARENTEAU

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCES AND RESOURCES: GEOLOGY

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The abstract and dissertation of Mary Nichole Parenteau for the Doctor of Philosophy in Environmental Sciences and Resources: Geology were presented May 9, 2007, and accepted by the dissertation committee and the doctoral program.

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ABSTRACT

An abstract of the dissertation of Mary Nichole Parenteau for the Doctor of Philosophy in Environmental Sciences and Resources: Geology presented May 9, 2007.

Title: Microbial biosignatures in high-iron thermal springs

Debate exists regarding whether abiotic or biotic mechanisms were responsible for the oxidation of Fe^{2+} and the subsequent accumulation of ferric (Fe^{3+}) iron assemblages in Precambrian Banded Iron Formations (BIFs). Direct paleontological evidence for a microbial role in the deposition of BIFs has been sought in the occurrence of microfossils and lipid biomarkers in these structures.

This study has characterized the formation of such biosignatures in modern iron deposits. The metabolic impact of microbes on Fe^{2+} oxidation in this system has previously been described (Pierson et al., 1999; Pierson and Parenteau, 2000). Cyanobacterial and filamentous anoxygenic phototrophic (e.g., Chloroflexus) microfossils, stromatolitic biofabrics, and lipid biomarkers were characterized using optical, scanning electron, and transmission electron microscopy; electron diffraction; X-ray diffraction; and lipid and compound-specific stable carbon isotope analyses.

TEM examination of the cyanobacterial cells revealed iron-mineralized carbonaceous microfossils that retained taxonomic features that allowed their identification to the species level. A robust suite of geologically significant lipid
biomarkers were identified and linked to the phototrophic source organisms. These biomarkers were found to survive microbial degradation and the earliest stages of diagenesis in the iron oxide deposits underneath the mats. Many of these modern biosignatures correlate with evidence preserved in the minerals and fabrics of ancient BIFs.

These biosignatures can be used to link modern microbial ecosystems to their fossilized equivalents preserved in the geological record. This type of fossil evidence can be used to infer the paleobiological role of microbes and paleoenvironmental conditions, and will establish a means to assess the microbial contribution to ancient iron deposits on Earth (e.g., BIFs) and, potentially, to those found on Mars.
This PhD dissertation is dedicated to Paul W. Parenteau, Mary M. Reynolds, and Eric A. Chin. Nothing can fill their absence.
I express my deep gratitude to the many people who have contributed to this dissertation by providing mentoring, assistance with equipment, and moral support. I thank my advisor, Sherry L. Cady, for her direction and insight while navigating the world of biosignatures. Her input was critical to the shaping and strengthening of this dissertation. I also thank my Committee Members, Michael L. Cummings, R. Benjamin Perkins, Radu Popa, and Kenneth Stedman, for their excellent suggestions as to how to best package the research for publication in the interdisciplinary field of Geomicrobiology. I thank Richard C. Hugo, Georg H. Grathoff, and R. Benjamin Perkins for their expertise and assistance with scanning and transmission electron microscopies, X-ray diffraction, and water chemistry analyses. I thank my undergraduate advisor Beverly K. Pierson for her continued excellent mentoring, my NASA Planetary Biology Internship advisor Linda L. Jahnke for introducing me to the world of lipids and stable carbon isotopes, and our collaborators Brian L. Beard and George W. Luther for enriching the project with their expertise in iron isotopes and aqueous geochemistry. I also owe a great debt of gratitude to my lab-mates Jessica Goin, Wendy Smythe, Hollie Oakes-Miller, Mary Dietrich, Zach Oestreicher and my office-mates Heather Easterly, Larry Robinson, Jon Ebnet, Pete Sniffen and my fellow graduate student Tom Lindsay for their wonderful and unending support. I would not have made it through the program without them. Finally, I thank my family and dear friend Kylie Kramer for always being there for me during this arduous journey.
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1.1 Iron Biogeochemistry on Modern Earth

Iron is the most abundant transition metal in the Earth’s crust and exists in two oxidation states, Fe$^{2+}$ and Fe$^{3+}$ (Rudnick and Gao, 2004). In the deep crust and mantle, iron exists largely as Fe$^{2+}$, creating a large redox potential when these settings are exposed to surface oxidizing conditions. Fe$^{2+}$ is soluble at circumneutral pH under low redox, anoxic conditions such as might be found in reducing groundwaters, restricted marine basins, stratified lakes, hot springs, or on early Earth when atmospheric O$_2$ concentrations were much lower than today (Stumm and Morgan, 1996; Kasting, 2001). In contrast, Fe$^{3+}$ is generally insoluble at neutral pH, and is soluble only under acidic conditions such as in acid mine drainage settings (Stumm and Morgan, 1996).

Biological processing of iron can exploit both oxidation states. Fe$^{2+}$ may act as an electron donor for chemolithotrophic growth of acidophilic and neutrophilic iron oxidizing bacteria such as *Thiobacillus* and *Gallionella* (e.g., Hallbeck and Pedersen, 1991; Ehrlich, 1996; Emerson and Moyer, 1997). *Thiobacillus ferrooxidans* is well known for oxidizing Fe$^{2+}$ under acidic conditions and contributing to the formation of acid mine drainage (Hallbeck and Pedersen, 1991). *Gallionella* and *Leptothrix*, the so-called “iron bacteria,” oxidize Fe$^{2+}$ in circumneutral iron seeps and are also responsible for the iron fouling of water pipes (e.g., Emerson and Ghiorse, 1992).
Fe$^{2+}$ can also donate electrons to support the photosynthetic growth of organisms. Purple and green phototrophs oxidize Fe$^{2+}$ in a type of anoxygenic photosynthesis known as photoferrotrophy (e.g., Widdel et al., 1993; Ehrenreich and Widdel, 1994a,b; Heising and Schink, 1998; Heising et al., 1999; Straub et al., 1999). These organisms are restricted to aquatic environments that are anoxic because oxygen inhibits anoxygenic phototrophic metabolism (Repeta et al., 1989; Falkowski and Raven, 1997). Consequently, and in contrast to the chemolithotrophic bacteria that require oxygen as a terminal electron acceptor, these phototrophs can mediate Fe$^{2+}$ oxidation in the absence of oxygen.

Although it has not yet been demonstrated, the filamentous green nonsulfur phototroph *Chloroflexus* has been hypothesized by Pierson and colleagues to oxidize Fe$^{2+}$ (Pierson et al., 1999; Hanada and Pierson, 2002). They suggest that Fe$^{2+}$ may donate electrons to the photosystem II (PSII)-type reaction center, similar to what occurs in the purple bacteria during photoferrotrophic growth. This possibility is intriguing because the family *Chloroflexaceae* is the most deeply branching phototrophic lineage on the 16S rRNA tree of life (Oyaizu et al., 1987), and may well indicate the antiquity of iron oxidation in supporting photoautotrophic growth.

Cyanobacteria have also been hypothesized to use Fe$^{2+}$ as an electron donor for photosynthesis (Cohen, 1984; 1989; Pierson et al., 1999). Fe$^{2+}$ oxidation in cyanobacteria is of interest because of the potential role that this reductant may have played in the evolution of H$_2$O-oxidizing photosynthesis that produces molecular oxygen as a by-product (Olson and Pierson, 1987; Pierson and Olson, 1989;
Blankenship and Olson, 2004; Olson 2006). Cyanobacteria typically use H₂O as an electron donor, which has a high reduction potential at +0.82 V, and harvest the redox energy using two photosystems (PSII and PSI) (Madigan and Martinko, 2006). They have also been shown to oxidize H₂S, which has a low redox potential at -0.28 V, using a single photosystem (PSI) (Cohen, 1986). This type of photosynthesis is much less complex and yields less energy than the H₂O-oxidizing photosynthesis and may have occurred in ancestral cyanobacteria utilizing H₂S on a mildly reducing early Earth (Cohen, 1986). It has been hypothesized that as these reductants were used up, the ancestral cyanobacteria began to oxidize the more widely available Fe²⁺, which has an intermediate reduction potential at +0.2 V, using a single photosystem (Olson and Pierson, 1987; Pierson and Olson, 1989). Then, as Fe²⁺ became scarce, the cyanobacteria evolved the ability to oxidize ubiquitous H₂O utilizing two photosystems. This type of photosynthesis is more complex and yields more energy because of the high reduction potential of H₂O, and is responsible for oxygenating the atmosphere (Kasting, 2001) and has been adopted by all green plants. If cyanobacteria are shown to oxidize Fe²⁺ in modern environments, it may confirm Fe²⁺ as an important intermediate reductant in the evolution of oxygenic photosynthesis in ancestral cyanobacteria as the early Earth transitioned from a mildly reducing environment to an oxidizing one (Olson and Pierson, 1987; Pierson and Olson, 1989; Blankenship and Olson, 2004; Olson 2006).

While it has not yet been conclusively demonstrated that cyanobacteria use Fe²⁺ as an electron donor for photosynthesis (Cohen, 1984; 1989), the oxygen
produced by these organisms has been shown to be the sole mechanism by which \( \text{Fe}^{2+} \) is oxidized in the anoxic waters flowing over hot spring microbial mats (Pierson et al., 1999; Trouwborst et al., in review).

These oxidative organisms also have reductive counterparts. Dissimilatory iron-reducing bacteria (DIRB) utilize \( \text{Fe}^{3+} \) as a terminal electron acceptor and generate \( \text{Fe}^{2+} \) as a metabolic by-product (e.g., Lovely, 1991; Nealson and Saffarini, 1994). These organisms are capable of reducing a range of \( \text{Fe}^{3+} \)-oxides from the poorly crystalline ferrihydrite to the more ordered phases such as goethite and hematite (Lovely, 1991). Much effort is directed at determining the factors that regulate the rate and extent of \( \text{Fe}^{3+} \)-oxide reduction, such as particle size, surface area, and crystallinity (Roden and Gorby, 2002).

1.2 Iron Cycle on Early Earth and Mars

The iron cycle on modern Earth has been well established, but we have only recently begun to elucidate the role of microbes in the biogeochemical cycling of this transition metal. Microbes may also have participated in the iron cycle on the early Earth, as evidenced by the presence of oxidized iron in Precambrian Banded Iron Formations (BIFs) during globally anoxic conditions (Cloud 1965; Hartman, 1984; Olson and Pierson, 1987; Holm, 1989; Widdel et al., 1993; Ehrenreich and Widdel, 1994a,b; Konhauser et al., 2002; Kappler et al., 2005). The recent discovery of oxidized iron on a once watery Mars also suggests the presence of an iron cycle (Squyers et al., 2004; Arvidson et al., 2005; Gendrin et al., 2005; Langevin et al.,
2005; Martinez-Frias et al., 2006; Griffès et al., 2007; Roach et al., 2007), and begs the question, were microbes involved in iron cycling on this planet as well?

The origin of the oxidized iron in Precambrian BIFs has been debated for decades. Several theories have been developed to account for the appearance of layers of oxidized iron, two of which involve the metabolic action of bacteria. The earliest (and most widely accepted) theory is that the oxygen produced by ancestral cyanobacteria indirectly oxidized the Fe\(^{2+}\) (Cloud, 1965, 1973). More recent theories suggest the direct oxidation of Fe\(^{2+}\) by anoxygenic photosynthetic bacteria in the absence of oxygen (Hartman, 1984; Kump, 1993; Pierson, 1994) or by chemotrophic bacteria in the presence of low levels or absence of oxygen (e.g., Hallbeck and Pedersen, 1991; Straub et al., 1996).

Liquid water is a fundamental requirement for life on Earth as we know it (Ball, 2005). This dictum has led to the strategy of “following the water” in the NASA mission-related goals of characterizing the habitability of Mars and determining whether life ever arose there. The recent discovery of liquid water-related minerals such as goethite by the NASA Mars Exploration Rover (MER), ESA Mars Express, and NASA Mars Reconnaissance Orbiter (MRO) missions has simultaneously enhanced the possibility of extinct or extant life on the red planet, as well as suggested a role of this life in generating the oxidized iron (Squyres et al., 2004; Arvidson et al., 2005; Gendrin et al., 2005; Langevin et al., 2005; Martinez-Frias et al., 2006; Griffès et al., 2007; Roach et al., 2007).
1.3 Biosignatures

Paleontological evidence of a microbial role in iron cycling on early Earth has been sought in the occurrence of biosignatures in these deposits. Specific evidence will be detailed in the following chapters. Iron deposits on Mars are also target sites for detection of biosignatures by the NASA Mars Science Laboratory (MSL) mission launching in 2009. Biosignatures provide fossil evidence of microbial life, and three main types of biosignatures form in mineralizing ecosystems: microbial fossils, chemofossils (biominerals, biomarkers), and organosedimentary structures (e.g. stromatolites) (Cady, 2001). Microfossils form by homogenous or heterogenous nucleation of minerals inside the microbial cells (permineralization) or on the exterior surfaces of cells (encrustation) (Fortin et al., 1997). Biominerals form by providing favored substrates for heterogeneous mineral precipitation, or by precipitating in proximity to the cell as a result of changes in pH, redox potential (Eh), or O_2 levels caused by the metabolic activities of microorganisms (Cady, 2001). Biomarkers are organic biosynthetic molecules indicative of different organisms (Cady, 2001), such as carbon skeletons of cellular components (e.g. lipids). Microbial mats and biofilms likely contribute to the construction of organosedimentary structures, but distinguishing the biotic from the abiotic contribution to these structures has proven problematic (Cady, 2001). By documenting how microbes and their environment contribute to the formation of primary biosignatures, it becomes possible to decipher biogenic inputs to the sedimentary rock record.
The paleontological interpretation of this suite of fossil information relies on comparison to the modern world. There are limitations to this approach because ancient ecosystems undoubtedly differed from current ones, but insight can be gained from assigning microbial fossils, chemofossils, and organosedimentary structures to extant organisms. This helps to infer the physiology of ancestral organisms and how their metabolism may have altered the paleomicro- and macroenvironment. Besides this comparative biology, knowledge of the geochemical constraints on the distribution and extent of microbes in modern environments helps to infer their distribution and ecological impact in paleoenvironmental settings.

Biosignatures in modern deposits also represent the maximum amount of paleobiological and paleoenvironmental information that can become preserved in the rock record. Studying biosignature formation in this setting allows for assessment of taphonomic changes and diagenetic mineral transformations that ultimately results in a loss of fossil information (Knoll and Walter, 1996). Understanding how these processes affect biosignatures in a modern environment improves our ability to detect and interpret paleontological information in ancient and extraterrestrial deposits (Cady, 2001).

Generation of such biosignatures can occur through the fossilization of microbes via entombment by chemical precipitates or burial in fine-grained detrital systems (Farmer and Des Marais, 1999). Rapid mineralization of cells via chemical precipitation can occur in environments such as sinter-depositing thermal springs and evaporite basins (Farmer and Des Marais, 1999; Cady et al., 2003).
1.4 Biosignatures in Modern Iron Deposits

The highly mineralizing environment of modern iron-depositing hot springs has been recognized as a sedimentary analog of some ancient iron deposits such as the ironstone bodies in the Barberton greenstone belt, South Africa, which formed from a subaerial spring system (Lowe and Byerly, 2007). While there is no direct modern analog for the majority of ancient iron deposits on early Earth such as Precambrian BIFs or for iron deposits on Mars, modern iron-depositing hot springs such as Chocolate Pots hot springs in Yellowstone National Park share certain key aspects. There is a hydrothermal supply of Fe$^{2+}$ and Mn$^{2+}$, the waters are devoid of H$_2$S and O$_2$, the hot spring is exposed to light, and is populated by cyanobacteria and anoxygenic phototrophs (Pierson et al., 1999; Pierson and Parenteau, 2000). Chocolate Pots hot springs is not a suitable structural model for the deposition of Precambrian BIFs, but can be considered an analog of a high iron anoxic environment exposed to light on early Earth. At the very least, it is a site in which to assess the impact of photosynthetic microbes on iron cycling and to examine the production and survival of their biosignatures in a modern iron deposit.

This study characterizes the biosignatures (microfossils, stromatolitic biofabrics, lipid biomarkers, and biominerals) forming in the modern iron-mineralized cyanobacterial mats at Chocolate Pots. This field site allows the unique opportunity to establish a link between (1) oxidation of Fe$^{2+}$ by a modern cyanobacterial community (2) production of biosignatures by the modern community and (3) survival of these biosignatures during the earliest stages of diagenesis in the geological record. Such a
biosignature database will establish a genetic relationship between the organisms and their metabolic impact on Fe$^{2+}$ oxidation in these modern deposits and, potentially, ancient ones on Earth and Mars. The results of this biosignature study may also aid in the search for evidence of past microbial life in iron deposits on Mars during the Mars Science Laboratory (MSL) and Astrobiology Field Lander (AFL) missions.
2.1 Introduction

Microbial biosignatures provide trace evidence of microbial life, past and present, and can be divided into three categories (Cady, 2001). The first category is comprised of microbially influenced biofabrics and sedimentary structures (e.g., microbialites and laminated biogenic stromatolites). The preservation of individual cells can generate the second category, carbonaceous bona fide microfossils. The last category is chemical fossils: remnants of cells can be preserved as lipid biomarkers, while isotopic signatures and biominerals provide evidence of microbial metabolism.

Microbial biosignatures provide fossil evidence of the origin and early evolution of life on this planet and aid in assessing the role of microbes in early biogeochemical cycles. The occurrence of microbial biosignatures in Precambrian Banded Iron Formations (BIFs) has been used to suggest a microbial role in early iron
transformations and deposition of these structures. The most well-known example of this is the description of microfossil remains of cyanobacteria in stromatolitic chert of the 1.9 billion year-old (Ga) Gunflint Iron Formation (Tyler and Barghoorn, 1954; Barghoorn and Tyler, 1965). Cloud (1965) confirmed the initial taxonomic descriptions of the microfossils discovered in the Gunflint Formation and postulated that the localized oxidation of dissolved iron and subsequent precipitation of ferric oxides by ancestral cyanobacteria occurred even before the well-documented and widespread oxygenation of the atmosphere at 2.3 Ga (Kasting, 2001).

Most of the microfossils in BIFs have been permineralized by silica and are found in bands of chert. The lack of bona fide (i.e., carbonaceous) microfossils in the iron-rich layers and, thus, the lack of a necessary spatial relationship between the microfossils and the iron mineral assemblages led Klein (2005) to conclude that microbes such as the Fe$^{2+}$-oxidizing purple phototrophs or chemolithotrophs did not play a direct role in BIF deposition. However, he stated that this did not exclude the possibility that oxygen produced by ancestral cyanobacteria, as hypothesized by Cloud (1968, 1972, 1973, 1983), could have provided the oxidizing power necessary for ferric oxide formation.

Our group has tested the Cloud hypothesis at a high-iron thermal spring in Yellowstone National Park that contains cyanobacteria and filamentous anoxygenic phototroph (Chloroflexus) microbial mats bathed in anoxic vent waters high in Fe$^{2+}$. Microelectrode studies at Chocolate Pots hot springs have revealed that oxygen produced by the cyanobacteria was solely responsible for oxidizing the Fe$^{2+}$ (Pierson
et al., 1999; Trouwborst et al., in review). The primary variable dictating the oxidation rate was determined to be light intensity, which controlled the photosynthetic production of oxygen (Trouwborst et al., in review). In addition to mediating $\text{Fe}^{2+}$ oxidation, the phototrophs played a physical role in the formation of the stromatolitic iron deposit by binding and trapping iron minerals (Pierson and Parenteau, 2000).

Chocolate Pots hot springs is not a suitable structural model for the deposition of Precambrian BIFs. However, it is one of the few modern environments that is anoxic, high in $\text{Fe}^{2+}$, devoid of sulfide, exposed to light, and is only slightly acidic, which allows phototrophs to flourish. These criteria make it a reasonable geochemical model for the oxidation of $\text{Fe}^{2+}$ by oxygenic and anoxygenic photosynthesis in an ancient anoxic world.

Our group's ongoing interdisciplinary study at this site provides the unique opportunity to integrate microbial physiology, geochemistry, and mineralogy to assess the metabolic impact of the phototrophs on $\text{Fe}^{2+}$ oxidation and link the source organisms to geologically significant biosignatures. Biosignature formation in a modern deposit represents the maximum amount of paleobiological and paleoenvironmental information that can become preserved in the rock record. We aim to characterize the taphonomic changes that occur to the biofabrics and microfossils during microbial degradative processes in the mats and during burial and earliest stages of diagenesis in the iron oxide deposits underneath the mats. Our biosignature database will establish a genetic relationship between the organisms and
their metabolic impact on Fe²⁺ oxidation in these modern deposits and, potentially, ancient ones on Earth (e.g., Precambrian BIFs).

2.2 Methods

Four types of phototrophic microbial mats which occur over different temperature ranges grow on the iron deposits of Chocolate Pots hot springs in Yellowstone National Park: Synechococcus-Chloroflexus spp. mat and Pseudanabaena sp. mat (50-54°C), narrow Oscillatoria sp. (36-45°C) and Oscillatoria cf. princeps (37-47°C) (Pierson et al., 1999; Pierson and Parenteau, 2000). We characterized the Synechococcus-Chloroflexus mat near the vent to assess biosignature formation in a higher temperature setting. We also characterized two variants of the lower temperature narrow Oscillatoria mats to compare the effect of hydrodynamic flow on biofabric formation. The microbial mats were sampled from 2001-2004 near the summer solstice to optimize photosynthetic activity.

2.2.1 Mapping

A map of Chocolate Pots hot springs was obtained July 2002 by recording the vent locations, the boundary of the iron oxide deposits, and topographical points using a Leica TC 805 L total station. A contour map of the 281 topographical points was created using ArcView GIS software. Surfaces were created of the vent and iron oxide deposit locations and overlain on the contour map. The extent of the iron oxide
deposits were drawn in using the stream digitizing extension and the boundary points as a guide.

2.2.2 Water Chemistry

The temperature of water flowing over the microbial mats was measured with a Fluke Model 52 K/J thermometer (Everett, WA). The pH was measured with a Fisher AccuTupH probe attached to an Accumet AP72 meter (San Diego, CA). The aqueous Fe\(^{2+}\) concentration was measured using the colorimetric ferrozine assay (Pierson et al., 1999) with a Hach DR/2400 portable spectrophotometer. To avoid Fe\(^{2+}\) oxidation, samples were not filtered prior to analysis and were analyzed immediately upon collection at the field site.

Water samples for cation and anion analysis were filtered (Nalgene surfactant-free cellulose acetate, 0.2 \(\mu\)m, Rochester, NY) and collected in nitric acid cleaned low-density polyethylene (LDPE) bottles. Cation samples were acidified to pH 0.9 with trace metal grade nitric acid (Fisher, Pittsburgh, PA) and stored at 4\(^{\circ}\)C until analysis by inductively-coupled plasma mass spectroscopy by XRAL Laboratories, Ontario, Canada. Anion samples were stored at 4\(^{\circ}\)C until analysis on a Dionex ICS-2500 ion chromatograph using IC Instrument Check Standard 6 (Lot #30-162AS) from SPEX CertiPrep (Metuchen, NJ).

2.2.3 X-Ray Diffraction (XRD)

A section of each mat type was prepared for powder X-ray diffraction (XRD)
analysis by air drying and then grinding them into a clay-size fraction using an agate mortar and pestle. The remaining organics were not digested prior to analysis because Cornell and Schwertmann (2003) state that while organics can bind to and increase the surface area of iron oxides, they have no effect on the amount of structural information obtained by XRD. The powdered samples were side-packed into aluminum holders using a frosted slide to ensure random orientation of the grains. Sample mounts were step scanned from 2.5 to 75° 2θ using a step size of 0.020° and a count time of 25 sec per step for a total of 25.5 hours per analysis. The samples were run on a Philips X'Pert PW3040 X-ray diffractometer at 40 kV and 30 mA using copper K-alpha radiation. The instrument contained an energy dispersive detector, so iron fluorescence was minimal. The spectra of samples run at similar conditions using cobalt K-alpha radiation did not contain any additional structural information about the hydrothermal iron precipitates (data not shown). The peaks on the powder XRD diffraction patterns were identified by comparing the measured sample d-spacings to previously published iron oxide d-spacings (Cornell and Schwertmann, 2003).

2.2.4 Elemental Analysis

Phototrophic mats were collected for total carbon (TC) and total organic carbon (TOC) analysis and immediately placed on dry ice in the field. A 2.5 cm deep core of the Synechococcus-Chloroflexus mat was removed, frozen, and sectioned with a sterile scalpel into two portions: the surface 0 - 2 mm containing only mat, and the underlying 2 – 25 mm containing iron oxides. Samples were kept frozen during transit
to the laboratory and then immediately freeze dried upon arrival. Iron oxide samples with no conspicuous mat on the surface were collected at the main iron deposit vent and along an outflow channel. Samples were air dried and stored at room temperature, then freeze-dried prior to analysis.

Freeze-dried mats and iron oxide samples were packed into tin capsules (Costech Analytical) and TC was determined using a Carlo Erba CHN EA1108 elemental analyzer. Acetanilide and EDTA (OAS, Elemental Microanalysis Limited) were used as standards. To determine TOC, the samples were exposed to an atmosphere of concentrated HCl (AR, Mallinckrodt) in a dessicator for 7 hours to liberate inorganic carbon as CO$_2$. The samples were then dried at 100°C for 1 hour to evaporate any remaining HCl and to reclaim any iron that may have gone into solution. Samples were packed in tin cups and analyzed as for TC.

2.2.5 Stereomicroscopy

The gross structure of the phototrophic mats was investigated in the laboratory using a Leica MZ9.5 stereomicroscope equipped with a Leica MPS60 camera. Kodak Ektachrome Elite II 100 and 200 ASA slide film was used to record photomicrographs.

2.2.6 Scanning Electron Microscopy (SEM)

Microbial mat samples measuring ~1 cm (l) x 1 cm (w) x 0.2 -10 mm (h) were excised in the field using a sterile scalpel. The mat slices were immediately preserved
in 2.5% (v/v) glutaraldehyde (EM grade, Ted Pella, Redding, CA) prepared with 0.2 μm filtered spring water, and stored on ice during transport back to the laboratory. Approximately 3 days from the time of collection, the field-fixed mat slices were rinsed twice in 0.1 M sodium cacodylate buffer, and post-fixed on ice for two hours using a 1% (v/v) osmium tetraoxide-0.1 M sodium cacodylate buffer solution. Samples were then rinsed twice in 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series, critical point dried (Pelco CP-2), and mounted on ethanol-cleaned aluminum stubs with carbon tape and colloidal graphite. The SEM samples were sputter coated with 150 Å gold-palladium or carbon (Gatan Model 682 PECS) and examined using a JEOL JSM 35-C SEM operating at 15-25 kV. The SEM was equipped with a KEVEX energy dispersive X-ray spectrometer (EDS). EDS spectra of the minerals encrusting the cells were collected at high enough magnifications (32,000-94,000 X) so that only the regions of interest were analyzed. EDS spectra were acquired at 25 kV for 120 sec with approximately 20% dead time (15 mm working distance and 45° sample tilt). The narrow Oscillatoria mats were also examined using a FEI Sirion high resolution SEM (HRSEM) operating at 5 kV and 5 mm working distance.

2.2.7 Transmission Electron Microscopy (TEM)

Microbial mat samples were preserved and embedded in resin as previously described (Pierson and Parenteau, 2000). Thin sections of transversely oriented mats (60 nm) were cut using a Sorval MT-2 ultramicrotome and collected on 400 mesh
copper grids and stained with 5% (w/v) uranyl acetate and 0.13% (w/v) lead citrate. Uncoated ultramicrotomed samples were examined with a JEOL 100CXII TEM operating at 100 kV accelerating voltage. Images were recorded on Kodak SO-163 film (Ted Pella, Rochester, NY). The TEM camera length was calibrated using a polycrystalline evaporated aluminum standard (EMS, Hatfield, PA).

2.3 Results and Discussion

2.3.1 Field Site

Chocolate Pots hot springs are comprised of a complex of 42+ vents along the Gibbon River approximately 5 km south of Norris Geyser Basin. There are eight main vents with major accumulations of iron oxides, 28+ minor seeps with lesser accumulations, and six vents in the river (Fig. 2-1). Most phototrophic mats populate the main vents and outflow channels, while chemotrophic communities occupy the areas around minor seeps that have cooled to ambient temperatures. In contrast to other silica and carbonate depositing springs that are surrounded by thick sinter deposits, the iron oxide precipitates at Chocolate Pots are flocculant and accumulate to only cm-scale thicknesses on outcrops of the 640,000-year-old Lava Creek ash-flow tuff member B (Christiansen, 2001). The Gibbon river has eroded through the tuff in this location, generating steep river banks from which the iron oxide deposits frequently slough.
Figure 2-1. Map of Chocolate Pots hot springs showing distribution of vents and extent of iron oxide deposits.
2.3.2 Water Chemistry

The temperature and geochemical composition of the anoxic vent fluids at Chocolate Pots hot springs have been documented since 1935 and have remained remarkably constant (Table 2-1) (Allen and Day, 1935; Rowe et al., 1973; Thompson and Yadav, 1979; Pierson and Parenteau, 2000). The slightly acidic pH (5.7 - 5.9) is due to the oxidation of H$_2$S to H$_2$SO$_4$ in subsurface hydrothermal system to generate acid-sulfate waters (Fournier, 1989). The high levels of Fe$^{2+}$ and Mn$^{2+}$ are likely generated by these acid-sulfate waters leaching iron from the rhyolite flows and welded tuffs. Chemical analyses of Lava Creek member B obsidian and devitrified densely welded tuff revealed FeO values of 0.17-.108 weight percent (Christiansen, 2001).

The chloride levels, which reflect the extent of water-rock interaction, are low (29 mg/L) compared to typical alkaline-chloride hot springs (~350 mg/L; Fournier, 1989), but are higher than typical steam-heated acid-sulfate boiling pools (1 mg/L; Fournier, 1989). These values suggest that the acid-sulfate waters are mixing with alkaline-chloride waters to generate acid-sulfate-chloride (ASC) springs, similar to the ones found in nearby Norris Geyser Basin (Inskeep and McDermott, 2005).

However, the pH at Chocolate Pots hot springs is not as acidic as the Norris iron-depositing springs (3.1; Inskeep and McDermott, 2005), which allows photosynthetic bacteria to flourish. Thermophilic cyanobacteria do not tolerate pH values below ~5, although at moderate temperatures eukaryotic algae can be found below pH 4 (Castenholz, 1988). Chocolate Pots is also unique in that the Fe$^{2+}$ levels
(5.5 mg/L) are approximately 10 times higher than at La Duke hot springs, another iron-depositing spring where phototrophs are found (Brown et al., 2005). The well-studied Octopus Spring contains 1.4-2.8 μg/L Fe$^{2+}$ in the vent waters (Stauffer et al., 1980).

Table 2-1. Geochemical composition of vent waters at Chocolate Pots hot springs.

<table>
<thead>
<tr>
<th>Species (mg/L)</th>
<th>Allen and Day, 1935</th>
<th>Rowe et al., 1973</th>
<th>Thompson and Yadav, 1979</th>
<th>Pierson and Parenteau, 2000</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp. (°C)</td>
<td>54</td>
<td>53</td>
<td>51</td>
<td>52.5</td>
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<tr>
<td>pH</td>
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<td>ND</td>
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<td>5.9</td>
<td>5.7</td>
</tr>
<tr>
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</tr>
<tr>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>248</td>
<td>ND</td>
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<td>21</td>
</tr>
<tr>
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<td>ND</td>
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<td>ND</td>
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<tr>
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<td>ND</td>
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</tr>
<tr>
<td>Nitrite nitrogen</td>
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<td>ND</td>
<td>ND</td>
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</tr>
<tr>
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<td>ND</td>
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<td>ND</td>
</tr>
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<td>159</td>
<td>141</td>
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<tr>
<td>Sodium</td>
<td>114</td>
<td>118</td>
<td>122</td>
<td>102</td>
<td>115</td>
</tr>
<tr>
<td>Sulfate</td>
<td>28</td>
<td>30</td>
<td>83</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>Sulfide</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not determined.
2.3.3 Distribution of Phototrophic Mats

Four types of phototrophic microbial mats grow on the iron deposits of Chocolate Pots hot springs. These mats are visually distinctive and have been observed to occur at the same locations and retain the same community composition since our group began working at the springs in 1992 (Pierson et al., 1999; Pierson and Parenteau, 2000; Trouwborst et al., in review). Our group previously identified the mat community members using in vivo pigment analyses, phase and epifluorescence microscopy (Pierson et al., 1999; Pierson and Parenteau, 2000) and phylogenetic analyses (unpublished data). In a study by Wade et al. (1999), cores of the iron deposit collected down an outflow channel for Mössbauer spectroscopy were found to contain encrusted bacterial filaments. However, the samples were not collected with relation to any mat community and therefore the identity, metabolic impact, and mineralization mechanism of the fossilized microbes could not be assessed.

Only two mat types were examined in this study. We compared the biosignatures forming in the higher temperature Synechococcus-Chloroflexus mat to the lower temperature narrow Oscillatoria mat. We also characterized two variants of the narrow Oscillatoria mat that experienced two distinct hydrodynamic flow regimes to assess how water flow affected biofabric formation.

The higher temperature Synechococcus-Chloroflexus mat (Table 2-2) was found (1) in the vent pool of the main iron deposit (Fig. 2-2); (2) in and approximately one meter down the outflow channel of the south satellite vent (Figs. 2-2, 2-3a); and (3) at two vents located farther south along the river bank, one located at the base of a
tree and one near the river.

Table 2-2. Geochemical and hydrologic conditions of water flowing over the phototrophic mats at Chocolate Pots hot springs.

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Fe²⁺ (mg/L)</th>
<th>%TC</th>
<th>%TOC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechococcus-Chloroflexus</em> mat</td>
<td>50-54</td>
<td>5.4-6.4</td>
<td>4.0-5.6</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Ferrihydrite underneath mat</td>
<td>50-54</td>
<td>5.4-6.4</td>
<td>4.0-5.6</td>
<td>0.8</td>
<td>0.2*</td>
</tr>
<tr>
<td>Narrow <em>Oscillatoria</em> mat terracette</td>
<td>39-46</td>
<td>7.3-7.7</td>
<td>0.6-1.3</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Narrow <em>Oscillatoria</em> mat channel</td>
<td>37-45</td>
<td>7.7-8</td>
<td>0.6-1.5</td>
<td>2.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Ferrihydrite MM Vent</td>
<td>51.8</td>
<td>5.6</td>
<td>5.7</td>
<td>0.7</td>
<td>0.3*</td>
</tr>
<tr>
<td>Ferrihydrite MM Traverse point 1</td>
<td>49.5</td>
<td>6.3</td>
<td>3.5</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Ferrihydrite MM Traverse point 2</td>
<td>46.3</td>
<td>6.7</td>
<td>1.9</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Ferrihydrite MM Traverse point 3</td>
<td>45.4</td>
<td>7.0</td>
<td>1.5</td>
<td>1.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* acid digest viscous

Figure 2-2. Distribution of three phototrophic mats examined in this study on main Chocolate Pots iron deposit on the east side of the river. The narrow *Oscillatoria* mat occurred in association with a red terracette structure on the steep face of the main iron deposit (A) and in the rapidly flowing vent outflow channels (B). The *Synechococcus-Chloroflexus* mat occurred at the south satellite vent and up to 1 m down the outflow channel (C).
Figure 2-3. Field and stereo microscope images of the three phototrophic mats examined in this study. All images are oriented so that water flow direction is from the top to the bottom. A) *Synechococcus-Chloroflexus* mat. B) Narrow *Oscillatoria* mat in outflow channel. C) Stereo microscope image of narrow *Oscillatoria* mat showing ridges that formed in the mat in the presence of rapid water flow. D) Narrow *Oscillatoria* mat in association with a red terracette structure on the steep face of the main iron deposit. Sample removed from center of image. E) Stereo microscope image of narrow *Oscillatoria* mat in terracette. Small botryoidal aggregates of ferrihydrite particles visible in the terracette pond (arrow).

The narrow *Oscillatoria* mat was widely distributed on the Chocolate Pots iron deposits. One variant occurred in association in a red terracette structure on the near-vertical face of the main iron deposit (Table 2-2; Figs. 2-2, 2-3d). The mat was bathed in a slow-moving, ~ 1 mm thin layer of water which we described as “sheet flow.” The other variant occurred in the lower reaches of the outflow channels on the main iron deposit and experienced rapid and continuous, voluminous flow (Table 2-2; Figs. 2-2, 2-3d).
To distinguish the two variants, we hereafter refer to them as the narrow Oscillatoria mat in the terracette and the narrow Oscillatoria mat in the channel.

Stereomicroscope images of the two hydrodynamic variants revealed two distinct iron-mineralized gross mat morphologies. The narrow Oscillatoria mat in the terracette appeared as a gelatinous light green material amongst botryoidal aggregates of iron oxide particles (Fig. 2-3c). The narrow Oscillatoria mat in the channel was much thicker and displayed ridges on the surface of the mat that were oriented with their axes parallel to the flow direction (Fig. 2-3c).

2.3.4 Minerals Associated with the Phototrophic Mats

Bulk random powder XRD spectra of the air-dried mats indicated that all of the mat types were mineralized by 2-line ferrihydrite with d-spacings of approximately 2.6 and 1.5 Å (Fig. 2-4). 2-Line ferrihydrite was also found in the surface deposits in the vents and outflow channels (data not shown).

SEM-EDS analyses of the mats revealed the presence of Si, Cl, and Fe in the minerals encrusting the cells. Slight variation in the Si:Fe ratio was attributed to slight variation in the interaction volume of the electron beam (~1 μm) with the sample: in some cases this volume likely extended beyond the filament or mineral aggregate being analyzed. Traces of Ca and Mn were detected in the narrow Oscillatoria mats, but not in the Synechococcus-Chloroflexus mat.

The association of trace amounts Mn with the narrow Oscillatoria mats can be linked to pH changes due to photosynthetic activity and hydrodynamics. In general,
pH affects the rate of Fe$^{2+}$ and Mn$^{2+}$ oxidation; above pH $\sim$5.8 Fe$^{2+}$ oxidation is greatly enhanced, while above pH $\sim$7.8 Mn$^{2+}$ oxidation is greatly enhanced (Stumm and Morgan, 1996). During photosynthesis, the pH in the pore spaces of mats increases due to photosynthetic CO$_2$ consumption (Pierson et al., 1999). Microelectrode measurements of the pH in the narrow *Oscillatoria* mat in the channel reveal values as high as 9, thus enhancing Mn$^{2+}$ oxidation (Pierson et al., 1999). The pH values in the narrow *Oscillatoria* mat in the terracettes also reflect photosynthetic CO$_2$ consumption, but we found that the thin sheet flow over the mats enhances CO$_2$ outgassing, which also drives up the pH. The thin *Synechococcus-Chloroflexus* mats do not build up a pH gradient because they are continually flushed by the low pH source water (Trouwborst et al., in review); consequently no Mn is found in the iron oxide minerals encrusting the cells.

![Figure 2-4](image)

Figure 2-4. Bulk random powder XRD spectra of air-dried iron-mineralized mat samples. The broad amorphous peaks of 2-line ferrihydrite are visible at 2.6 and 1.5 Å. Spectra were offset by -10 to 30 counts/s to fit on the same graph. A) narrow *Oscillatoria* mat in terracette. B) *Synechococcus-Chloroflexus* mat. C) narrow *Oscillatoria* mat in outflow channel.
2.3.5 Carbon Content

The total carbon (TC) and total organic carbon (TOC) content of the microbial mats ranged from 2.9 - 0.8 wt% and 2.2 - 0.4 wt%, respectively (Table 2-2). Use of EDTA as an internal standard revealed that there was 0.3 - 2.2 % error in the analyses. The TOC values were low and reflected the extent of mineralization of the mats. The narrow *Oscillatoria* mat embedded in the terracettes was the most mineralized and contained the lowest TOC (0.4 wt%). For comparison, the relatively unmineralized floating streamers of the *Pseudanabaena* mat contained 28.8 wt% TOC. There was a loss of organic carbon with burial of the mats. The *Synechococcus-Chloroflexus* mat contained 1.3 wt% TOC, whereas the iron oxide underneath the mat contained 0.2 wt% TOC, representing a 85% loss of organic carbon over a scale of approximately two centimeters.

The low organic carbon content of well-preserved, very low-grade metamorphosed BIFs is thought to have precluded microbial involvement in their deposition (Klein, 2005). The siderite-rich BIFs in the Transvaal Supergroup contain 0.041 - 0.203 wt% organic carbon, while the oxide-rich BIFs contain 0.008 – 0.017 wt% organic carbon (Buekes et al., 1990). Kaufman et al. (1990) reported organic carbon values of 0.032 – 0.108 wt% in the quartz-magnetite-hematite-siderite-greenalite-stilpnomelane assemblage of the Dales Gorge Member of the Hamersley Range. Surprisingly, the most heavily iron-mineralized mats at Chocolate Pots fall within the upper end of the range of these well-preserved BIF sequences. Because the mats were immediately frozen in the field to prevent degradation of organic carbon,
the reported values can be considered 100%. The inevitable further loss of organic carbon upon diagenesis (as was evidenced by the 85% loss of organic carbon upon burial of the *Synechococcus-Chloroflexus* mat) would lower the organic carbon contents of the mats further within the range reported for BIFs. Such a study suggests that the relatively low organic carbon contents of well-preserved BIFs do not preclude phototrophs from having played a role in their deposition.

2.3.6 Biofabrics

2.3.6.1 *Synechococcus-Chloroflexus* mat

A cohesive microbial mat ~0.2-2 mm thick formed on top of the ferrihydrite sediments (Figs. 2-3a, 2-5a). Rods of *Synechococcus* sp. (1.5 μm diameter) and ovoid to coccoid cells of the cyanobacterium *Cyanothece minervae* (3-4 μm diameter) dominated the uppermost unmineralized surface of the mat (Figs. 2-5c, d). A few filaments of motile *Chloroflexus* sp. (0.6-0.7 μm diameter) and other filamentous anoxygenic phototrophs (FAPs) (~1.5 μm diameter) were also visible at the mat surface and their proportion increased with depth. These organisms were identified in a previous ecological and physiological study of the mats (Pierson et al., 1999; Pierson and Parenteau, 2000).

Mineral precipitates were observed just beneath the mat surface as iron-silica colloids that encrusted the rods, filaments, and strands of mucilage (Fig. 2-5e). No preferential encrustation of the cells by colloids could be discerned, though it was easier to identify mineralized *Chloroflexus* filaments because of their increasing
abundance with depth and because the morphology of the rods and cocci became obscured by the iron-silica colloids.

Figure 2-5. Biofabric of higher temperature *Synechococcus-Chloroflexus* mat. A) Transverse vertical section showing dark, cohesive mat on ferrihydrite sediments. B) Ferrihydrite aggregates (arrows) trapped in biofabric of air-dried mat sample. C) Surface of mat composed mainly of rods of *Synechococcus* (S.) and a few filaments of *Chloroflexus* (C.) and other bacteriochlorophyll-containing filamentous anoxygenic phototrophs (FAP). D) Higher magnification of mat surface. E, F) Mat fabric becoming progressively encrusted with iron-silica colloids.

In the lowermost section of the mat, just above the iron deposit, the iron-silica colloids began to coalesce and infill the pore spaces between the encrusted cells and strands of mucilage (Fig. 2-5f). Though it was more difficult to identify cells in heavily mineralized sections of the mat, *Chloroflexus* filaments could still be recognized because of their elongate nature.

While most stromatolitic biofabrics have been associated with gliding filamentous microorganisms, it appears that the motile *Synechococcus* rods at Chocolate Pots hot springs also contribute to the formation of such structures.
Large aggregates of ferrihydrite occasionally became entrained in the mat (Fig. 2-5b). This is most likely due to the flocculation of precipitates that had formed upstream. The entrainment of these aggregates at depth within the mat indicates that the cells were able to reestablish colonies on the surface of these aggregates and generate a successive mat layer. Motility has been demonstrated in thermophilic Synechococcus sp. (0.1-0.3 μm/s) (Ramsing et al., 1997) and Chloroflexus aurantiacus (0.01–0.04 μm/sec) (Pierson and Castenholz, 1974) and is a likely mechanism to escape the reduced light intensity due to the accumulation of ferrihydrite aggregates on the surface of the mats.

2.3.6.2 Narrow Oscillatoria mat in terracette

The narrow Oscillatoria sp. filaments (1.5 μm diameter) formed a thin (~5-30 μm) mat in the ponds of the terracettes on the steep face of the main iron deposit (Figs. 2-3d, e). In general, the motile filaments were oriented parallel to the water flow direction and were distributed between protruding botryoidal aggregates of ferrihydrite particles in the terracette ponds (Figs. 2-6a, b). The thin gelatinous mat contained abundant strands of mucilage. Occasionally rods of Synechococcus were found on the mat surface (Fig. 2-6b).

The diameter of the ferrihydrite aggregates in the terracette ponds ranged from 70-200 μm when measured in air-dried samples, and from 10-30 μm in glutaraldehyde-fixed mat samples. The difference in particle size resulted from the filaments obscuring the edges of the structures in the fixed mat samples. The
ferrihydrite aggregates were observed to occasionally group together into larger cauliflower-shaped aggregates that measured 400-500 μm in diameter.

Figure 2-6. Biofabric of narrow Oscillatoria mat in terracette. A) Looking down on surface of air-dried mat sample that was composed of numerous small botryoidal aggregates of ferrihydrite particles (arrow). These were similar to those in the field photo in Fig. 2-2e. Pond lip visible on left. B) Ferrihydrite aggregates in preserved mat sample. Filaments are narrow Oscillatoria (n.O.) and rods are Synechococcus. C) Transverse section of preserved mat revealed that ferrihydrite aggregates displayed a dendritic-like structure (arrow). D) Transverse cut of deeper layers of air-dried mat demonstrated that the dendritic-like structures persisted with depth (arrow). E) Surface of air-dried mat in (A) where structures became top heavy, causing them to break off at their base (arrow). F) Close-up of base of structure (arrow in E) revealed the presence of an encrusted filament that had degraded, leaving an empty microfossil. G) Less well-developed structures broken off at base also revealed filament encrustation (arrow). H) These "proto-"dendritic-like structures (arrows) were more fine-grained than well-developed structures. I) Proto-structures (arrows) were visible in the top of the ferrihydrite aggregate from (A).
A transverse section of the narrow Oscillatoria mat in a terracette revealed that the ferrihydrite aggregates were actually the tops of dendritic-like structures (Fig. 2-6c). The diameter of the widest part of the structures ranged from 20-120 μm, but most were 20-40 μm. Strands of mucilage and horizontally oriented encrusted narrow Oscillatoria filaments were wrapped around and between the dendritic-like structures.

These structures, which formed discrete layers up to 1 mm thick, were observed up to 3 mm below the surface of the thin mat (Fig. 2-6d). The discrete layers were delineated by dense, randomly oriented aggregates of ferrihydrite particles and encrusted cells that capped the tops and bottoms of the structures (Fig. 2-6d). The deeper layers of the dendritic-like structures were not infilled with mineral precipitates or other detrital material, which allowed the fabric to remain visible (Fig. 2-6d).

Examination of a dendritic-like structure broken off at its base revealed that it formed by encrustation on a single vertically oriented narrow Oscillatoria filament, though the cell appeared to have fully degraded (Figs. 2-6e, f). The inner diameter of the resulting microfossil of the filament (~1.2 μm) approximated the measured diameter of a live sheathed narrow Oscillatoria filament (~1.5 μm).

Various stages of development of the dendritic-like structures were observed. "Proto"-dendritic-like structures were more fine-grained and growth began at one end of the encrusted filament as an enlarged club (Fig. 2-6h). As mineral precipitation proceeded, botryoidal aggregates of ferrihydrite particles were visible on the tops and sides of the structures (Fig. 2-6g). The structures continued to grow upward and outward and began to bifurcate (Figs. 2-6c, d, e). Examination of the bottoms of
exposed dendritic-like structures in all stages of development revealed encrustation on narrow *Oscillatoria* filaments (Figs. 2-6g, h). These structures were only observed to be associated with the narrow *Oscillatoria* mats in the terracettes.

It does not appear that the cyanobacteria played a role in the formation of the terracettes. These structures are commonly observed on inclines in thermal spring outflows and appear to be related to the hydrodynamics of the thin sheet flow on a slope (e.g., Jamtveit et al., 2005). However, it does appear that the cyanobacteria were responsible for the distinct dendritic biofabric forming in this iron deposit. Our SEM analysis revealed that this biofabric formed as a result of ferrihydrite encrustation on vertically oriented narrow *Oscillatoria* filaments as shown in Fig. 2-7. Three stages of dendritic-like structure growth were documented. Primary encrustation on the filaments, which was fine-grained and produced a smooth surface, manifested itself as an enlarged club on the terminal end of the vertical filament. Secondary encrustation entailed the growth of botryoidal aggregates of ferrihydrite particles on top of the smooth primary encrustation with most of the growth concentrated on the enlarged terminal end. Tertiary encrustation led to continued upward growth and eventual bifurcation of the structure. The predominantly upward growth of these structures likely occurred via evaporative wicking of the hydrothermal fluid up the filament and deposition of ferrihydrite at the terminal end of the filament at the air/water interface. Once encrusted, the light necessary to sustain photosynthesis was effectively blocked, so the filaments died and degraded, leaving empty external molds. The dendritic-like structures were not infilled at least 3 mm below the surface of the mat. Though the
formation of manganese and iron-rich structures in thermal travertine deposits, termed arborescent shrubs, appear visually similar at the SEM scale, they were populated by densely packed rods and cocci (Chafetz et al., 1998) rather than by mineralization of individual filaments as observed in our study.

![Diagram of morphogenesis model of dendritic-like structures in narrow Oscillatoria mat in terracette.](image)

Figure 2-7. Morphogenesis model of dendritic-like structures in narrow Oscillatoria mat in terracette. A) Unencrusted narrow Oscillatoria filament displaying a characteristic hooked end. B) Fine-grained primary (1°) encrustation on the filament. C) Secondary (2°) encrustation and growth of botryoidal aggregates of ferrihydrite particles on the sides and top of structure. D) Tertiary (3°) encrustation and growth leading to bifurcation of the structure. Illustration roughly to scale of measured averages of dendritic-like structures.

2.3.6.3 Narrow Oscillatoria mat in channel

The mat growing in the outflow channel was found to be up to 1 cm thick (Fig. 2-3b). It was dominated by motile narrow Oscillatoria filaments (1.5 μm diameter), although rods of Synechococcus and ovoid-to-coccoid cells of C. minervae as well as a few spirochetes were occasionally observed throughout the mat. The gelatinous mat
contained abundant strands of mucilage that bound the cells and loose ferrihydrite aggregates together.

Ridges formed on the surface of the mat, and their axes were oriented roughly parallel to the direction of the water flow (Figs. 2-3c, 2-8a). Narrow Oscillatoria filaments on the top of the ridges were generally oriented parallel to the flow direction as well (Figs. 2-8b, c). Occasionally the filaments appeared as a meshwork on the ridges and in the troughs.

Ferrihydrite encrusted the narrow Oscillatoria filaments located just beneath the surface of the mat (Figs. 2-4, 2-8). The encrustation tended to preserve the
orientation of the filaments, whether they were parallel (Fig. 2-8c) or in a meshwork (Figs. 2-8e, f). In deeper layers of the mat, the mineralized meshwork of filaments was not infilled and it was still visible 0.2-0.3 cm underneath the surface of the mat. In other areas, the meshwork became totally obscured as more ferrihydrite precipitated.

In addition to the observed encrustation, the mat was further mineralized by abundant detrital ferrihydrite. This detritus was deposited on the surface of the mat and was likely generated from an upstream disturbance of the flocculant ferrihydrite. Some motile narrow Oscillatoria filaments were observed to glide up onto the ferrihydrite aggregates to escape the reduced light intensity, while others remained trapped beneath the ferrihydrite layer. The aggregates were effectively attached to the surface of the mat by the gliding filaments. In this way, the motility of the narrow Oscillatoria filaments played a definitive role in the formation of a laminated biofabric. Our SEM observations are in agreement with the observations made using confocal laser scanning microscopy (CLSM) and the mat morphogenesis model suggested by Pierson and Parenteau (2000).

2.3.7 Microfossils

2.3.7.1 Synechococcus-Chloroflexus mat

Analytical SEM examination of the Synechococcus-Chloroflexus mat revealed that the cells were encrusted by colloids (Fig. 2-9) composed of iron and silica. The size of these colloids ranged from 0.1-1 μm in diameter, but most were 0.2-0.5 μm.
Figure 2-9. Microfossils forming in higher temperature *Synechococcus-Chloroflexus* mat. A) Iron-silica colloids beginning to coat *Chloroflexus* filament (C.); *Synechococcus* (S.) and *Cyanothece minervae* (C.m.) rods also present. B) *Chloroflexus* filament becoming progressively more encrusted with iron-silica colloids. C) Nearly fully encrusted *Chloroflexus* filament.
Chloroflexus filaments were observed to become progressively encrusted with these colloids, which eventually coalesced and encased the entire filament and preserved the gross morphology of the cell (Figs. 2-9b, c). The cells appeared to degrade during partial and full encrustation, leaving an external mold of the filaments. This phenomenon was also observed on the rods of Synechococcus and ovoid-to-coccoid cells of C. minervae, though it was more difficult to distinguish these cells because the coalescing colloids obscured their morphology.

TEM examination of the mat revealed distinctive ultrastructural features of the community members. Transverse sections of C. minervae cells revealed a characteristic arrangement of the photosynthetic membranes. As shown in Fig. 2-10d, stacks of thylakoids were arranged in a triangular orientation around the center of the cell and along the periphery of the cell wall, which allowed for clear identification of the organism.

Deeper in the mat, these cells were permineralized by iron oxides. The cell walls retained a high degree of integrity, and in some cases there was excellent preservation of the thylakoid membranes (Fig. 2-10c). The ability to identify C. minervae was possible due to the excellent preservation of the characteristic triangular orientation of the photosynthetic membranes in partially and fully permineralized cells (Figs. 2-10c, d). Although it appeared that shrinkage of the membranes had occurred, the triangular orientation was still recognizable. Permineralized Chloroflexus filaments were identified on the basis of size comparisons of the diameter of the permineralized cell to those of unmineralized cells (Fig. 2-10d). field of cells
Figure 2-10. TEM micrographs of narrow *Oscillatoria* mat in outflow channel (A, B) and *Synechococcus-Chloroflexus* mat (C, D). A) Oblique cross section of *Cyanothecae minervae* cell containing ferrihydrite in periplasmic space (arrows) and thylakoid membranes (T). Inset image displays ferrihydrite (Fe) in periplasmic space and S-layer (S). B) *Cyanothecae minervae* cell containing periplasmic ferrihydrite (arrow) and secondary ferrihydrite encrustation exterior to the outer membrane. C) Incomplete permineralization of *Cyanothecae minervae* cell showing excellent preservation of thylakoid membranes (T). D) Field of cells completely permineralized with 2-line ferrihydrite (see Fig. 2-10). Thylakoid membranes (T) of *Cyanothecae minervae* (C.m.) visible in center permineralized cell, allowing positive identification by comparison to adjacent unmineralized cell. Identification of *Chloroflexus* sp. (C.) was based on size comparisons as the chlorosomes weren’t visible.
completely infiltrated with minerals in Fig. 2-10d. Typically *Chloroflexus* is identified by chlorosomes lining the cell wall, but their small size makes them difficult to capture in an ultramicrotomed section of the cell.

Selected area electron diffraction (SAED) patterns of the mineralized thylakoid membranes in Fig. 2-10c were dominated by the high intensity of the transmitted beam and the amorphous carbon in the resin, but the secondary ring of 2-line ferrihydrite was identified at 1.5 Å (see also Greffié et al., 2001). 2-Line ferrihydrite can be distinguished from amorphous carbon because of the narrow sharp character of its electron diffraction ring patterns as opposed to the broad, diffuse rings produced by amorphous carbon (Janney et al., 2000; Mavrocordatos and Fortin, 2002).

SAED of the field of cells completely infiltrated with minerals in Fig. 2-10d revealed 2-line ferrihydrite and another faint ring at 2.25 Å, which was attributed to more ordered 6-line ferrihydrite (Fig. 2-11d). Permineralization of single cells and clusters of cells was often observed in the mat, but was not uniform throughout it.

In general, TEM micrographs of the *C. minervae* cells in the *Synechococcus-Chloroflexus* mat revealed excellent preservation of the characteristic photosynthetic membranes. This allowed for positive identification of the cells and documentation of several time steps during permineralization. As the mineralizing fluid precipitated intracellularly, the photosynthetic thylakoid membranes retained their characteristic architecture and appear to have acted as scaffolding that held the cell together. Full permineralization of the cell appeared to have occurred without cell lysis due to the high degree of fidelity displayed by the cell walls, regardless of the cell orientation in
the TEM thin section. It has been suggested, though not proven, that intracellular permineralization occurs via cell lysis in silica mineralizing ecosystems (e.g., Konhauser et al., 2003).

Figure 2-11. Selected area electron diffraction (SAED) patterns of mineralized cells in the phototrophic mats. A) Broad, diffuse rings of amorphous carbon in resin. These rings partially obscured the SAED patterns of amorphous iron minerals. B) SAED of minerals encrusting a cell in the *Synechococcus-Chloroflexus* mat. The amorphous rings have d-spacings of ~2.5 and 1.5 Å, which correspond to 2-line ferrihydrite. C) SAED of permineralized thylakoid membranes in Fig. 2-10c. Amorphous carbon in the resin obscured all but the 1.5 Å ring of 2-line ferrihydrite. D) SAED of permineralized cells in the *Synechococcus-Chloroflexus* mat. 2-Line ferrihydrite is present, as well as a ring at 2.25 Å, which can be attributed to the more ordered 6-line ferrihydrite.

2.3.7.2 Narrow Oscillatoria *mat in terracette*

Examination of microfossils in the narrow *Oscillatoria* mat in the terracettes with analytical SEM and XRD revealed that the cells were encrusted by 2-line ferrihydrite with appreciable amounts of silica (Figs. 2-4, 2-12). The size of the
aggregates of ferrihydrite particles initially encrusting the filaments was 25-50 nm in diameter (Fig. 2-12a). The size of ferrihydrite aggregates on the interior of hollow microfossils measured 30-50 nm in diameter, with some as large as 100 nm on the exterior surfaces (Figs. 2-6f, 2-12b).

We observed successive stages of encrustation on the filaments within the mat. The initial thin encrustation rind, which outlined the morphology of the cell, was visible in air-dried samples like the one shown in Fig. 2-12a. The cellular material appears to have degraded once the encrustation rind thickened, leading to the formation of hollow microfossils like those observed at the bottoms of the dendritic-like structures (Figs. 2-6f, 2-12b). Where ferrihydrite continued to precipitate onto the microfossils, secondary growth along the length of the filaments occurred (Fig. 2-12c). Microfossils of *C. minervae* were also present, though they were more difficult to distinguish with an SEM than the narrow *Oscillatoria* filaments (Fig. 2-12d). When encrustation of the cell was not complete prior to the sample becoming dessicated during air-drying, the remainder of the cell collapsed and only a partial microfossil was visible.
Figure 2-12. Microfossils forming in narrow Oscillatoria mat in red terracettes on steep face of main iron deposit. A) Air-dried sample in Fig. 2-6a showing a very thin layer of ferrihydrite coating surface of narrow Oscillatoria filaments. B) Base of proto-dendritic-like structure in Fig. 2-5h revealing empty microfossil. C) Secondary ferrhydrite precipitation along the length of narrow Oscillatoria (n.O.) filament in preserved mat sample. S., Synechococcus. D) Air-dried sample of partially encrusted Cyanothece minervae cell. Organic matrix (arrow) is dehydrated and collapsed cell.
2.3.7.3 Narrow Oscillatoria mat in channel

TEM examination of the narrow Oscillatoria mat in the channel revealed that in addition to the narrow Oscillatoria filaments, cells of C. minervae were also present. This is the same cyanobacterium that was detected in the Synechococcus-Chloroflexus mat.

The cell wall of these organisms displayed characteristics of both Gram – and Gram + cells and was composed of an S-layer, sheath, outer membrane, peptidoglycan layer, and cell membrane (Fig. 2-10a) (e.g., Hoiczyk and Hansel, 2000; Gromov et al., 1986). Though the thickness of the S-layer and sheath material was observed to vary from ~50nm to ~100 nm in our specimens, the thickness of the peptidoglycan layer (12-13 nm) did not. Large polyhedral carboxysomes, which displayed a granular texture, were found in the cells (Fig. 2-10a). We observed that 30-40% of cells contained carboxysomes of various sizes.

Our TEM investigation revealed that mineral grains were deposited within the periplasmic space between the peptidoglycan layer and the outer membrane of the C. minervae cells (Fig. 2-10a). When the diameter of the mineral grains increased, the peptidoglycan layer tended to become compressed against the cell membrane, and the outer membrane occasionally was distended. We could not obtain SAED patterns using conventional TEM analysis from individual grains within the periplasmic space; however, we observed variation in the contrast of these grains, which could be caused by differences in grain thickness, crystallinity, or orientation. SAED patterns produced by several grains within the periplasmic space indicated the presence of 2-
line ferrihydrite. However, the faint intensity of the ring patterns produced only by the
grains in the periplasmic space was obscured by the diffuse rings produced by the
amorphous carbon in the resin and the high intensity of the transmitted beam (data not
shown). Periplasmic precipitation of ferrihydrite was not observed in C. minervae
cells in the Synechococcus-Chloroflexus mat.

We also observed that the thylakoid membranes were well preserved during
the initial stages of permineralization and encrustation. Figure 10b revealed that
ferrihydrite permineralization was initiated within the periplasmic space of C.
minervae cell walls (Fig. 2-3), while encrustation appeared to be initiated in the S-
layer of the microbes. Schultze-Lam et al. (1992) demonstrated that the S-layer of
freshwater Synechococcus acts as a template for mineral precipitation by providing
discrete, regularly arranged nucleation sites for the critical initial events in the
mineralization process.

2.3.8 Iron Preservation of Phototrophic Cells

Although permineralization by silica is considered to result in microfossils
with the highest cellular fidelity, our investigation of the preservation of phototrophic
cells at Chocolate Pots hot springs suggests that iron permineralization may also result
in exceptionally well-preserved microfossils. We found that the cyanobacterial cells
retained taxonomic features that allowed for positive identification to the genus and
sometimes species level. This is in contrast to previous studies of iron mineralized
microbial mats in subaerial thermal springs which demonstrated poor preservation of
microbes by iron (Konhauser and Ferris, 1996; Reysenbach et al., 1999; Konhauser et al., 2003).

Our TEM observation that the cellular and thylakoid membranes retained a high degree of integrity upon iron mineralization indicates that persistent preservation during the earliest stages of fossilization is favored in this unique high iron system. In a related study examining the production and fate of lipid biomarkers in these mats, we found that membrane-associated cyanobacterial fatty acids survived microbial degradative processes to persist in the ferrihydrite underneath the mats (Chapter 3). These fatty acids are typically rapidly degraded after cell death (Harwood and Russell, 1984). However, it has been suggested that iron may inhibit cellular autolytic enzymes (Herbold and Glaser, 1975; Leduc et al., 1982), which would lead to the enhanced preservation of iron-mineralized cells.

We have shown via X-ray and electron diffraction that 2-line, and possibly 6-line, ferrihydrite encrust and permineralize the cells. The formation of ferrihydrite is favored in systems where Fe$^{2+}$ is rapidly oxidized or Fe$^{3+}$ is rapidly hydrolyzed (Cornell and Schwertmann, 2003). No other diagenetic phases were detected in the mats or surface deposits despite the steep oxygen and pH gradients that occur in the mats and the continuous supply of Fe$^{2+}$ in the vent waters. SEM-EDS analyses revealed an abundance of Si in the mineral-encrusted cells. It is possible that the relatively high concentration of silica in the vent waters (~150 mg/L) may stabilize the ferrihydrite by blocking surface nucleation sites, a mechanism that would prevent its transformation to more ordered forms (e.g., Carlson and Schwertmann, 1981;
Schwertmann et al., 1984; Cornell et al., 1987; Zhao et al., 1994). Such a process may enhance the period of time during which the primary biosignatures may survive and, in effect, delay diagenetic phase transformations, though the time scale of this process is not known. Although Wade et al. (1999) identified the diagenetic phases goethite, hematite, siderite, and nontronite in lithified cores, these phases were not detected during this study because we focused on the primary precipitates in the vents, outflow channels, and microbial mats.

2.3.9 Paleoecological and Paleobiological Significance

Chocolate Pots hot springs, a subaerial thermal spring, is not a suitable structural model for deposition of Precambrian BIFs in deep ocean basins or shallow platformal areas. However, this hot spring ecosystem with anoxic vent waters high in Fe$^{2+}$ can provide insight into the possible roles of cyanobacteria and filamentous anoxygenic phototrophs (Chlorolectus sp.) in the oxidation of Fe$^{2+}$, iron biomineralization, and biosignature formation and preservation.

Our group has observed the impact of oxygenic photosynthesis on Fe$^{2+}$ oxidation, distinct from the influence of atmospheric O$_2$ and anoxygenic photosynthesis (Pierson et al., 1999; Trouwborst et al., in review). Trouwborst et al. (in review) have calculated Fe$^{2+}$ oxidation rates per cyanobacterial cell and related them to Fe$^{2+}$ oxidation requirements for BIF deposition. Comparison of the in situ Fe$^{2+}$ oxidation rates to those calculated by Konhauser et al. (2002) demonstrates a significant difference in the efficiency of oxic vs. anoxic oxidation. Based on a
deposition rate of $4.53 \times 10^{12}$ moles of iron per year needed to form an annual varve in the Hamersley Basin BIF, Trouwborst et al. (in review) calculated that $2.3 \times 10^{21}$ cyanobacterial cells with an annual iron oxidation rate of $2.35 \times 10^9$ moles of iron per cell would be needed. This number is two orders of magnitude less than the $5.7 \times 10^{23}$ cells of the purple anoxygenic phototroph and $4.3 \times 10^{23}$ cells of chemolithotrophs needed to oxidize the same amount of iron (Konhauser et al., 2002). The *in situ* studies at Chocolate Pots hot springs confirm that localized cyanobacterial oxygen production is a biologically efficient way to oxidize ferrous iron in the deposition of Precambrian BIFs, as was suggested by Cloud (1965, 1973).

Our group's *in situ* geochemical modeling of oxidation of Fe$^{2+}$ by cyanobacterial oxygenic photosynthesis lends support to the Cloud hypothesis. However, there must be unequivocal paleontological evidence to support a bacterial role in BIF deposition, whether by cyanobacteria, purple and green anoxygenic phototrophs, or chemolithotrophs. Currently there is more paleontological evidence to support a cyanobacterial role. The lack of microfossils in the iron-rich layers and, thus, the lack of a necessary spatial relationship between the microfossils and the iron mineral assemblages led Klein (2005) to conclude that the anoxygenic phototrophs and chemolithotrophs did not directly oxidize Fe$^{2+}$ and mediate the deposition of BIFs. However, he stated that this did not exclude the possibility that oxygen produced by ancestral cyanobacteria, as hypothesized by Cloud (1968, 1972, 1973, 1983), could have provided the oxidizing power necessary for ferric oxide formation. Cyanobacteria are free from this spatial constraint as the photosynthetically produced
oxygen can conceivably locally diffuse into a nearby basin to oxidize Fe$^{2+}$. Nearly all microfossils are found in bands of chert (Barghoorn and Tyler, 1965; Walter et al., 1976; Knoll and Simonson, 1980; Klein et al., 1987), and some have been identified as cyanobacteria (Barghoorn and Tyler, 1965).

The role of cyanobacteria in BIF deposition is supported by another class of microbial biosignatures: lipid biomarkers. Brocks et al. (1999) detected the hydrocarbon derivative (2-methylhopanoid) of the cyanobacterial biomarker 2-methylbacteriohopanepolyol (Summons et al., 1999) in the 2.7 to 2.5 Ga shales of the Hamersley Basin in the Pilbara Craton. These shales were interbedded with the oxide facies BIFs in the Hamersley Group, which suggests that formation of the oxide facies was due to oxygen produced by cyanobacteria (Brocks et al., 1999). We have also found 2-methylbacteriohopanepolyol in the cyanobacterial mats at Chocolate Pots hot springs (Chapter 3), a site where cyanobacteria have been shown to mediate Fe$^{2+}$ oxidation (Trouwborst et al., in review).

Our recent findings regarding the excellent preservation of cells by iron, while detected in cyanobacteria, may renew the search for iron permineralized cells in the iron-rich layers in BIFs, no matter the organism. While Klein (2005) did state that there is a distinct lack of microfossils in the iron layers, in a review of the paleontology of BIFs, Walter and Hoffman (1983) suggest that there is paucity of paleontological and geochemical research to arrive at a convincing interpretation of their origin.
While our group's interdisciplinary *in situ* studies have found support for a cyanobacterial role in BIF deposition, Trouwborst et al. (in review) point out that it is likely that no single mechanism, abiotic or biotic, completely explains the deposition of all BIFs, or even any particular formation. What our study does offer is a suite of microbial biosignatures that provides a framework for deciphering biogenic roles in ferric oxide deposition in the sedimentary rock record (e.g., Precambrian BIFs).

2.4 Acknowledgments

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3.1 Introduction

Banded Iron Formations (BIFs) are widespread Precambrian sedimentary deposits, the least metamorphosed of which are composed of chert, magnetite, hematite, carbonates, greenalite, stilpnomelane, riebeckite and, locally, pyrite (Klein, 2005, and references therein). Major occurrences deposited during the late Archean (2.7-2.5 Ga) and early Proterozoic (2.5-1.9 Ga) accumulated in deep ocean basins or shallow platformal areas with inputs of reduced iron (Fe$^{2+}$) and silica (SiO$_2$) from deep ocean hydrothermal activity (e.g., Fryer, 1983; Klein & Beukes, 1989; Beukes & Klein, 1992; Morris, 1993).
Debate exists regarding whether abiotic or biotic mechanisms were responsible for the oxidation of Fe\(^{2+}\) and the subsequent accumulation of ferric (Fe\(^{3+}\)) iron assemblages in BIFs. Several possibilities have been set forth. The higher UV flux present during the Precambrian could have resulted in the abiotic oxidation of iron, either directly through photo-oxidation of dissolved iron or indirectly by the photodissociation of atmospheric water vapor (e.g., Cairns-Smith, 1978; Braterman et al., 1983). Biotic iron oxidation could have occurred indirectly due to photosynthetic production of oxygen by cyanobacteria. Indeed, the abundance of ferric iron in BIFs was suggested by Cloud (1965, 1973) to signal the origin of oxygenic photosynthesis and the resulting oxygenation of the atmosphere.

Other metabolic types could have directly oxidized Fe\(^{2+}\) and precipitated the primary iron oxides that likely accumulated during BIF formation. Modern purple and green phototrophs oxidize Fe\(^{2+}\) in a type of anoxygenic photosynthesis known as photoferrotrophy (e.g., Widdel et al., 1993; Ehrenreich & Widdel, 1994; Heising & Schink, 1998; Heising et al., 1999; Straub et al., 1999). Chemolithotrophic bacteria also oxidize Fe\(^{2+}\) directly and use the redox energy generated during the process for growth (e.g., Hallbeck & Pedersen, 1991; Ehrlich, 1996; Emerson & Moyer, 1997; Straub et al., 1996; Straub et al., 2004).

Evidence for a microbial role in the deposition of BIFs has been sought in the occurrence of lipid biomarkers in the deposits. Lipid biomarkers can link modern microbial ecosystems to the geological record, and this type of fossil evidence can be used to infer the paleobiological role of microbes and paleoenvironmental conditions.
Lipid biomarkers have been particularly instrumental in advancing the hypothesis that cyanobacteria played a role in Precambrian Fe\(^{2+}\) oxidation. In a survey of cyanobacterial cultures and mats, Summons et al. (1999) determined that 2-methylbacteriohopanopolyol is a biomarker for cyanobacteria and oxygenic photosynthesis. Brocks et al. (1999) detected the hydrocarbon derivatives of these compounds (2-methylhopanoids) in the 2.7 to 2.5 Ga shales of the Hamersley Basin in the Pilbara Craton. These shales were interbedded with the oxide facies BIFs in the Hamersley Group, which suggests that formation of the oxide facies was due to oxygen produced by cyanobacteria (Brocks et al., 1999). The Hamersley Basin biomarkers also indicate that oxygenic photosynthesis evolved by 2.7 Ga (Brocks et al., 1999; Summons et al., 1999), well before the oxygenation of the atmosphere at 2.3 Ga (Kasting, 2001).

Since 1992, our group has investigated the classical scenario of Fe\(^{2+}\) oxidation by cyanobacteria and the formation of an iron deposit at Chocolate Pots hot springs in Yellowstone National Park. Extensive microelectrode analyses demonstrated that cyanobacterial oxygen production mediated the oxidation of Fe\(^{2+}\) in the anoxic waters, and carbon-14 uptake experiments revealed a stimulation of photosynthesis in the presence of Fe\(^{2+}\) (Pierson et al., 1999; Trouwborst et al., in review). Confocal laser scanning microscopy revealed that the phototrophs played a physical role in the formation of the iron deposit by binding and trapping iron minerals (Pierson and Parenteau, 2000).
Chocolate Pots hot springs is not a suitable model for the deposition of Precambrian BIFs. However, the cyanobacterial mats in the anoxic vent waters may be a reasonable geochemical model for the oxidation of Fe$^{2+}$ by oxygenic photosynthesis in mats or the water column in an ancient anoxic world (Trouwborst et al., in review). The springs also provide a setting in which to examine the formation and diagenesis of primary Fe$^{3+}$ precipitates, iron biomineralization, and biosignature formation.

We are currently investigating biosignature formation in the modern iron-mineralized phototrophic mats with the aim of determining the maximum amount of paleobiological and paleoenvironmental information that survives microbial degradative processes in these mats and the earliest stages of diagenesis in the iron oxide deposits underneath the mats. Specifically, we are using lipid biomarkers to (1) describe the community structure of the phototrophic mats and (2) determine how lithification by 2-line ferrihydrite, the primary iron phase of the sinter, affects the biomarker signature of the communities.

3.2 Methods

3.2.1 Field Measurements

The temperature of water flowing over the microbial mats was measured with a Fluke Model 52 K/J thermometer (Everett, WA). The pH was measured with a
Fisher AccuTupH probe attached to an Accumet AP72 meter (San Diego, CA). The aqueous Fe$^{2+}$ concentration was measured using the colorimetric ferrozine assay (Pierson et al., 1999) with a Hach DR/2400 portable spectrophotometer. To avoid Fe$^{2+}$ oxidation, samples were not filtered prior to analysis and were analyzed immediately upon collection at the field site.

Field site photo was acquired with an Olympus C3040Z camera; no image processing occurred (Fig. 3-1). Mat photos were acquired using Kodak Elite Chrome 100 ASA slide film and scanned on a Nikon Super Coolscan 9000 in the Adobe RGB color space (Fig. 3-2). To match the scanned images to the slides, Adobe Photoshop 6.0 was used to adjust the levels histogram so that the darkest and lightest pixels were mapped to black and white, thereby increasing the tonal range of the image. The saturation was also increased by 15 in three images (Fig. 3-2 a, c, e) to reflect the saturation of the slides.

3.2.2 Sample Collection

Microbial mat samples for lipid analyses were collected from Chocolate Pots hot springs, a series of high iron thermal springs located along the banks of the Gibbon River southwest of Norris Geyser Basin in Yellowstone National Park. Four distinct mat types which were identified in previous studies (Pierson et al., 1999; Pierson and Parenteau, 2000), 1) *Synechococcus-Chloroflexus* sp., 2) *Pseudanabaena*, 3) *Oscillatoria* cf. *princeps*, and 4) narrow *Oscillatoria*, were collected in July 2003 and immediately placed on dry ice in the field (Figs. 3-1 and 3-2). A 2.5 cm deep core of
the *Synechococcus-Chloroflexus* mat was removed, frozen, and sectioned with a sterile scalpel into two portions: the surface 0 - 2 mm containing only mat, and the underlying 2 – 25 mm containing iron oxides. A 2 cm deep core of iron oxides with no conspicuous mat on the surface was collected adjacent to the *Synechococcus-Chloroflexus* mat to act as a control (Fig. 3-2). Frozen samples were stored on dry ice during transit to the laboratory where they were subsequently freeze-dried. Freeze-dried samples were stored at 4°C until lipid analyses were performed in August 2003.

Figure 3-1. Distribution of three phototrophic mats examined in this study on main Chocolate Pots iron deposit on the east side of the river. The *Pseudanabaena* mat occurred at the north satellite vent (NSV). The narrow *Oscillatoria* mat occurred in association with a red terracette structure on the steep face of the main iron deposit and experienced thin, sheet-like flow over the mat. It also occurred in a different hydrodynamic regime in the rapidly flowing vent outflow channel. The *Synechococcus-Chloroflexus* mat occurred at the south satellite vent (SSV), but was collected for lipid analyses at a vent further south (not visible). The *Oscillatoria* *cf. princeps* mat occurred in areas of low flow (not pictured). MMV; main mound vent.
Figure 3-2. Field images of the four phototrophic mats examined in this study. a. The *Synechococcus-Chloroflexus* mat grew in the vent pool, but mainly occurred as a thin, cohesive mat up to ~1m down the outflow channel. A core of the mat was removed (upper arrow) and sectioned into the mat and ferrihydrite underneath the mat. A ferrihydrite core with no conspicuous mat on the surface was also collected (lower arrow). b. Narrow *Oscillatoria* mat in rapid outflow channel. Mat was thicker and more gelatinous and was frequently embedded with iron particles, giving it an olive color. Note pine cone in bottom right of image for scale. c. Narrow *Oscillatoria* mat in association with a red terracette structure on the steep face of the main iron deposit. Mat was evident as a very thin green film in the terracette ponds. d. Floating streams of the *Pseuderamabaena* mat near the north satellite vent. e. The *Oscillatoria* cf. *princeps* mat (arrow) in association with reflective iron film that fractured upon handling.
3.2.3 X-Ray Diffraction (XRD)

The extracted mat and iron oxide cores were prepared for powder X-ray diffraction (XRD) by air drying and grinding into a clay-size fraction using a mortar and pestle. The remaining organics were not digested prior to analysis because Cornell and Schwertmann (2003) state that while organics can bind to and increase the surface area of iron oxides, they have no effect on the amount of structural information obtained by XRD. The powdered samples were side-packed into aluminum holders using a frosted slide to ensure random orientation of the grains. Sample mounts were step scanned from 2.5 to 75° 2θ using a step size of 0.020° and a count time of 25 sec per step for a total of 25.5 hours per analysis. The samples were run on a Philips X’Pert PW3040 X-ray diffractometer at 40 kV and 30 mA using copper K-alpha radiation. The instrument contained an energy dispersive detector, so iron fluorescence was minimal. The peaks on the powder XRD diffraction patterns were identified by comparing the measured sample d-spacings to previously published values (Cornell & Schwertmann, 2003).

3.2.4 Lipid Extraction, Separation, and Derivatization

Lipids were extracted using a modified Bligh and Dyer procedure to generate a total lipid extract (TLE) (Fig. 3-3) (Jahnke et al., 1992). L-α-phosphatidylcholine, dibehenoyl (Sigma) was added as an internal extraction standard and the TLE was separated into polar and neutral fractions using a cold acetone precipitation. The polar precipitate was separated into glycolipid (GL), phospholipid (PL), and
bacteriohopanepolyol (BHP) by preparative thin layer chromatography (TLC) on Silica gel G plates (Merck) using an acetone-benzene-water (91:30:8) solvent system. GL- and PL-derived fatty acid methyl esters (FAME) were prepared by mild alkaline methanolysis (MAM) (Jahnke et al. 2001; 2004). The bond position of the monounsaturated FAME was determined by generating dimethyl disulfide (DMDS) adducts (Yamamoto et al., 1999). Hopanols (C_{31}, C_{32} and 2-methylhomologs) were prepared by treatment of the BHP using an oxidation-reduction procedure and
analyzed as acetate derivatives (Jahnke et al. 2004; Rohmer et al. 1984).

The neutral supernatant was separated into hydrocarbons, wax esters, pigments, and glycolipids by preparative TLC on Silica gel G plates (Merck) by subsequent development in methylene chloride and then hexane (Jahnke et al., 2004). Neutral GL-derived FAME and chlorophyll-derived phytol were prepared by mild alkaline methanolysis (Jahnke et al. 2001; 2004). The monounsaturated bond position of the neutral GL FAME was determined using DMDS adducts as described above. Phytols were hydrogenated prior to GC analysis (Jahnke et al., 2004).

3.2.5 Gas Chromatography

FAME, DMDS-adducts, BHP hopanol-acetates, hydrocarbons, wax esters, and hydrogenated phytols were analyzed by gas chromatography (GC) using flame ionization (FID) and mass spectrometry (MS) detectors as previously described (Jahnke et al. 2004). The FAME was quantified on the GC-FID using methyl tricosanoate (Sigma) as an internal standard and reported as µg/g TOC. The hydrocarbons and phytol were quantified using n-tricosane (Sigma), while the wax esters were quantified using 5α-cholestane (Sigma). For ease of comparison between mats, the quantified hydrocarbons, phytol, and wax esters were reported as relative abundance. An insufficient quantity of mat material was extracted to quantify the BHP, so it was reported as an unquantified relative abundance.
3.2.6 Lipid Nomenclature

Lipids were named according to the delta convention $X:Y\Delta Z$, where $X$ is the number of carbon atoms in the chain, $Y$ is the number of unsaturations, and $Z$ is the position of the unsaturation relative to the carboxyl carbon. Branching at C2 and C3 is designated relative to the methyl end as iso ($i$) and anteiso ($a$), respectively, while mid-chain branching is specified relative to the carboxyl end (e.g., 10Me). Straight chain compounds lacking branching are designated normal ($n$). Cyclopropyl compounds are indicated by the prefix cy.

3.3 Results

3.3.1 Gross Description of Mats

The *Synechococcus-Chloroflexus* mat and the *Pseudanabaena* mat were located closest to the vents and consequently experienced the highest temperature and levels of Fe$^{2+}$ (Figs. 3-1 and 3-2, Table 3-1). The *Oscillatoria* cf. *princeps* mat occurred in areas of low flow and was associated with a reflective iron film that fractured upon handling (Fig. 3-2, Table 3-1). The fourth mat type, narrow *Oscillatoria*, was the most widely distributed on the iron deposits and occurred as two morphotypes: one in a terrace on the steep face of the main iron deposit and the other in a rapidly flowing channel (Figs. 3-1 and 3-2, Table 3-1).
Table 3-1. Geochemical measurements of water flowing over the phototrophic mats at Chocolate Pots hot springs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Fe²⁺ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechococcus-Chloroflexus</em> mat core (a)</td>
<td>52.8</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Ferrihydrite underneath mat (b)</td>
<td>52.8</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Ferrihydrite core adjacent to mat</td>
<td>51.5</td>
<td>6.0</td>
<td>5.9</td>
</tr>
<tr>
<td><em>Pseudanabaena</em> mat</td>
<td>50.4</td>
<td>5.5</td>
<td>5.8</td>
</tr>
<tr>
<td><em>Oscillatoria cf. princeps</em> mat</td>
<td>41.4</td>
<td>5.8</td>
<td>ND</td>
</tr>
<tr>
<td>Narrow <em>Oscillatoria</em> mat terrace</td>
<td>40.1</td>
<td>7.3</td>
<td>0.4*</td>
</tr>
<tr>
<td>Narrow <em>Oscillatoria</em> mat channel</td>
<td>39.6</td>
<td>7.3</td>
<td>0.2*</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not determined.
* Measured above mat types in 2005

3.3.2 Mineralogy

XRD analysis revealed that both the upper mat and lower iron oxide sections of the extracted *Synechococcus-Chloroflexus* mat core were comprised of 2-line ferrihydrite with d-spacings of 2.6 and 1.5 Å (Fig. 3-4) (mat diffraction pattern not shown). The adjacent control core with no conspicuous mat was composed of 2-line ferrihydrite with a slight amount of the more ordered 6-line ferrihydrite present (2.2 Å peak) (Fig. 3-4).

![Figure 3-4. Random powder XRD diffraction patterns of the iron oxide underneath the *Synechococcus-Chloroflexus* mat (gray) and the adjacent iron oxide core with no conspicuous mat on the surface (black). The two broad amorphous peaks of 2-line ferrihydrite are visible at 2.6 and 1.5 Å. Also visible in the lower pattern is the 2.2 Å peak of the slightly more ordered 6-line ferrihydrite.](image-url)
3.3.3 Community Structure of Microbial Mats

3.3.3.1 Ester-linked fatty acids

The phospholipid fraction of the surface *Synechococcus-Chloroflexus* mat was dominated by the $n$-16 and $n$-18 ester-linked fatty acids (Fig. 3-5). Four fatty acids ($n$-16:1, $n$-16, $n$-18:1, and $n$-18) comprised 80% of the total phospholipid fatty acid (PLFA) recovered. Polyunsaturated fatty acids (PUFA), primarily 18:2, were also present. Branched fatty acids, the iso-, anteiso- and mid-chain isomers, accounted for approximately 10% of the total. Of the terminally branched PLFA, the $i$-17 was the most plentiful.

![Phospholipid FAME profile of *Synechococcus-Chloroflexus* mat, ferrihydrite core underneath mat, and adjacent ferrihydrite core lacking a conspicuous mat on the surface. Graphs were plotted on a log scale to be able to compare the subtleties of the trends.](image-url)

Figure 3-5. Phospholipid FAME profile of *Synechococcus-Chloroflexus* mat, ferrihydrite core underneath mat, and adjacent ferrihydrite core lacking a conspicuous mat on the surface. Graphs were plotted on a log scale to be able to compare the subtleties of the trends.
The polar glycolipid fraction of the *Synechococcus-Chloroflexus* mat was similarly dominated by \(n\)-16 and \(n\)-18 ester-linked fatty acids (Fig. 3-6). The \(n\)-16:1, \(n\)-16, \(n\)-18:1, and \(n\)-18 fatty acids comprised 82\% of the total polar glycolipid fatty acid (PGLFA) recovered. The 18:2 PUFA was more abundant than in the phospholipid fraction, while the terminally branched fatty acids were less abundant. Mid-chain branched fatty acids were not detected.

![Figure 3-6](image)

Figure 3-6. Polar glycolipid FAME profile of *Synechococcus-Chloroflexus* mat, ferrihydrite core underneath mat, and adjacent ferrihydrite core lacking a conspicuous mat on the surface. Graphs were plotted on a log scale to be able to compare the subtleties of the trends.

The neutral glycolipid fraction of the *Synechococcus-Chloroflexus* mat was again dominated by \(n\)-16 and \(n\)-18 (Fig. 3-7). The \(n\)-16:1, \(n\)-16, \(n\)-18:1, and \(n\)-18 fatty acids comprised 83\% of the total neutral glycolipid fatty acid (NGLFA). The proportion of 18:2 PUFA was greatest in this fraction. Terminally and mid-chain branched NGLFA were again low in abundance.
The double bond position of the polar glycolipid fatty acid 18:1, determined as DMDS adducts, from the *Synechococcus-Chloroflexus* mat revealed that 89% occurred at C9 (Δ9), 10% at Δ11 and 1% at Δ13. In contrast, the neutral glycolipid fatty acid contained 18:1 with 97% of the double bond in the Δ9 position and 3% in the Δ11 position. DMDS adducts of the unsaturated phospholipids were not prepared.

The phospholipid, polar glycolipid, and neutral glycolipid fractions of the *Pseudanabaena*, the *Oscillatoria* cf. *princeps*, and the narrow *Oscillatoria* mats displayed similar trends. All mats were dominated by n-16 and n-18 fatty acids, contained low levels of terminally and mid-chain branched fatty acids, and contained 18:2 PUFA (data not shown).
3.3.3.2 Hydrocarbons, phytol, and bacteriohopanepolyol (BHP)

The hydrocarbon fraction of the *Synechococcus-Chloroflexus* mat was dominated by normal straight chain alkanes (C_{14}-C_{31}) (Table 3-2). Only one alkene, \( n-C_{31:3} \), was detected. Short chain monomethylalkanes (MMA) (7-methyl, 6-methyl, and 5-methylheptadecanes) and one dimethylalkane (DMA) (7,11-dimethylheptadecane) were also recovered (Fig. 3-8).

![Gas chromatogram of hydrocarbon fraction of *Synechococcus-Chloroflexus* mat displaying short chain monomethylalkanes (7-methyl, 6-methyl, and 5-methylheptadecanes) and one dimethylalkane (7,11-dimethylheptadecane).](image)

The *Pseudanabaena*, *Oscillatoria* cf. *princeps*, and narrow *Oscillatoria* mats contained short chain normal alkanes (C_{13} to C_{19:23}) but lacked the \( n-C_{31:3} \) alkene (Table 3-2). These mats displayed a greater range in the monomethylalkanes (7-methyl to 3-methyl), but lacked any dimethylalkanes (Table 3-2). Phytol was recovered from all mats.
The *Synechococcus-Chloroflexus* mat contained the greatest variety of BHP products (Table 3-2). A homohopanol (C\textsubscript{31}) and bishomohopanol (C\textsubscript{32}) were detected, as well as traces of their 2-methyl homologues (2-methyl-C\textsubscript{31} and 2-methyl-C\textsubscript{32}). The homo- and bishomohopanols were also found in the *Pseudanabaena* and narrow *Oscillatoria* mats (Table 3-2). The narrow *Oscillatoria* mat in the terrace on the face of the main iron deposit also contained a trace of the 2-methyl-C\textsubscript{31} BHP product.

3.3.3.3 Wax esters

Straight chain saturated normal, normal (n,n-) wax esters dominated the wax ester profile of the *Synechococcus-Chloroflexus* mat (Table 3-2). The n,n-C\textsubscript{34} and n,n-C\textsubscript{32} were most abundant, with somewhat greater C34. Wax esters with one and two iso-methyl moieties as iso, normal- (i,n-) and iso, iso- (i,i-), respectively, were also detected. The *Pseudanabaena* mat displayed a similar profile, except that in this mat, the n,n-C\textsubscript{32} wax ester was more abundant than the C34. Wax esters were not detected in the *Oscillatoria cf. princeps* or in the narrow *Oscillatoria* mats.
Table 3-2. Diagnostic lipid biomarkers found in phototrophic mats, ferrihydrite underneath mats, and ferrihydrite adjacent to mats at Chocolate Pots hot springs. See Methods text for lipid nomenclature.

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3.3.4 Community Structure of Ferrihydrite Underneath Synechococcus-Chloroflexus Mat

3.3.4.1 Ester-linked fatty acids

The FAME recovered from the ferrihydrite core material underneath the Synechococcus-Chloroflexus mat was 31 times less abundant than in the surface mat. The phospholipid, polar glycolipid, and neutral glycolipid profiles were dominated by \( n-16 \) and \( n-18 \) fatty acids, reflecting the same distribution as was observed in the mat (Figs. 3-5, 3-6, and 3-7). The proportion of 18:2 PUFA was highest in the neutral glycolipid fraction, as in the surface mat. The relative abundance of iso-, anteiso- and mid-chain branched fatty acids also reflected what was observed in the mats.

The DMDS adducts of the 18:1 neutral glycolipid fatty acid from the ferrihydrite core material underneath the mat revealed that 94% occurred at \( \Delta 9 \), a
slight decrease from what was observed in the mat. The double bond occurred at Δ11 and Δ13 in the remaining 5% and 1% of the fatty acid, respectively. Preparation of DMDS adducts of the phospholipids and polar glycolipids was unsuccessful due to incomplete reaction of the DMDS with the double bond.

As a control, an adjacent ferrihydrite core with no conspicuous surface mat was analyzed. The concentration of fatty acids recovered was 50 times less than in the *Synechococcus-Chloroflexus* mat, and 1.6 times less than in the ferrihydrite underneath the mat. This control core was also dominated by *n*-16 and *n*-18 fatty acids in all phospholipid and glycolipid fractions (Figs. 3-5, 3-6, and 3-7). The percentage of 18:2 PUFA in the phospholipid and polar glycolipid fractions were the same as in the mat (~4% and ~8%, respectively), and increased from ~10% to ~18% in the neutral glycolipid fraction. Most notably, the *iso*-, *anteiso*-, and mid-chain branched fatty acids in the phospholipid fraction increased relative to that in the mat. The fatty acids of the polar glycolipid fraction displayed a similar trend, although no mid-chain branched compounds were detected.

The DMDS adducts of the monounsaturated neutral glycolipids from the adjacent ferrihydrite core revealed similar relative amounts of the Δ9-18:1 fatty acid as in the *Synechococcus-Chloroflexus* mat (97%). Preparation of DMDS adducts of the phospholipids and polar glycolipids was unsuccessful.

3.3.4.2 Hydrocarbons, phytol, BHP, and wax esters

The hydrocarbons detected in the *Synechococcus-Chloroflexus* mat were also
present in the ferrihydrite core material underneath the mat and in the adjacent ferrihydrite core with no conspicuous surface mat (Table 3-2). Most notably, the mono- and dimethylalkanes survived lithification by ferrihydrite in both cores. The C_{31:3} alkene was not detected in the core material under the mat, nor were any BHP hopanol products. However, a trace amount of bishomohopanol (C_{32}) product was detected in the adjacent core. Phytol was recovered from the ferrihydrite under and adjacent to the mat as well.

The wax esters were also present in the ferrihydrite cores beneath and adjacent to the *Synechococcus-Chloroflexus* mat. The relative compositions were similar to the mat with the exception of the iso-, iso-branched compounds, which were in the lowest abundance in the mat and not detected in the cores (Table 3-2).

3.4 Discussion

3.4.1 Community Structure of Microbial Mats

3.4.1.1 Ester-linked fatty acids

All microbes contain n-16 and n-18 saturated and unsaturated fatty acids to some degree. However, in hot spring systems, these compounds have been found to dominate the fatty acid profiles of cyanobacteria and *Chloroflexus* isolates (Kenyon & Gray, 1974; Fork et al., 1979; Knudsen et al., 1982; Ward et al., 1989). More specific fatty acid biomarkers for cyanobacteria include PUFA and the Δ9 positional isomer of
PUFA are often found in cyanobacteria (Kenyon, 1972; Kenyon et al., 1972) and are produced by few other prokaryotes. The \( n-18:1 \Delta 9 \) is considered of cyanobacterial origin, particularly when associated with the glycolipid fractions (Sakamoto et al., 1994). The preponderance of \( n-18:2 \) and \( n-18:1 \Delta 9 \) in the four microbial mats at Chocolate Pots hot springs clearly indicate that they were dominated by phototrophs, specifically cyanobacteria. The detection of wax esters in these mats demonstrates the presence of *Chloroflexus* sp. This lipid data supports previous initial microscopic observations, *in vivo* pigment analyses (Pierson & Parenteau, 2000), and physiological characterization (Pierson et al., 1999; Trouwborst et al., in review) of this high iron system.

The determination of bond position in the monounsaturated fatty acids aids the assessment of the relative proportion of cyanobacteria to other bacteria in these relatively simple microbial mats. The double bond position of cyanobacterial \( n-18:1 \) is different from that of most other bacteria because the biosynthetic pathway involves an \( O_2 \)-dependent desaturation mechanism (Bloch, 1971) rather than the chain-elongation, dehydration system extensively described for bacteria (Cronan & Rock, 1996). These different biosynthetic mechanisms result in \( 18:1 \Delta 9 \) in cyanobacteria and \( 18:1 \Delta 11 \) in most other bacteria. Additionally, the membrane lipids of cyanobacteria are primarily glycolipids: monogalactosyl diacylglycerol (MG), digalactosyl diacylglycerol (DG), sulfoquinovosyl diacylglycerol (SQ). Only one phospholipid, phosphatidylglycerol (PG), is present (e.g., Fork et al., 1979; Jurgens & Weckesser, 1985; Miller et al., 1988; Ward et al., 1994). The monounsaturated C\(_{18}\) fatty acids
display a double bond at C9 (18:1Δ9) due to the enzyme Δ9 acyl-lipid desaturase (Sakamoto et al., 1994). *Chloroflexus* also contains MG, DG, and PG (Kenyon & Gray, 1974; Knudsen, 1982), and as in cyanobacteria, the C₁₈ unsaturation occurs at Δ9 (Kenyon & Gray, 1974). Based on the DMDS adducts of glycolipid FAME, cyanobacteria and *Chloroflexus* sp. were confirmed as the major components (89-97%) of the higher temperature microbial mat.

The presence of low levels of branched fatty acids suggests the presence of other bacteria in the Chocolate Pots mats. Terminally branched *iso-* and *anteiso-*fatty acids are common in Gram positive bacteria and sulfate-reducing bacteria. Mid-chain branched phospholipid fatty acids such as 10-methylhexadecanoate are considered indicative of the *Desulfobacter* spp. and Actinomycetes (Boschker & Middelburg, 2002). However, numerous sulfide and scanning voltammetric microelectrode measurements of these mats in the light and the dark revealed no detectable sulfide (Pierson et al., 1999; Trouwborst et al., in review). Additionally, no sulfur-containing minerals were detected by XRD or EDS (Parenteau and Cady, in preparation) further suggesting no sulfur cycle. Therefore, Actinomycetes are the most probable source of the 10-methyl fatty acids and were likely derived from the soil immediately adjacent to the hot springs. Thermophilic chemolithotrophic iron oxidizers are probably not present in this system because microscopic examination of surface samples taken at the vent and 1 m intervals away from the vent did not reveal obvious morphological evidence of iron oxidizers (Emerson and Weiss, 2004). Attempts at isolating the organisms using gradient and liquid enrichment techniques were also unsuccessful.
Cyanobacteria do not contain iso- and anteiso- fatty acids. *Chloroflexus auranticus* also does not contain these branched fatty acids (Knudsen, 1982), although the presence of numerous branched wax esters in *Chloroflexus*-containing mats throughout the thermal features of Yellowstone (e.g., van der Meer et al., 2003; Jahnke et al., 2004) strongly suggests that some species do synthesize branched fatty acids, necessary biosynthetic precursors to these compounds.

### 3.4.1.2 Phototrophic biomarkers

Short chain MMA and DMA are considered biomarkers for cyanobacteria (Shiea et al., 1990; Kenig et al., 1995; Jahnke et al., 2004). Mid-chain branched MMAs have been documented in a large number of cyanobacterial mats and cultures (Shiea et al., 1990, and references therein; Zeng et al., 1992; Kenig et al., 1995; Jahnke et al., 2004). Although DMA are found extensively in cyanobacterial mats throughout the world (Robinson & Eglinton, 1990; Shiea et al., 1990; Kenig et al., 1995; Jahnke et al., 2004), they have been characterized in only a few cyanobacterial cultures (Summons et al., 1996; Köster et al., 1999; Jahnke et al., 2004). In Yellowstone, DMA have only been reported in the Fountain Paint Pots *Phormidium* mats by Jahnke et al. (2004). Consequently, this report of 7,11-dimethylheptadecane in the *Synechococcus-Chloroflexus* mat of Chocolate Pots is only the second documentation of DMAs in Yellowstone.

The $C_{31,3}$ alkene and wax esters are considered biomarkers for *Chloroflexus*.
(Shiea et al., 1991; van der Meer et al., 1999; Knudsen et al., 1982). Their detection in the higher temperature Synechococcus-Chloroflexus mat confirms the previous identification of Chloroflexus in this mat by phase and near-infrared epifluorescence microscopy and in vivo pigment analyses (Pierson & Parenteau, 2000). However, the diverse assemblage of wax esters indicates that in addition to Chloroflexus, there are other green nonsulfur or filamentous anoxygenic phototrophs (FAPs) present in the mat. Lipid analysis of cultures has shown that Chloroflexus synthesizes both saturated and unsaturated wax esters (Knudsen et al., 1982), while Roseiflexus contains only straight chain wax esters (van der Meet et al., 2002). Those culturable FAPs do not contain the types of wax recovered from the Chocolate Pots mats.

The presence of other FAPs is supported by the detection of larger (~1.5 μm diameter) filaments in enrichment cultures whose bacteriochlorophyll autofluoresced in the near-infrared (unpublished data). Boomer and colleagues (Boomer et al., 2000; Boomer et al. 2002) performed a survey throughout Yellowstone National Park for bacteriochlorophyll a (Bchl a)-containing filaments and found that all FAP-like clones were most similar to Roseiflexus castenholzii. The FAPs of the Boomer studies were associated with distinct red layers that are not overtly present in the phototrophic mats at Chocolate Pots. However, our microscopic observations coupled with wax ester data suggests that the FAPs present in the Synechococcus-Chloroflexus mat may be taxonomically diverse.

Some lipids in the mats can be attributed to detrital material. Cyanobacteria only produce short chain alkanes (Zeng et al., 1992), so the long chain alkanes found
in the *Synechococcus-Chloroflexus* mat were likely derived from insect waxes (Kenig et al., 1995, and references therein), or from epicuticular waxes of terrestrial higher plants (Eglinton & Hamilton, 1967). Grass, pine needles, and other detritus have been observed in the mats and outflow channels at Chocolate Pots.

Bacteriohopanepolyol (BHP) are pentacyclic triterpanoids with a hopane skeleton linked to a variety of polyhydroxylated side chains (Ourisson et al., 1987; Summons et al., 1999). They are synthesized by a wide variety of cultured aerobic bacteria (Rohmer et al., 1984), although oxygen doesn’t appear to be required for biosynthesis (Fischer et al., 2005). Modification of the hopanoid skeleton by alkyl substituents is limited so far to specific groups of organisms; for example, methylation at C2 is considered a biomarker for cyanobacteria (Summons et al., 1999). The 2-methyl-C31 and 2-methyl-C32 BHP recovered from the Chocolate Pots mats were also found in the *Phormidium* mats studied by Jahnke et al. (2004).

3.4.2 Community Structure of Ferrihydrite Underneath Synechococcus-Chloroflexus Mat

3.4.2.1 Ester-linked fatty acids

This portion of the study aimed to address how lithification by 2-line ferrihydrite, the primary iron phase of the sinter, affected the lipid signature of the phototrophic community. In general, the lipid signature of the phototrophs was preserved in the ferrihydrite core material underneath the *Synechococcus-Chloroflexus* mat and was not replaced by a chemotrophic one. Examination of the fatty acids
allowed for assessment of the entire bacterial community. The fatty acid profiles in the ferrihydrite core underneath the mat were remarkably similar to the mat, albeit lower in abundance. The slight 3% increase in the mid-chain branched phospholipids suggests that proportionally there were more chemotrophs present in the ferrihydrite than in the mat. However, the high percentage of 18:1Δ9 and the presence of 18:2 PUFA indicate that the signature of the cyanobacteria is strongly preserved in the ferrihydrite.

The adjacent ferrihydrite core with no conspicuous mat on the surface also retained the phototrophic fatty acid signature, most likely from previously existing mat. This core was used as a control for the scanning voltammetric microelectrode measurements and the oxygen profile reflected atmospheric diffusion (Trouwborst et al., unpublished data). Even though the microelectrode measurements indicated that there were no active phototrophs, the ferrihydrite was once likely covered by a mat.

The recovery of cyanobacterial and Chloroflexus fatty acids from the ferrihydrite underneath the mat was unexpected. Membrane lipids and associated fatty acids are typically rapidly degraded after cell death (Harwood & Russell, 1984) and for this reason have become indicators of cellular viability in natural environments (White et al., 1997). It has been suggested that iron may inhibit cellular autolytic enzymes (Herbold & Glaser, 1975; Leduc et al., 1982), leading to the enhanced preservation of iron-mineralized cells (Ferris et al. 1988). We have observed through transmission electron microscopy (TEM) that the cellular and thylakoid membranes of the Chocolate Pots phototrophs retained a high degree of integrity upon iron
mineralization (Parenteau & Cady, in preparation). The permineralization of these cells and impregnation of the membranes by ferrihydrite appears to preserve lipid composition. We are also currently investigating the inhibition of phototrophic autolytic enzymes by iron.

3.4.2.2 Phototrophic biomarkers

In addition to the cyanobacterial fatty acids, the cyanobacterial biomarkers MMA and DMA survived in the ferrihydrite underneath and adjacent to the *Synechococcus-Chloroflexus* mat. These recalcitrant hydrocarbons have been found in Holocene cyanobacterial mats (Kenig et al., 1995). Attempts have been made to correlate these compounds to those found in ancient sediments and oils, but have been met with limited success due to a discrepancy in chain lengths (Kenig et al., 1995). Additionally, abundant short-chain wax esters similar to those present in the surface mat also provide support for the survival of *Chloroflexus* lipids.

The C$_{32}$ BHP, which was the most abundant BHP product, also survived in the ferrihydrite underneath the mat. The 2-methyl homologue (which was produced in the mat) was not detected in the ferrihydrite, probably due to its lower relative sample abundance and the relatively small ferrihydrite sample available for extraction. The hydrocarbon derivative of 2-methyl-BHP, 2-methylhopane, is a robust biosignature. This geohopane was found in 2.7 to 2.5 Ga shales of the Hamersley Basin in the Pilbara Craton and used to date the origin of oxygenic photosynthesis at 2.7 Ga (Brocks et al., 1999; Summons et al., 1999), well before the oxygenation of the
atmosphere at 2.3 Ga (Kasting, 2001). Also significant was the occurrence of this biomarker in shales interbedded with the oxide facies BIF in the Hamersley Group, which suggests that the formation of the oxide facies was due to oxygen produced by cyanobacteria (Brocks et al., 1999). The detection of 2-methyl-BHP in the modern iron-mineralized mats at Chocolate Pots is significant because the oxygen produced by the cyanobacteria has been shown to mediate Fe\(^{2+}\) oxidation (Trouwborst et al., in review). This serves as a modern ground-truthing of the Brocks et al. (1999) suggestion that oxygen from cyanobacteria was responsible for the deposition of ferric oxides in BIFs from which the 2-methylhopane was recovered.

Trouwborst et al. (in review) used a voltammetric microelectrode to simultaneously measure O\(_2\), Fe\(^{2+}\) and Mn\(^{2+}\) in situ at 0.1 mm resolution in the *Synechococcus-Chloroflexus* mat to demonstrate that the oxygen produced by cyanobacteria was the sole mechanism by which the Fe\(^{2+}\) was oxidized in the anoxic water flowing over the mats. The investigators also used cell suspensions prepared from homogenized mat to estimate the total iron oxidation rate on a cellular basis in closed-system experiments. They quantified the cyanobacterial oxidation activity relative to iron oxidation requirements for formation of BIFs, and compared it to rates obtained for purple phototrophs and chemotrophic bacteria. These studies suggest that localized cyanobacterial oxygen production appears to be a biologically efficient way to oxidize ferrous iron in the deposition of Precambrian BIFs, as was suggested by Cloud (1965, 1973).
Chocolate Pots hot springs is not a suitable depositional model for Precambrian BIFs. However, the cyanobacterial mats in the anoxic vent waters may be a reasonable geochemical model for the oxidation of Fe$^{2+}$ by oxygenic photosynthesis in mats or the water column in an ancient anoxic world. These springs may also serve as an analog for potential hydrothermal systems on Mars, such as the outflow channel Dao Vallis near the volcano Hydriaca Patera (Crown, et al., 2004). At the very least, these springs provide an appropriate setting in which to examine the roles of cyanobacteria and filamentous anoxygenic phototrophs (e.g., *Chloroflexus*) in Fe$^{2+}$ oxidation, iron biomineralization, and biosignature formation. We have found geologically-significant cyanobacterial lipid biomarkers in the ferrihydrite and linked them to the source organisms in these modern iron-mineralized phototrophic mats whose metabolic impact on Fe$^{2+}$ oxidation has been quantified. Such biosignatures can be used to assess the biological contribution to ancient iron deposits on Earth and, potentially, to those found on Mars.

3.5 Acknowledgments

This work was supported by NASA Exobiology Grant NAG5-12328. Additional support was generously provided by a NASA Oregon Space Grant Graduate Fellowship and a NASA Planetary Biology Internship to M.N. Parenteau. We thank the National Park Service for allowing us to conduct research in
Yellowstone National Park, and thank Kendra Turk, University of California Santa Cruz, Mike Kubo, SETI Institute, and Georg Grathoff, Portland State University, for technical assistance. We also thank Beverly Pierson for helpful discussions.
Chapter 4. Carbon Isotopic Fractionation Patterns in Iron-Mineralized *Synechococcus-Chloroflexus* Mats at Chocolate Pots Hot Springs, Yellowstone National Park, USA

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4.1 Introduction

The family *Chloroflexaceae* is the most deeply branching phototrophic lineage on the 16S rRNA tree of life (Oyaizu et al., 1987). The most well characterized member, *Chloroflexus aurantiacus*, is metabolically diverse (Pierson and Castenholz, 1974) and capable of growing photoautotrophically using sulfide or hydrogen as an electron donor in cultures and hot spring microbial mats (Castenholz, 1973; Holo and
Sirevåg, 1986; Giovannoni et al., 1987). Although it has not yet been demonstrated, Pierson and colleagues have hypothesized that *Chloroflexus* oxidizes Fe$^{2+}$ in a type of anoxygenic photosynthesis known as photoferrotrophy (Pierson et al., 1999; Hanada and Pierson, 2002). The widespread abundance of reduced iron on the early Earth prior to the appearance of oxygen would have made it particularly suitable as an electron donor for photosynthesis. Such an ancestral filamentous anoxygenic phototroph (FAP) may have oxidized Fe$^{2+}$ during the Precambrian and, like the purple bacteria (e.g., Kump, 1993), mediated iron transformations in the absence of oxygen and contributed to the deposition of Banded Iron Formations (BIFs). Pierson et al. (1993) also point out that the oxidized iron products of this type of photosynthesis would have provided substantial protection from UV radiation for surface-dwelling phototrophs prior to the development of an ozone shield.

We have been investigating the possibility of Fe$^{2+}$ oxidation by *Chloroflexus* sp. in the *Synechococcus-Chloroflexus* mats at Chocolate Pots hot springs, a high-iron hot spring in Yellowstone National Park. Uptake experiments using $^{14}$C-labeled bicarbonate revealed a stimulation of photosynthetic CO$_2$ fixation by the mat community members in the light and in the presence of Fe$^{2+}$ (Pierson et al., 1999). The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an herbicide that inhibits photosystem II (PSII) in cyanobacteria and may inhibit the PSII-type reaction center in *Chloroflexus*, repressed CO$_2$ fixation (Pierson et al., 1999). Control experiments with other reductants, such as ascorbate and thioglycollate, did not stimulate fixation. This suggests that Fe$^{2+}$ was not simply functioning to reduce the
amount of free oxygen that builds up during oxygenic photosynthesis, which can potentially become inhibitory (Weller et al., 1975; Mouget et al., 1995; Pearl, 1996). These data suggest that photoferrotrophy is potentially occurring in these mats.

Because these $^{14}$C uptake experiments were performed with a mixed mat population, the possibility exists that either *Chloroflexus* sp. or cyanobacteria could be directly oxidizing Fe$^{2+}$. We propose to determine whether *Chloroflexus* is growing photoautotrophically, likely using Fe$^{2+}$ as an electron donor (no sulfide or hydrogen has been detected at Chocolate Pots), or photoheterotrophically using cyanobacterial photosynthate by performing compound-specific isotope analyses (CSIA) of the lipid biomarkers. Comparison of the stable carbon isotopic compositions of the *Chloroflexus* and cyanobacterial lipid biomarkers will reveal whether *Chloroflexus* is growing autotrophically by fixing carbon via the 3-hydroxypropionate pathway ($\delta^{13}$C ~ -14%; Holo and Sirevåg, 1986; Strass and Fuchs, 1993) or photoheterotrophically by consuming cyanobacterial carbon compounds fixed via the Calvin Cycle ($\delta^{13}$C -20 to -35%; Schidlowski 1988; Mojzsis et al., 1996).

4.2 Methods

4.2.1 Sample Collection

Samples of the *Synechococcus-Chloroflexus* mat were removed with a sterile scalpel and placed in 100% ethanol-cleaned aluminum foil that had been baked at
400°C for 3 hours to remove organics. The aluminum foil packets containing the mat were immediately placed on dry ice in the field and kept frozen until lyophilization and lipid extraction.

4.2.2 Microscopy

Stereomicroscope images of fresh mat were collected with a Nikon SMZ800 equipped with a Leica DFC480 camera. Compound microscope phase contrast and epifluorescence images were collected on a Leica DMRX equipped with a Leica DFC480 camera. Samples for TEM analysis were collected and prepared as previously described (Parenteau and Cady, in preparation), and viewed on a JEOL 100CXII TEM operating at 100 kV accelerating voltage.

4.2.3 Water Chemistry

The temperature, pH, and aqueous Fe$^{2+}$ concentration of the vent pool and water immediately above the mat were measured as previously described (Parenteau and Cady, in preparation). Water samples for cation analysis were filtered (Nalgene surfactant-free cellulose acetate, 0.2 µm, Rochester, NY) into nitric acid cleaned low-density polyethylene (LDPE) bottles and acidified to pH 0.9 with trace metal grade nitric acid (Fisher, Pittsburgh, PA) and stored at 4°C until analysis. Samples were analyzed for Fe and Mn within a month on a HP-4500 Inductively Coupled Plasma Mass Spectrometer using a Claritas PPT standard (Lot #26-110AS, SPEX CertiPrep, Metuchen, NJ). Aqueous silica was measured immediately upon collection in the field.
using the USGS molybdate blue colorimetric assay (Fishman and Friedman, 1989) and a Hach DR/2400 portable spectrophotometer. Ca and Fe are known to interfere with the reaction and were removed by complexing with disodium dihydrogen ethylenediamine tetraacetate (Na₂EDTA) according to the USGS protocol. A solution of Na₂SiF₆ was used as a standard.

Water samples for dissolved inorganic carbon (DIC) analysis were removed using a 60 ml syringe and immediately filtered through Whatman GF/F filters baked at 400°C for 3 hours into pre-evacuated 25 ml serum bottles sealed with silicone stoppers and containing 15 μl of a saturated HgCl₂ solution to inhibit bacterial growth. The bottles were immediately placed on ice in the field and then stored at 4°C until analysis by flow injection analysis (Hall and Aller, 1992).

4.2.4 Lipid Extraction, Separation, Derivatization, and Analysis

Lipids were extracted and separated into the following classes and derivatized as previously described: phospholipids, polar and neutral glycolipids, bacteriohopanepolyol (BHP), hydrocarbons, wax esters, and pigments (Chapter 3). Lipids were analyzed using gas chromatography-mass spectroscopy (GC-MS) as previously described in Chapter 3, except the column was changed to a 60 m DB-5ms (J&W Scientific).
4.2.5 Lipid Nomenclature

Lipids were named according to the delta convention X:YΔZ, where X is the number of carbon atoms in the chain, Y is the number of unsaturations, and Z is the position of the unsaturation relative to the carboxyl carbon. Mid-chain branching is specified relative to the carboxyl end (e.g., 10Me). Straight chain compounds lacking branching are designated normal (n).

4.2.6 Isotopic measurements

Serum bottles of DIC were collected for isotope analysis at each site (vent and water immediately above the mat). Samples were withdrawn and acidified, and the liberated CO$_2$ was purified and collected on a vacuum line for isotopic analysis on a Nuclide 6-60RMS mass spectrometer modified for small samples (Hayes et al., 1977; Summons et al., 1994). Biomass was determined using a Carlo Erba CHN EA1108 elemental analyzer interfaced to a Finnigan Delta Plus XL isotope ratio mass spectrometer (EA-IRMS) (Jahnke et al., 2001). Compound-specific isotope analyses (CSIA) were carried out on an HP 6890 GC equipped with a 60 m DB-5ms column (J&W Scientific) and the Finnigan Delta Plus XL IRMS (Jahnke et al., 2004). Chromatographic conditions were the same as for the GC-MS analyses. All isotopic analyses occurred within a month of lipid extraction and all samples were analyzed at least in triplicate.
4.3 Results

4.3.1 Gross Description of Microbial Mat

The *Synechococcus-Chloroflexus* mat is thin (0.2 – 2 mm) and cohesive and grows on top of the ferrihydrite deposits in the vent pools and outflow channels at Chocolate Pots hot springs (Fig. 4-1a). The anoxic waters flowing over the mat are high in Fe$^{2+}$, Mn, and SiO$_2$ (Table 4-1). The DIC content is also very high at 11.3 mM (Table 4-1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>DIC (mM)</th>
<th>Fe (mg/L)</th>
<th>Fe$^{2+}$ (mg/L)</th>
<th>Mn (mg/L)</th>
<th>SiO$_2$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vent</td>
<td>53.2</td>
<td>5.8</td>
<td>15.2</td>
<td>5.3</td>
<td>3.5</td>
<td>1.6</td>
<td>131.2</td>
</tr>
<tr>
<td><em>Synechococcus-</em></td>
<td>50.7</td>
<td>5.9</td>
<td>11.3</td>
<td>5.1</td>
<td>3.3</td>
<td>1.6</td>
<td>128.9</td>
</tr>
<tr>
<td><em>Chloroflexus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The phototrophic members of the mat community, *Synechococcus* sp. and *Chloroflexus* sp., were initially identified by *in vivo* pigment analyses and phase and epifluorescence microscopy (Pierson et al., 1999; Pierson and Parenteau, 2000). Further stereomicroscopic examination of the mat revealed an additional member of the phototrophic community that was not readily apparent in the gross morphology of the mat in the field (Fig. 4-1b). We observed patches of brown material that were spatially discrete from the patches of green *Synechococcus-Chloroflexus* material. These brown patches contributed substantial biomass to the mats (~50% in some
Figure 4-1. *Synechococcus-Chloroflexus* mat at Chocolate Pots hot springs in Yellowstone National Park. a. Vent showing thin, cohesive mat growing on ferricydrite sediments in outflow channel. b. Stereomicroscopic image of mat showing green patches of *Synechococcus* and *Chloroflexus* and brown patches of *Cyanothece minervae* and *Chloroflexus*. Brown color is due to the accessory pigment phycocyanin. c. Phase contrast image of *Synechococcus* rods (1.5 μm diameter) and *Chloroflexus* filaments (0.6 – 0.7 μm diameter). d. Epifluorescence image of *Synechococcus* rods displaying red Chlorophyll a autofluorescence and *Cyanothece minervae* cocci displaying yellow phycoerythrin autofluorescence. Diameter of *Cyanothece minervae* cocci is 3-4 μm).
samples). Phase and epifluorescence microscopy of the brown patches revealed the presence of masses of the cyanobacterium *Cyanothece minervae*. The ovoid to coccoid cells, which were 3 - 4 μm in diameter, contained the accessory pigment phycocyanin, which autofluoresced yellow (Fig. 4-1d) and was responsible for the brown color of the mat (Fig. 4-1b). *Chloroflexus* filaments were found intermingled with *C. minervae* in the brown patches.

TEM examination of the unmineralized surface of the phototrophic mat revealed distinctive ultrastructural features of the community members. *Chloroflexus* filaments (~0.6 μm in diameter) were encased by a narrow sheath ~0.15 μm thick (Fig. 4-2a). Chlorosomes, which house the photosynthetic apparatus and line the interior of the cell wall, were often difficult to image due to their small size. Cross sections of *C. minervae* cells revealed photosynthetic membranes comprised of stacks of thylakoids (Fig. 4-2b). When viewed in a transverse cross section, a characteristic arrangement of the photosynthetic membranes was revealed. Stacks of thylakoids were arranged in a triangular orientation around the center of the cell and along the periphery of the cell wall. The cells also contained large polyhedral carboxysomes that house ribulose-1,5-bisphosphate carboxylase (RuBisCo), the enzyme that catalyzes the first major step of carbon fixation in the Calvin Cycle (Fig. 4-2b).

4.3.2 Stable Carbon Isotopic Compositions

The stable carbon isotopic compositions of the DIC, biomass, and individual compounds are summarized in Table 4-2. In addition to the δ^{13}C values, fractionation
Figure 4-2. TEM micrographs of *Synechococcus-Chloroflexus* mat. a. Slightly oblique cross section and oblique longitudinal sections of *Chloroflexus* sp. filament displaying a thin sheath (Sh). b. Oblique cross section of *Cyanothecae minervae* cell containing large polyhedral carboxysome (C) and stacks of thylakoids (T). Cell wall is comprised of a cell membrane (CM), peptidoglycan layer (P), outer membrane (OM) and an S-layer (S) (inset). TEM micrograph of *Synechococcus* sp. not shown.

Factors relative to the source CO$_2$ (as determined by equilibration with DIC) were reported ($e_b$, defined in Table 4-2).

The $\delta^{13}C$ values of the DIC in the water flowing over the *Synechococcus-Chloroflexus* mat was approximately the same as the $\delta^{13}C$ values of the DIC in the vent, which was ~0.5 m upstream from the mat. The $\delta^{13}C$ values of the biomass in the mat collected in 2004 was -23.2‰ vs. Pee Dee Belemnite (PDB) limestone, reflecting a depletion in $^{13}C$ of 19.3‰ relative to the source CO$_2$. The ferrihydrite under the mat...
was isotopically heavier and enriched in $^{13}$C relative to the mats by 8.9‰. The $\delta^{13}$C values biomass in the mat collected in 2005 was slightly lighter than the 2004 mat by -2.7‰.

For individual compounds, biosynthetic fractionation relative to biomass was not reported because the mat biomass was comprised of organics contributed by both cyanobacteria and Chloroflexus. Rather, biosynthetic fractionation relative to source CO$_2$ was calculated ($\varepsilon_b$, defined in Table 4-2). The cyanobacterial acetogenic (straight chain) lipid biomarkers ranged from -28.5 to -29.3‰ in the 2004 mat and -37.3 to -38.4‰ in the 2005 mat. The cyanobacterial polyisoprenoid lipid biomarkers synthesized from the isoprene monomer (e.g., phytol) was -34.3‰ in the 2005 mat. The Chloroflexus wax esters ranged from -23.3 to -25.5‰ in the 2004 mat and -28.5 to -30.0, while the acetogenic long chain tri-unsaturated alkene ($n$-C$_{31,3}$) was -27.1‰ in the 2005 mat. The general acetogenic ester-linked fatty acids ranged from -35.6 to -39.0‰.
Table 4-2. Carbon isotopic compositions of DIC, biomass, and individual compounds.

<table>
<thead>
<tr>
<th></th>
<th>2004 Synechococcus-Chloroflexus</th>
<th>2005 Synechococcus-Chloroflexus</th>
<th>( \varepsilon_8 )</th>
<th>( \varepsilon_7 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mat ( \delta^{13}C ) (‰) v. PDB</td>
<td>( \varepsilon_8 )</td>
<td>mat ( \delta^{13}C ) (‰) v. PDB</td>
<td>( \varepsilon_7 )</td>
</tr>
<tr>
<td>DIC vent</td>
<td>-2.1 ± 0.1</td>
<td>0</td>
<td>-0.9 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>DIC water above mat</td>
<td>-2.0 ± 0.1</td>
<td>0</td>
<td>-1.0 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Biomass</td>
<td>-23.2 ± 0.8</td>
<td>-21.2</td>
<td>-25.8 ± 0.5</td>
<td>-24.8</td>
</tr>
<tr>
<td>Extracted biomass</td>
<td>-22.2 ± 0.1</td>
<td>-20.3</td>
<td>-24.3 ± 0.2</td>
<td>-23.4</td>
</tr>
<tr>
<td>Ferricydrite under mat</td>
<td>-14.5 ± 1.2</td>
<td>-12.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Extracted ferricydriteunder mat</td>
<td>-14.4 ± 0.4</td>
<td>-12.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chloroflexus biomarkers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n-C_{31:3} ) alkene</td>
<td>—</td>
<td>—</td>
<td>-28.1 ± 0.9</td>
<td>-27.1</td>
</tr>
<tr>
<td>( n,n-C_{32} ) wax ester</td>
<td>-26.3 ± 0.3</td>
<td>-24.4</td>
<td>-29.5 ± 1.0</td>
<td>-28.5</td>
</tr>
<tr>
<td>( n,n-C_{33} ) wax ester</td>
<td>-27.4 ± 0.2</td>
<td>-25.5</td>
<td>-30.9 ± 0.9</td>
<td>-30.0</td>
</tr>
<tr>
<td>( n,n-C_{34} ) wax ester</td>
<td>-25.2 ± 1.7</td>
<td>-23.3</td>
<td>-30.2 ± 0.5</td>
<td>-29.2</td>
</tr>
<tr>
<td>Cyanobacterial biomarkers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n-C_{37} ) alkane</td>
<td>-31.2 ± 0.2</td>
<td>-29.3</td>
<td>-39.4 ± 3.1</td>
<td>-38.4</td>
</tr>
<tr>
<td>7-Me, 6-Me ( C_{37} ) alkane</td>
<td>-31.0 ± 0.2</td>
<td>-29.1</td>
<td>-38.3 ± 0.8</td>
<td>-37.3</td>
</tr>
<tr>
<td>7,11-DiMe ( C_{17} ) alkane</td>
<td>-30.4 ± 4.8</td>
<td>-28.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>( n-C_{18} ) polar glycolipid</td>
<td>—</td>
<td>—</td>
<td>-38.3 ± 1.5</td>
<td>-37.3</td>
</tr>
<tr>
<td>phytol</td>
<td>—</td>
<td>—</td>
<td>-35.2 ± 0.7</td>
<td>-34.3</td>
</tr>
<tr>
<td>General lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n-C_{16} ) phospholipid</td>
<td>—</td>
<td>—</td>
<td>-35.6 ± 0.4</td>
<td>-34.7</td>
</tr>
<tr>
<td>( n-C_{18:1} ) phospholipid</td>
<td>—</td>
<td>—</td>
<td>-39.0 ± 0.8</td>
<td>-38.0</td>
</tr>
<tr>
<td>( n-C_{18} ) phospholipid</td>
<td>—</td>
<td>—</td>
<td>-37.0 ± 1.7</td>
<td>-36.0</td>
</tr>
<tr>
<td>( n-C_{16} ) neutral glycolipid</td>
<td>—</td>
<td>—</td>
<td>-35.9 ± 1.9</td>
<td>-35.0</td>
</tr>
<tr>
<td>( n-C_{18:1} ) neutral glycolipid</td>
<td>—</td>
<td>—</td>
<td>-38.6 ± 1.1</td>
<td>-37.6</td>
</tr>
<tr>
<td>( n-C_{18} ) neutral glycolipid</td>
<td>—</td>
<td>—</td>
<td>-38.8 ± 0.7</td>
<td>-37.8</td>
</tr>
<tr>
<td>( n-C_{16} ) polar glycolipid</td>
<td>—</td>
<td>—</td>
<td>-38.2 ± 1.2</td>
<td>-37.3</td>
</tr>
<tr>
<td>( n-C_{18:1} ) polar glycolipid</td>
<td>—</td>
<td>—</td>
<td>-38.3 ± 1.5</td>
<td>-37.3</td>
</tr>
<tr>
<td>( n-C_{18} ) polar glycolipid</td>
<td>—</td>
<td>—</td>
<td>-38.0 ± 1.6</td>
<td>-37.0</td>
</tr>
</tbody>
</table>

\( \varepsilon_8 = 1000(\alpha_8 - 1), \) where \( \alpha_8 = (\delta_{\text{biomass or lipid}} + 1000)/(\delta_{\text{CO}_2} + 1000) \)
4.4 Discussion

4.4.1 Photoheterotrophy vs. Photoautotrophy in Chloroflexus sp.

*Chloroflexus* sp. preferentially grow photoheterotrophically in hot spring systems using cyanobacterial photosynthate (Castenholz and Pierson, 1995), and therefore inherit the δ^{13}C signatures of the cyanobacteria (van der Meer et al., 2003). Cyanobacteria fix CO₂ via the Calvin Cycle to yield organic carbon that is depleted in ^{13}C by -20% to -35% relative to the source CO₂ (Schidlowski 1988; Mojzsis et al., 1996). Consequently, *Chloroflexus* lipid biomarkers such as wax esters display δ^{13}C values similar to that of the Calvin Cycle (van der Meer et al., 2003).

However, *Chloroflexus* is metabolically diverse and has been shown to grow photoautotrophically using sulfide or hydrogen as an electron donor in cultures and in microbial mats at Mammoth hot springs (Castenholz, 1973; Holo and Sirevåg, 1986; Giovannoni et al., 1987). It’s thought that the high level of sulfide at Mammoth poisons the cyanobacteria, allowing pure mats of *Chloroflexus* to develop that oxidize sulfide (Giovannoni et al., 1987). Autotrophic growth of *Chloroflexus* occurs via the 3-hydroxypropionate pathway and yields organic carbon that is ~14‰ depleted in ^{13}C relative to the source CO₂ (Holo and Sirevåg, 1986; Strass and Fuchs, 1993).

While photoautotrophic growth has been demonstrated, it has been shown that photoheterotrophy is the preferred growth mode in cultures (Pierson and Castenholz, 1992) and even in the pure *Chloroflexus* mats at Mammoth (Castenholz and Pierson, 1995). The mats at Mammoth are laminated and the surface layers grow
photoautotrophically using sulfide while the underlayers grow photoheterotrophically by consuming the surface photosynthate (Castenholz and Pierson, 1995).

That being said, recent stable carbon isotopic investigations by van der Meer and colleagues (van der Meer et al., 2000; van der Meer et al., 2003, van der Meer et al., 2005) have demonstrated that Chloroflexus grows both photoautotrophically and photoheterotrophically not only in the sulfidic mats at Mammoth, but also in alkaline silica-depositing springs such as Octopus. This conclusion was initially reached by comparing the $\delta^{13}C$ values of Chloroflexus lipid biomarkers (wax esters and the C$_{31:3}$ alkene) to cyanobacterial lipid biomarkers ($n$-C$_{17}$ alkane). It was noted that the wax esters were enriched in $^{13}C$ relative to the C$_{17}$ alkane, leading the authors to conclude that the isotopically “heavier” signatures were due to photoautotrophic growth via the 3-hydroxypropionate pathway (van der Meer et al., 2000; 2003). Subsequent experiments using $^{13}C$- and $^{14}C$-labeled bicarbonate and acetate revealed a diel variation in the Chloroflexus carbon metabolisms (van der Meer et al., 2005). During the morning, Chloroflexus was found to grow photoautotrophically utilizing the biogenic sulfide and hydrogen that had accumulated in the mats during the night. As the light intensity increased, cyanobacterial oxygen production oxidized all of the sulfide and hydrogen and Chloroflexus switched to a photoheterotrophic mode and consumed the cyanobacterial metabolites (van der Meer et al., 2005).
4.4.2 Chocolate Pots Hot Springs Chloroflexus *sp.*

Initial comparison of the lipid biomarkers in the *Synechococcus-Chloroflexus* mats at Chocolate Pots hot springs also revealed that the *Chloroflexus* wax esters and C\textsubscript{31:3} alkene were enriched in $^{13}$C relative to the cyanobacterial acetogenic alkanes and C\textsubscript{18:1} polar glycolipid. However, the biosynthetic fractionation relative to biomass of the different lipid classes, acetogenic and polyisoprenoid, must be taken into account when comparing the isotopic compositions of lipid biomarkers. For instance, in a study of the unicellular freshwater cyanobacterium *Synechocystis*, the acetogenic lipids were found to be depleted in $^{13}$C by 9.1\% relative to total biomass (Sakata et al., 1997). Taking into account that fractionation factor, the $\delta^{13}$C values of the acetogenic alkanes and C\textsubscript{18:1} polar glycolipid are similar to the *Chloroflexus* wax esters and C\textsubscript{31:3} alkene, as well as the total biomass. The similarity of these data suggest that *Chloroflexus* is not oxidizing Fe\textsuperscript{2+} and is growing photoheterotrophically at Chocolate Pots by consuming the cyanobacterial photosynthate and thus inheriting the Calvin Cycle isotopic signature.

This conclusion is supported by in situ scanning voltammetric microelectrode measurements of the *Synechococcus-Chloroflexus* mats (Trouwborst et al., in review). Filters were placed over the mats in a light-tight apparatus to restrict the wavelengths of light to the near-infrared (NIR) so that only bacteriochlorophylls *a* and *c* in *Chloroflexus* could function. Simultaneous measurement of O\textsubscript{2}, Fe\textsuperscript{2+}, Mn\textsuperscript{2+}, H\textsubscript{2}S, polysulfides, and thiosulfate revealed that when exposed to NIR, no O\textsubscript{2} was evolved and no Fe\textsuperscript{2+} oxidation occurred, suggesting that anoxygenic photoferrotrophy by
Chloroflexus was not occurring in these mats (Trouwborst et al., in review).

4.4.3 New Photoferrotrophic Candidate

Because we have eliminated Chloroflexus as the potential photoferrotrophic organism in the Synechococcus-Chloroflexus mat, that leaves a cyanobacterial candidate to explain the stimulation of $^{14}\text{C}$-bicarbonate uptake in the presence of Fe$^{2+}$ (Pierson et al., 1999). Based on the observed deposition of iron oxides (ferrihydrite) in its periplasmic space (Parenteau and Cady, in preparation), Cyanothece minervae has become the candidate photoferrotroph. Although the mechanism of ferrihydrite deposition is not known, one of our working hypotheses is that the cells walls become “leaky” due to the action of lytic enzymes during the earliest stages of cell death. Another working hypothesis is that Fe$^{2+}$ diffuses into the cell through the outer membrane porin channels, which only allows the passage of small solutes such as ions (Hoiczyk and Hansel, 2000). Once inside the periplasmic space, the cyanobacterium could use the Fe$^{2+}$ as an electron donor for photosynthesis.

Although deposition of ferrihydrite in the periplasmic space would seem detrimental to the cell, the presence of very large carboxysomes may alleviate the impairment of transport across the cell wall. Carboxysomes store the Calvin Cycle enzyme RuBisCo (Shively et al., 1973). Amassing large amounts of RuBisCo facilitates rapid fixation of CO$_2$ in carbon-limited environments (Kaplan et al., 1989; Price and Badger, 1991). However, the water flowing over the Synechococcus-Chloroflexus mat is very high in DIC (11.3 mM) relative to other hot springs (Jahnke
et al., 2004), so the cells are not carbon-limited. The abnormally large carboxysomes may facilitate rapid CO₂ fixation as the periplasmic space becomes mineralized via Fe²⁺ oxidation.

### 4.4.4 Cyanobacterial Photoferrotrophy

Cohen has suggested (1984, 1989) that cyanobacteria may be capable of directly oxidizing Fe²⁺ without producing oxygen. It is not clear from Cohen’s early findings whether cyanobacteria use Fe²⁺ as an electron donor for photosystem I (PSI) or photosystem II (PSII) (1984, 1989). Cohen and colleagues (1986) did demonstrate that some mat-forming cyanobacteria, such as marine *Microcoleus* sp. and some *Oscillatoria* species from hot springs use sulfide to sustain anoxygenic photosynthesis. Sulfide appears to donate electrons exclusively to PSI. Both sulfide and Fe²⁺ can donate electrons to PSII-related RC2 reaction centers in purple bacteria (Widdel et al., 1993; Ehrenreich and Widdel, 1994a,b). It is plausible that Fe²⁺ can donate electrons to the cyanobacterial PSII, and has been postulated that such a mechanism was part of an evolutionary process leading to the water-oxidizing PSII in cyanobacteria (Olson and Pierson, 1987; Pierson and Olson, 1989; Olson, 2006). The widespread abundance of reduced iron on the early Earth prior to the appearance of oxygen would have made it particularly suitable as an electron donor for photosynthesis. Such an organism would join the purple and green bacteria and chemotrophs in potentially mediating Fe²⁺ oxidation in the deposition of Precambrian BIFs in an ancient anoxic world. We are currently testing photoferrotrophy in our candidate cyanobacterium.
4.5 Acknowledgments

This work was supported by NASA Exobiology Grant NAG5-12328 to S.L. Cady. Additional support was generously provided by a NASA Oregon Space Grant Graduate Fellowship and a NASA Planetary Biology Internship to M.N. Parenteau. We thank the National Park Service for allowing us to conduct research in Yellowstone National Park and R.C. Hugo and R.B. Perkins, Portland State University, for technical assistance.
Chapter 5. Characterization of Natural 2-line and 6-line Ferrihydrite from a High-Iron Thermal Spring

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5.1 Introduction

Amorphous iron oxides (ferrihydrites) are important minerals found in many natural environments. They are very difficult to characterize due to their susceptibility to beam damage, so their structure and chemical formula is still under investigation (Eggleton and Fitzpatrick, 1988; Janney et al., 2000a, 2000b; Janney et al., 2001; Garvie et al., 2004; Martin et al., 2005). Synthetic and natural ferrihydrites display a range in order and can be categorized on the basis of their XRD patterns as 2-line ferrihydrite and the slightly more crystalline 6-line ferrihydrite (Carlson and Schwertmann, 1981; Cornell and Schwertmann, 1996). These ferrihydrites are not truly amorphous, but contain extremely small coherent scattering domains, the size of which identifies them as 2-line or 6-line (Drits et al., 1993). These ferrihydrites are
also thermodynamically unstable and are thought to transform to more ordered Fe$^{3+}$ oxides such as goethite or hematite via dissolution and re-precipitation or a solid state transformation, respectively (Feitknecht and Michaelis 1962; Schwertmann and Murad 1983). Questions remain as to whether 2-line ferrihydrite transforms to 6-line ferrihydrite (Kukkadupu et al., 2003) or whether they precipitate as two separate phases (Schwertmann et al., 1999; Schwertmann and Cornell, 2000).

5.1.1 Ferrihydrite Structure

The initial description of ferrihydrite structure came not from direct observation, but from modeling bulk XRD patterns. Drits et al. (1993) suggested that ferrihydrite is composed of hexagonal unit cells with the dimensions of $a = 2.96$ Å and $c = 9.40$ Å. The iron was found to be in octahedral coordination, but whether there was corner-, edge-, or face-sharing between octahedra was unknown (Manceau and Drits, 1993). The water content of the ferrihydrite, whether it was adsorbed or structural, was also unknown.

High resolution transmission electron microscopy (HRTEM) provided information about the structure of ferrihydrite that was not available from bulk analyses. Particles of ferrihydrite dispersed on a holey carbon supporting film revealed the presence of lattice fringes in individual crystallites (Janney et al., 2000a). However, these regions were only 1-3 nm in diameter and were scattered amongst areas that lacked lattice fringes. No intersecting lattice fringes were observed, which led the authors to suggest that there was no three-dimensional order in the sample.
Selected area electron diffraction (SAED) of ferrihydrite revealed two amorphous rings at 2.5 and 1.5 Å (Janney et al., 2000a). Individual crystallites were oriented along their zone axis and electron nanodiffraction patterns were obtained from areas <1 nm across. Rather than spots, the resulting pattern was composed of diffuse streaks (Janney et al., 2000a, 2000b). This supported the HRTEM observations that there was only two-dimensional structure to the ferrihydrite. Comparison of the experimental nanodiffraction patterns to simulated ones suggested that the two-dimensional structure was composed of hexagonal and cubic stacking of closed packed layers of O$^{2-}$ and OH$^{-}$ ions with Fe randomly distributed (Janney et al., 2000b).

### 5.1.2 Transformation Pathways

Diagenetic transformation of ferrihydrite to more ordered iron minerals such as goethite or hematite occurs readily by one of two mechanisms: dissolution and re-precipitation or a solid state process of internal atomic rearrangements (Feitknecht and Michaelis 1962; Schwertmann and Murad 1983). These competing mechanisms are thought to be controlled by solution pH with goethite being favored at pH 4 and 11 and hematite at pH 8 (Schwertmann and Murad, 1983).

Recent work by Banfield et al. (2000) has revealed aggregation-based crystal growth as an additional transformation mechanism. HRTEM was used to examine 2-3 nm particles of ferrihydrite attached to the negatively charged cell surface of
**Gallionella.** The particles rotated due to a variety of forces (such as Brownian motion) so that they lined up in a low-energy, parallel fashion in three dimensions. This gradual alignment lead to a polycrystalline material of larger particle size. In some of the samples, goethite was intergrown within regions of aligned ferrihydrite. It was suggested that goethite could be formed from ferrihydrite by relocating iron atoms into adjacent face-sharing octahedral sites and small displacements of every fourth oxygen plane (Banfield et al. 2000).

Questions remain regarding the initial steps in the transformation mechanism of 2-line ferrihydrite to more ordered forms. Specifically, there is conflicting data whether 2-line ferrihydrite recrystallizes to 6-line ferrihydrite en route to goethite or hematite (or as a final product) (Fredrickson et al., 2001; Rancourt et al., 2001; Kukkadupu et al., 2003), or whether 2-line and 6-line precipitate as two separate phases under different conditions (Schwertmann et al., 1999; Schwertmann and Cornell, 2000). In an examination of synthetic ferrihydrites, Kukkadupu et al. (2003) observed that particles of 2-line ferrihydrite aggregated to form 6-line ferrihydrite during a three year ageing experiment. In contrast, Schwertmann and Cornell (2000) detail protocols for 2-line and 6-line ferrihydrite synthesis in the laboratory by varying the rate of hydrolysis of Fe$^{3+}$ salt solutions at room temperature, or by oxidizing Fe$^{2+}$ salt solutions in the presence of various concentrations of dissolved silica.

**5.1.3 Examination of Natural 2-line and 6-line Ferrihydrite**

Chocolate Pots hot springs in Yellowstone National Park are a series of high
iron thermal springs that are precipitating 2-line and 6-line ferrihydrite in a unique layered deposit. We characterized these natural ferrihydrites to determine whether they precipitated as two separate phases under different solution chemistries, or whether the 2-line re-crystallized to 6-line. Examination of these two mechanisms in a natural system may help elucidate the transformation pathway of these poorly crystalline, thermodynamically unstable phases to more ordered Fe\textsuperscript{3+} oxides.

5.2 Methods

5.2.1 Sample Collection

Samples of a distinct black mineralogical facie were collected from the steep face of the main iron deposit at Chocolate Pots hot springs in Yellowstone National Park (Fig. 5-1). The “black streak” samples were actually a layered deposit of alternating black and orange material. Samples measuring approximately 2 cm x 2 cm were excised with an ethanol-cleaned spatula and immediately placed in sealed Tupperware containers to prevent desiccation during transport to the laboratory. The moist samples were then air-dried at room temperature to avoid inducing phase transformations. Samples were not collected anoxically under an atmosphere of nitrogen because the Fe\textsuperscript{2+} in the water is rapidly oxidized by microbial mats supersaturated with O\textsubscript{2} as well as by atmospheric oxygen mixing in the outflow channels (Pierson et al., 1999; Trouwborst et al., in review). Exposing the samples to atmospheric oxygen levels upon removal would not alter redox state of sample.
5.2.2 Water Chemistry

The temperature, pH, and aqueous Fe\textsuperscript{2+} concentration of the vent pool and water immediately above the sample were measured as previously described (Parenteau and Cady, in preparation). Water samples for cation analysis were filtered (Nalgene surfactant-free cellulose acetate, 0.2 \(\mu\text{m},\) Rochester, NY) into nitric acid cleaned low-density polyethylene (LDPE) bottles and acidified to pH 0.9 with trace metal grade nitric acid (Fisher, Pittsburgh, PA) and stored at 4°C until analysis. Samples were analyzed for Fe and Mn within two months by inductively coupled plasma mass spectroscopy (ICP-MS) by XRAL Laboratories, Ontario, Canada and on a HP-4500 ICP-MS at Portland State University using a Claritas PPT standard (Lot #26-110AS, SPEX CertiPrep, Metuchen, NJ). Aqueous silica was measured immediately upon collection in the field using the USGS molybdate blue colorimetric assay (Fishman and Friedman, 1989) and a Hach DR/2400 portable spectrophotometer. Ca and Fe are known to interfere with the reaction and were removed by complexing with disodium dihydrogen ethylenediamine tetraacetate (Na\textsubscript{2}EDTA) according to the USGS protocol. A solution of Na\textsubscript{2}SiF\textsubscript{6} was used as a standard.

5.2.3 X-Ray Diffraction (XRD)

The individual black and orange phases of the “black streak” layered deposit were separated by physically scraping the orange material from the hard, black material. These two fractions were each subsequently purified by allowing them to
settle in a graduated cylinder filled with 18MΩ H₂O for 45 minutes. The <2 μm Stokes’ size fraction of the orange material was collected and air-dried. The black material, which instantly settled to the bottom, was collected and sonicated for 30 seconds to remove any remaining orange material and re-suspended. The purified black settled material was collected and air-dried and ground into a clay-size fraction using an agate mortar and pestle.

The powdered samples were side-packed into aluminum holders using a frosted slide to ensure random orientation of the grains. Sample mounts were step scanned from 2.5 to 75° 2θ using a step size of 0.020° and a count time of 25 sec per step for a total of 25.5 hours per analysis. The samples were run on a Philips X’Pert PW3040 X-ray diffractometer at 40 kV and 30 mA using copper K-alpha radiation. Samples were also analyzed using cobalt K-alpha radiation from 2.5 to 85° 2θ using the same parameters listed above. The peaks on the powder XRD diffraction patterns were identified by comparing the measured sample d-spacings to previously published iron oxide d-spacings (Cornell and Schwertmann, 2003).

5.2.4 Light Microscopy

Stereomicroscope images of air-dried samples were collected with a Nikon SMZ800 equipped with a Leica DFC480 camera. Compound microscope transmitted light images were collected on a Leica DMRX equipped with a Leica DFC480 camera.
5.2.5 Scanning Electron Microscopy (SEM)

The air-dried black and orange layered deposit was prepared for SEM analysis in two different ways. Grain mounts were prepared by placing the black and orange fractions examined on the XRD in 100% ethanol and placing a drop of each suspension on ethanol-cleaned aluminum stubs. The layered deposit was also prepared as a petrographic thin section with an electron microprobe polish by Spectrum Petrographics, Vancouver, WA. The thin section was affixed to the slide using a heat soluble epoxy (Crystalbond 509 thermoplastic cement). Samples were examined on a JEOL JSM 35-C SEM operating at 15-25 kV. Samples were not coated prior to viewing. The SEM was equipped with a KEVEX energy dispersive X-ray spectrometer (EDS). EDS X-ray maps of Fe and Si were acquired at 25 kV for 120 sec with approximately 20% dead time using a 15 mm working distance and 45° sample tilt.

5.2.6 Transmission Electron Microscopy (TEM)

A core of the petrographic thin section of the layered deposit was removed by placing a slotted Cu grid over a region of interest containing both the black and orange phases. This area was heated to melt the epoxy and the material surrounding the Cu grid was cut away using an ethanol-cleaned razor blade. The Cu grid containing the core of the thin section was thinned to electron transparency using a Gatan Model 691 Precision Ion Polishing System operated at 4 kV accelerating voltage and 4° ion gun tilt. Sample was ablated for approximately 200 minutes to achieve electron
transparency. Sample was examined with a JEOL 100CXII TEM operating at 100 kV accelerating voltage. Images were recorded on Kodak SO-163 film (Ted Pella, Rochester, NY). The TEM camera length was calibrated using a polycrystalline evaporated aluminum standard (EMS, Hatfield, PA) for calculation of d-spacings on the electron diffraction patterns.

5.2.7 Electron Microprobe Analyses

The electron microprobe polished petrographic thin section of the layered deposit was carbon coated to a depth of 40 nm using an Edwards E306A Thermal Evaporator at the University of Oregon. The thin section was analyzed with a Cameca SX 50 electron microprobe at the University of Oregon using a 5 µm and 20 µm spot size and 10 nA beam. Sample damage occurred with the 5 µm spot size, so that data was discarded. A sample traverse across microbands of the black (23 spot analyses) and orange phases (11 spot analyses) was conducted (Fig. 5-5). A zonation rim between the two phases was also examined (5 spot analyses). Magnetite and a volcanic glass were used as standards. An amphibole standard examined as an unknown indicated there was 4-4.9% error in the analyses.

5.2.8 Diagenesis Experiments

Samples of the black and orange streaks were collected from the steep face of the main iron deposit and from a vent near the river. Samples were kept moist during transport to the laboratory. One split of the samples was air-dried and analyzed on the
XRD as "raw" sample. The two other splits were incubated for a month at 65°C and 150°C to determine whether heating induced phase transformations.

5.3 Results and Discussion

5.3.1 Mineralogical Facies

Chocolate Pots hot springs are a series of high iron thermal springs that flow into the Gibbon River approximately 5 km south of Norris Geyser Basin (Parenteau and Cady, in preparation). The emerging anoxic vent waters are very high in Fe²⁺, which is oxidized by oxygen produced by cyanobacterial mats in the vent pools as well as by turbulent mixing with atmospheric oxygen in the outflow channels (Pierson et al., 1999; Trouwborst et al., in review). Colorful iron sinter deposits accumulate along the steep riverbank. Iron oxides and oxyhydroxides form distinct streaks of color down the steep face of the iron deposits and range from 5 - 90 cm in width (Fig. 5-1). Three separate mineralogical facies have been identified. A hard, deep burgundy to black-colored deposit that appears as vertical black streaks is found exclusively in outflow channels that experience rapid and voluminous flow (Figs. 5-1, 5-2, 5-3, 5-4). A soft orange ochre precipitate alternates with the black streaks and experiences more moderate flows (Figs. 5-1, 5-3). A red terrace material experiencing sheet flow also alternates with the black streak and orange ochre and is consistently populated by a cyanobacterial microbial mat comprised of filaments of narrow
*Oscillatoria* (Parenteau and Cady, in preparation) (Fig. 5-2). The orange ochre and the red terrace have been identified as 2-line ferrihydrite in a previous study (Parenteau and Cady, in preparation). 2-Line ferrihydrite is the primary precipitate in the outflow channels and is the only mineral associated with the cyanobacterial mats (Parenteau and Cady, in preparation).

Figure 5-1. Field image of Chocolate Pots hot springs showing two distinct mineralogical facies on the steep face of the main iron deposit. A black-colored precipitate occurs as vertical streaks is found exclusively in outflow channels that experience rapid and voluminous flow. A soft orange ochre precipitate (ferrihydrite) experiences more moderate flows.
Figure 5-2. Field image of black streak (BS) and a third mineralogical facie, a red terrace (RT) material. This red terrace material (also ferrihydrite) is consistently populated by a cyanobacterial microbial mat.

Figure 5-3. Field image of black streak (BS) and ferrihydrite (Fh).
5.3.2 Black Streak Layered Deposit

Examination of the surface of the black streak in detail revealed that two shades of dark material were present, black and burgundy (Fig. 5-4a). Stereomicroscopic images of the black streak demonstrated that the shades were not two different mineralogical phases, but represented varying thickness of the black material (Figs. 5-4b, c). The burgundy color resulted from a very thin deposition of the black material on orange ferrihydrite, while the black color resulted from a thicker deposition.

Hand and petrographic thin sections revealed that the black streak was actually a layered deposit comprised of alternating black material and orange ferrihydride.
The isotropic black material appeared blood red in transmitted light, while the orange ferrihydrite material was opaque (Figs. 5-5a, b). The interface between the two regions was marked by a rim that tended to fracture vertically along its length (Figs. 5-5b, c).

A backscattered SEM micrograph of the area shown in Fig. 5-5b revealed that the blood red material appeared brighter than the opaque areas and thus had a higher average atomic number (Fig. 5-5c). Magnification of the sample revealed very fine micro-banding within each phase (Fig. 5-6a). X-ray mapping showed a slightly higher abundance of Fe in the blood red areas (Fig. 5-6b). This was confirmed by EDS spot analyses which also showed relatively more Fe in the blood red bands. The Fe:Si ratio in these areas averaged 2.5, and averaged 1.9 in the opaque ferrihydrite bands.
Grain mounts of the fine-grained black/blood red phase showed conchoidal fracturing (Fig. 5-7a), while the opaque ferrihydrite appeared as aggregates of small particles (Fig. 5-7b).

![Figure 5-6](image_url)

Figure 5-6. Backscattered SEM micrograph of the petrographic thin section of the black streak layered deposit. The black material, which appears red in transmitted light, is the light area higher in atomic number. The orange ferrihydrite, which appears opaque in transmitted light, is the darker material. X-ray mapping shows a higher relative concentration of Fe (B) and Si (C) in the black/blood red material.

Interestingly, XRD identified the black/blood red phase as 2-line ferrihydrite and the opaque ferrihydrite phase as the more crystalline 6-line ferrihydrite (Fig. 5-8). Minor peaks of 6-line ferrihydrite were present in the black/blood red phase as well and may be attributed to incomplete purification of the two phases. To confirm the assignment of 2-line and 6-line ferrihydrite to the specific layers within the deposit, electron diffraction (ED) was performed on area 3 in Fig. 5-5c of the ion-milled thin section. 6-Line ferrihydrite with d-spacings of 2.6 – 1.46 Å was confirmed as the identity of the orange material that appeared opaque in transmitted light (Fig. 5-9).
Figure 5-7. Secondary electron SEM micrograph of the purified phases of the black streak layered deposit analyzed by XRD. A. Black material (2-line ferrihydrite) displayed conchoidal fracturing. B. Orange ferrihydrite (6-line) appeared as aggregates of small particles.

Electron microprobe analyses corroborated the backscatter, X-ray mapping and EDS spot analyses and also demonstrated that the black/blood red 2-line ferrihydrite was comprised of a higher concentration of Fe and Si than the 6-line ferrihydrite (Table 5-2). Because the structural formula of ferrihydrite is not currently agreed upon, the chemical composition of the ferrihydrites could not be calculated from the
oxide data, so the results of the analyses are reported as weight percent. Additionally, the totals were less than 100% and may be a reflection of beam damage even though the spot size was increased from 5 to 20 μm. This beam damage may have resulted in a loss of water from these highly hydrated samples because the oxygen totals decreased along the traverse from the 2-line to 6-line ferrihydrite.

Figure 5-8. Powder XRD pattern of the purified phases of the black streak layered deposit. The black, conchoidally fracturing phase was identified as 2-line ferrihydrite (lower grey pattern), while the orange phase was identified as 6-line ferrihydrite (upper black pattern).

Table 5-2. Electron microprobe analyses of petrographic thin section of layered deposit. Areas of analyses are numbered in Fig. 5c.

<table>
<thead>
<tr>
<th>Elemental WT%</th>
<th>Area 1</th>
<th>Area 2</th>
<th>Area 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black material</td>
<td>Alteration rim</td>
<td>Orange ferrihydrite</td>
</tr>
<tr>
<td>Ca</td>
<td>0.66</td>
<td>0.54</td>
<td>0.31</td>
</tr>
<tr>
<td>K</td>
<td>0.02</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Fe</td>
<td>43.57</td>
<td>31.74</td>
<td>34.59</td>
</tr>
<tr>
<td>Mn</td>
<td>0.28</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>Mg</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Na</td>
<td>0.09</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>Al</td>
<td>1.65</td>
<td>0.98</td>
<td>1.09</td>
</tr>
<tr>
<td>Ti</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Si</td>
<td>8.05</td>
<td>9.04</td>
<td>6.32</td>
</tr>
<tr>
<td>O</td>
<td>42.38</td>
<td>30.22</td>
<td>27.75</td>
</tr>
<tr>
<td>Cl</td>
<td>0.01</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>S</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>P</td>
<td>0.02</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td>96.8</td>
<td>73.2</td>
<td>70.7</td>
</tr>
</tbody>
</table>
It is unexpected that the 2-line ferrihydrite contained a higher abundance of Fe than the 6-line ferrihydrite. 6-Line ferrihydrite is more crystalline with larger coherent scattering domains (Drits et al., 1993), which should reflect a higher spatial concentration of Fe atoms. However, the SEM micrographs display conchoidal fracturing of the 2-line ferrihydrite, suggesting that this fine-grained siliceous material may be more densely packed than the more porous 6-line ferrihydrite.

The higher concentration of silica in the 2-line ferrihydrite as well as the dense packing of Fe atoms suggest that these natural ferrihydrites precipitated as two separate phases under different solution chemistries, supporting the assertions of Schwertmann and Cornell (1999, 2000). Indeed, in a study of synthetic ferrihydrite synthesis, oxidation of Fe$^{3+}$ salts solutions in the presence of various concentrations of dissolved silica generated a range of ferrihydrites (Schwertmann and Cornell, 2000).
2-Line ferrihydrite formed at the highest concentrations of silica tested, while 6-line formed at medium concentrations and lepidocrocite/goethite at the lowest concentrations (Schwertmann and Cornell, 2000).

5.3.3 Solution Chemistry Controls Ferrihydrite Formation?

The concentration of silica in the waters at Chocolate Pots hot springs is approximately 135 mg/L (Table 5-1). The solubility of silica in hydrothermal systems is predominantly controlled by pH (Govett, 1961). The pH of the waters flowing over the different mineralogical facies can differ on a cm-scale due to flow rate, which controls CO₂ outgassing. For example, the pH of the water flowing over the black streak in Fig. 5-2 is 6.5 (Table 5-1). The pH of the water flowing over the red terrace ~20 cm away is 7.5. This increase of one pH unit is due to the thin sheet flow over the red terrace, which allows for more extensive CO₂ outgassing and concomitant increase in pH. This is supported by measurements of dissolved inorganic carbon (DIC) in the vents and along outflow channels, which decreases with distance from the vent (Chapter 4).

Similarly, the pH above the black streak is typically more acidic than the pH above the orange ferrihydrite and is related to flow characteristics and CO₂ outgassing (Fig. 5-3). The flow rate and volume change rapidly over these facies due to perturbations upstream, and the consequent change in pH may affect silica solubility, which in turn controls whether 2-line or 6-line ferrihydrite is precipitated.
Table 5-1. Geochemical measurements of thermal waters at Chocolate Pots hot springs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Fe$^{2+}$ (mg/L)</th>
<th>Fe (mg/L)</th>
<th>Mn (mg/L)</th>
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<td>5.4*</td>
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Abbreviations: SMR, South Mound River; MM, Main Mound. *Fe and Mn analyzed by XRAL Laboratories, Ontario, Canada. All other analyses were performed in the field or at Portland State University in the Trace Element Laboratory.

The alternation of these facies in the layered deposit may be due to the changes in flow mentioned above. To test this hypothesis, the solution chemistries of the waters flowing over adjacent black streak and orange ferrihydrite facies were analyzed during the summers of 2004, 2005, and 2006 (Table 5-1). The data indicate that there is not a strong correlation between the range of pH values measured (5.6 -7.5) and silica concentration. Even though this is a change of 2 pH units, it still falls within the range where silica solubility is stable (1-8) (Govett, 1961). It is only above pH 9 that the
solubility changes. This indicates that at Chocolate Pots hot springs, flow rate, CO₂ outgassing, and concomitant changes in pH do not affect silica solubility to determine whether 2-line or 6-line ferrihydrite precipitates.

5.3.4 Surface Complexation of Silica

Ferrihydrite has a very large reactive surface area that can adsorb or form coprecipitates with a wide variety of organics, cations, and anions (Martin et al., 2005). In a study of the surface structure of ferrihydrite by X-ray absorption fine structure (XAFS), Zhao et al. (1994) described H₂O adsorbed to the surface of the ferrihydrite, which completes the surface Fe coordination. When this water is removed, the vacancy is considered coordination unsaturated and consequently becomes a crystallization site for the phase transformation of ferrihydrite to hematite at low temperatures (Zhao et al., 1994). Impurities such as the silicate ion (SiO₄²⁻) can block these sites and prevent the transformation of ferrihydrite to more ordered forms (Zhao et al., 1994; Cornell and Schwertmann, 2003).

Field and stereomicroscopic images of the black streak revealed that the black siliceous 2-line ferrihydrite coated all fresh orange ferrihydrite deposits where co-occurring (Fig. 5-4). The coating began as a thin film that appeared burgundy in color and gradually increased in thickness until it appeared black. This is apparent in Figs. 5-4 b and c where a fresh deposit of ferrihydrite settled on the black streak and was promptly coated in the black 2-line ferrihydrite, which graded to burgundy at the distal edge where fresh ferrihydrite was still visible. This observation suggests that within
the black streak layered deposit, the siliceous 2-line ferrihydrite may be coating and saturating the flocculant 6-line ferrihydrite. This 6-line ferrihydrite, which may be favored to form in the elevated temperatures of the vent waters (Schwertmann and Cornell, 2000), is likely sent downstream in pulses from the frequent disturbances upstream. The layered deposit may be generated from the alternation of these phenomena.

However, HRTEM analyses of the micro-banding of this deposit, as shown in Fig. 5-6a, is needed to examine the distribution of silica. It is a possibility that once the surface crystallization sites are saturated by the silicate ion that ferrihydrite begins to precipitate again. Structural and chemical analysis of this potential phenomenon need to take place on a scale relevant to the small crystallite size of the ferrihydrites using electron nanodiffraction and nanometer-scale electron energy loss spectroscopy (EELS), respectively. Whether or not this surface complexation phenomenon is occurring, it is clear that 2-line ferrihydrite is not transforming to 6-line ferrihydrite in these natural samples because all of the crystallization sites in the siliceous 2-line ferrihydrite are blocked. Samples heated for one month at 65°C and 150°C show now diagenetic transformations (Fig. 5-10). These data support the observations of Schwertmann and Cornell (1999, 2000).
Figure 5-10. Powder XRD patterns of heating experiments. Black patterns are the air-dried raw samples, dark grey patterns are the samples heated for 1 month at 65°C, light grey patterns are the samples heated for 1 month at 150°C. A. Black streak layered deposit from main iron deposit. B. Ferrihydrite from main iron deposit. C. Black streak layered deposit from a small vent near the river termed “South Mound River” vent.
5.4 Acknowledgments

This work was supported by NASA Exobiology Grant NAG5-12328 to S.L. Cady. Additional support was generously provided by a NASA Oregon Space Grant Graduate Fellowship and a NASA Planetary Biology Internship to M.N. Parenteau. We thank the National Park Service for allowing us to conduct research in Yellowstone National Park and R.C. Hugo, R.B. Perkins, and G. Grathoff, Portland State University, for technical assistance.
6.1 Iron Biogeochemistry in Modern Environments

6.1.1. Phototrophic iron cycling

The ongoing interdisciplinary study at Chocolate Pots hot springs is the first in situ study of Fe$^{2+}$ oxidation mechanisms of phototrophs (Pierson et al., 1999; Pierson and Parenteau, 2000; Trouwborst et al., in review) and the first description of iron mineralization and biosignature formation in the phototrophic mats (this study). Our collaborators assessed the relative contributions of oxygenic photosynthesis, which produces O$_2$ as an oxidizing waste product, and anoxygenic photosynthesis in which Fe$^{2+}$ is directly oxidized, to the oxidation of Fe$^{2+}$ in the anoxic waters of the hot spring (Trouwborst et al., in review). They observed that O$_2$ produced by the cyanobacteria was the sole mechanism by which the Fe$^{2+}$ was oxidized, distinct from atmospheric O$_2$ and anoxygenic photosynthesis (Trouwborst et al., in review). This demonstrates the efficiency of oxic versus anoxic oxidation, and suggests that the kinetics of Fe$^{2+}$ oxidation are not favorable for anoxygenic phototrophs in this system.

Other studies have examined Fe$^{2+}$ oxidation using laboratory cultures of purple and green anoxygenic phototrophs (Widdel et al., 1993; Ehrenreich and Widdel, 1994a,b; Heising and Schink, 1998; Heising et al., 1999; Straub et al., 1999). However, it is widely recognized that laboratory cultures are optimized for the fastest
growth rates and do not reflect the environmental conditions from which they were isolated (Madigan and Martinko, 2006). Therefore, in situ studies, while they are much more tedious to perform, are worthwhile in assessing the metabolic impact of organisms in natural, complex settings.

6.1.2 Iron Mineralization

Our collaborators have examined Fe$^{2+}$ oxidation in the phototrophic mats at Chocolate Pots hot springs and have shown that while cyanobacteria are performing photosynthesis in the light, Fe$^{2+}$ removal is rapid and complete (Trouwborst et al., in review). This companion study has examined the resulting mineralization of the mats by the Fe$^{3+}$ oxidation products to generate a suite of biosignatures. Two modes of mineralization were observed: encrustation and permineralization.

Encrustation results from interaction with the Fe$^{3+}$ assemblages with the exterior of the cell. Specifically, functional groups present on the cell wall and sheath such as carboxyl, phosphoryl, or amine deprotonate with increasing pH and thus impart a net negative surface charge (Phoenix et al., 2002). Cations such as Fe$^{3+}$ interact electrostatically with the negatively charged functional groups and these points act as nucleation sites for mineral precipitation. This is in contrast to silica mineralization where the negatively charged silicic acid displays a low affinity for the negatively charged cell surface, regardless of whether the solution is supersaturated or undersaturated with silica (Phoenix et al., 2003; Yee et al., 2003). It is thought that silica mineralization of cyanobacteria in hot springs is purely an abiotic process with
the cells surface playing no active role (e.g., Benning et al., 2005). Iron mineralization of cyanobacteria in hot springs, however, is comprised of two active processes (1) Fe$^{2+}$ oxidation by the organisms and (2) active interaction of the resulting Fe$^{3+}$ with the cell surface.

Permineralization results from nucleation of the Fe$^{3+}$ assemblages inside of the cells. It is likely that the Fe$^{3+}$ enters the cell during the earliest stages of cell death as the integrity of the cell wall is compromised due to the action of lytic enzymes such as peptidoglycan hydrolases (Ferris et al., 1986). Once inside the cell, Fe$^{3+}$ interacts electrostatically with the functional groups present in the periplasmic space as well as those present on the photosynthetic membranes, and iron mineral precipitation proceeds.

It has been well documented that silica permineralization generates cellularly preserved microfossils in chert layers in the rock record (e.g., Barghoorn and Tyler, 1965; Walsh and Lowe, 1985). Consequently, many studies have been aimed at characterizing the silicification of modern microbial communities in thermal springs (e.g., Cady and Farmer, 1996; Jones et al., 1998). Laboratory studies focus on the experimental silicification of model organisms to elucidate the mechanisms of silica permineralization (e.g., Phoenix et al., 2002; Konhauser et al., 2004). It is permineralization, rather than encrustation, which leads to microfossils with the highest fidelity.

In contrast to silica, it is thought that iron does not preserve cells well. Studies to date of iron mineralized microbial mats in subaerial thermal springs (Konhauser and
Ferris, 1996; Reysenbach et al., 1999) have demonstrated poor preservation of the microbes with cellular details obscured by the iron precipitates (Konhauser et al., 2003). However, this study has shown that the iron permineralized photosynthetic mats at Chocolate Pots hot springs displayed excellent microfossil fidelity and retained taxonomic features that allowed for positive identification of some of the phototrophs.

6.1.3 Biosignatures

These two modes of iron mineralization have resulted in the formation of distinct phototrophic biofabrics and microfossils at Chocolate Pots hot springs. The formation of dendritic biofabrics was dependent on the presence of vertically oriented cyanobacterial filaments which provided nucleation sites where encrustation was initiated. As noted above, iron permineralization of cells generated cellularly preserved microfossils. Geologically significant lipid biomarkers such as mono- and dimethylalkanes and 2methyl-BHP were produced in the mats.

These biosignatures also survived with depth in the ferrihydrite underneath the mats. Bulk TOC values provide a general estimate that 15% of the organics survived microbial degradative processes and the early stages of burial, while quantification of phospholipids and glycolipids revealed that 2 - 10% of these membrane components survived. These data indicate that there is excellent preservation of organic material by iron.

As previously noted, a subaerial thermal spring is not a suitable global model for BIF deposition, however it does provide a site to study the interaction of
hydrothermal fluids rich in reduced iron, silica, and carbonates with cyanobacteria and other anoxygenic phototrophs.

6.2 Iron Biogeochemistry in Ancient Environments

6.2.1 Phototrophic Iron Cycling in Ancient Environments

Our collaborators extrapolated their \textit{in situ} voltammetric microelectrode measurements of Fe$^{2+}$ oxidation by phototrophs in a natural system to the Precambrian to assess the potential impact of oxygenic photosynthesis on ancient iron cycling. They compared their rates of \textit{in situ} biological Fe$^{2+}$ oxidation to the actual geological iron oxide accumulations of BIFs of the Hamersley Group in the Pilbara Craton, Western Australia (Trouwborst et al., in review). Trendall (1983) suggested that iron precipitation in the Hamersley Basin was due to oxidation of Fe$^{2+}$ in the basin water by oxygen produced by cyanobacteria. U-Pb dating of zircon grains in the finely laminated BIF units of the Hamersley Group indicates that iron accumulated at rates of 1 mm hematite ($\sim 4.53 \times 10^{12}$ moles iron) per year to generate an annual varve (Morris, 1993; Barley et al., 1997). A comparison between the isotopically dated accumulation rate of BIF iron oxides and the \textit{in situ} Fe$^{2+}$ oxidation rates of a planktonic suspension of cyanobacterial cells from Chocolate Pots hot springs revealed that $2.3 \times 10^{21}$ cells would be needed to accumulate annually a 1 mm thick layer of hematite (Trouwborst et al., in review). The concentration of cells ($5.35 \times 10^6$
cells/ml) is comparable to that of modern populations of bacteria and plankton in the photic zone of marine coastal waters (10^6 cells/ml) (Azam et al., 1983).

Comparison of our collaborator’s *in situ* Fe^{2+} oxidation rates to those calculated by Konhauser et al. (2002) using laboratory cultures of *Gallionella*, a chemolithotroph (Emerson and Revsbech, 1994), and *Chromatium*, a purple anoxygenic phototroph (Ehrenreich and Widdel, 1994a,b), revealed that 4.3 x 10^{23} cells of *Gallionella* and 5.7 x 10^{23} cells of *Chromatium* would be needed to oxidize the same amount of iron. These numbers are two orders of magnitude greater than cyanobacterial cells and demonstrate that localized cyanobacterial oxygen production is a biologically efficient way to oxidize Fe^{2+} in the chemical deposition of some Precambrian BIFs, as was suggested by Cloud (1965, 1973).

6.2.2 Biosignatures in Ancient Environments

Walter and Hofmann (1983) in a review of the paleontology of Precambrian BIFs acknowledge that very little effort has been spent on studying the paleontology and paleoecology of ancient iron formations. They note that there is a considerable chance that such research would solve some of the more significant problems of BIF deposition.

There are limited reports of biosignatures in ancient iron deposits since that review which have provided some additional evidence of a microbial role in their deposition. Microfossils of cyanobacteria have been found in the BIFs of the ~2.1 Ga Gunflint Formation in Canada. Most of the microfossils have been found in bands of
chert (Barghoorn and Tyler, 1965), however Allen et al. (2001) found filaments, rods, and spheres associated with iron oxides. In addition, iron mineralized polymeric substances were detected, prompting the authors to suggest that microbial mats may have played a roll in iron oxide precipitation and BIF formation (Allen et al., 2001, Schelble et al., 2001). Microfossils of nanobacteria have also been observed in the iron-rich layers of BIFs from Carajas Province and Urucum District in Brazil (Avila et al., 2001). The cyanobacterial biomarker 2α-methylhopane has been found in 2.7 billion year-old shales interbedded with the oxide facies BIFs in the Hamersley Group (Brocks et al., 1999; Summons et al., 1999), supporting the assertion by Trendall (1983) that oxygen produced by cyanobacteria was responsible for the chemical deposition of this BIF.

This study has demonstrated that iron preserves organic material in the form of lipid biomarkers and bacterial cells, and generates distinctive biofabrics by cell encrustation. This “proof of concept” study indicates that it is worthwhile to search for these types of biosignatures in ancient iron deposits. Detection of such biosignatures in the iron layers of BIFs is critical for demonstrating the necessary spatial relationship between the microfossils or lipid biomarkers and the iron mineral assemblages and to prove that the microbes were active in Fe$^{2+}$ oxidation.

Based on the type and quality of biosignatures detected in the iron mineralized phototrophic mats at Chocolate Pots hot springs, cellurally preserved microfossils and lipid biomarkers appear to provide the most fossil information. The iron permineralized C. minervae cells demonstrate that taxonomic features are retained
which aid in identification of the cell, and because the organics are preserved within the cell, the isotopic signatures can be measured using high resolution instrument such as an ion microprobe (e.g., Orphan et al., 2001). However, even though microfossils were abundant in the modern iron mineralizing environment at Chocolate Pots, it is rare to find cellurally preserved microfossils in the rock record because of the unique set of conditions that must be met in order for them to form (Cady, 2001). Demonstrating their biogenicity has also proven to be problematic (Schopf, 1993; Brasier et al., 2002; Schopf et al., 2002).

While cellurally preserved microfossils provide multiple lines of evidence that aid in identification and assessment of their carbon fixation pathways, lipid biomarkers also provide similar information and their preservation may not require such a specific set of circumstances to retain the integrity of the cell. The observations at Chocolate Pots indicate that even if the cell dies and lyses and the chance of microfossil formation is lost, iron may still function to inhibit enzymes that degrade the lipid biomarkers. The lipid biomarkers may also be intimately associated with the amorphous ferrihydrite, which may confer protection from UV exposure, oxidative processes, and microbial consumption. It is the author’s opinion that extracting the iron oxide facies in un- or low-grade metamorphosed BIFs and measuring the carbon isotopic composition of any biomarkers may be the most rapid and diagnostic way to search for phototrophic biomarkers and assess potential Fe²⁺ oxidation and carbon flow within the communities (i.e. photoautotrophy or photoheterotrophy) to determine whether they may have functioned in BIF deposition.
While the limitations of this study have been noted regarding Chocolate Pots hot springs as a geochemical analog for the Fe$^{2+}$ oxidative processes in an ancient anoxic world, the relation of \textit{in situ} Fe$^{2+}$ oxidation rates by cyanobacterial oxygen production to iron oxidation requirements for the chemical deposition of the Hamersley BIF by our collaborators is not unreasonable. The biosignatures described in this study are linked to those quantitative Fe$^{2+}$ oxidation measurements and together they provide a powerful suite of information for interpreting the role of microbes in BIF deposition should such biosignatures be found in the iron facies BIFs.


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Appendix A. Future Work

While many questions have arisen from this initial study, it is worthwhile as an exhaustive report of biosignatures in a natural iron mineralizing setting. Such a descriptive study is necessary to establish a baseline of observations regarding iron preservation of cells and lipids so that the fate of these biosignatures upon diagenesis and the specific mechanisms of preservation can be addressed in a controlled laboratory setting.

This study has characterized the microbial biosignatures (microfossils, stromatolitic biofabrics, lipid biomarkers, and biominerals) forming in the modern iron-mineralized cyanobacterial mats at Chocolate Pots hot springs. These biosignatures represent the maximum amount of paleobiological and paleoenvironmental information being produced in this system. This study has characterized some of the taphonomic changes that occur to the biofabrics and microfossils and have also quantified the survival of organic carbon and lipid biomarkers during microbial degradative processes in the mats and during burial and earliest stages of diagenesis in the iron oxide deposits underneath the mats. However, this project could be enhanced by further examining the fate of the biosignatures upon diagenetic changes (microbial degradation and heating) to make the biosignature database more relevant to the rock record.
1.1 Lipid Biomarkers

The discovery of cyanobacterial membrane ester-linked fatty acids and other cyanobacterial and Chloroflexus lipids in the iron oxides under the mats was unexpected and suggests that iron may inhibit cellular autolytic enzymes, such as phospholipases and other lipases, of cyanobacteria, anoxygenic phototrophs (Chloroflexus), and heterotrophs at Chocolate Pots hot springs. Inhibition of the cell’s own lytic enzymes, or those of heterotrophs, may promote the survival of lipids during cell death and heterotrophic grazing and thus increase the yield of lipids potentially entering the rock record. The possibility that this mechanism could enhance the preservation of lipids during the earliest stages of diagenesis is significant because it is generally thought that 99.9% of all organic matter is rapidly re-mineralized back to inorganic carbon and escapes incorporation into sediments (Des Marais, 2001). Some lipid biomarkers are robust biosignatures and it is important to understand under which conditions their production and preservation are favored.

To determine whether Fe (and Mn) inhibit lipases, commercially available lipases can be used to act as proxies for those found in cyanobacteria and Chloroflexus. Specifically, phospholipase C or an acylhydrolase, potentially in combination with galactosidase and/or glucosidase can be used to simulate the degradation of polar membrane lipids.

Additionally, a culture of heterotrophs can be established to enrich for in situ extracellular lipases. Extracellular lipases are produced by many heterotrophic bacteria, which are the probable origin of membrane lipid degradation in the
Chocolate Pots sedimentary layers. A complex medium can be used to enrich a heterotrophic sub-population. The enriched cells can be used directly or a crude homogenate can be prepared by cell lysis as a *in situ* source of lipid degrading enzymes for determination of the potential Fe and Mn inhibition associated with lipid preservation.

An alternate explanation for the detection of cyanobacterial membrane ester-linked fatty acids and other cyanobacterial and *Chloroflexus* lipids in the iron oxides under the mats is that the lipids are preserved within the amorphous structure of the primary iron oxide precipitates (ferrihydrite). Incorporation or intimate association of the lipids with the amorphous (i.e., nanocrystalline) ferrihydrite may lead to enhanced preservation of the organics by shielding them from UV exposure, oxidative processes, and microbial consumption. Characterizing the preservation of lipid biomarkers in terrestrial iron deposits may aid in the detection of organics in ancient iron deposits on Earth as well as the iron deposits on Mars by the SAM instrument package on the MSL.

To assess iron preservation of lipids, extracted "naked" lipids can be mineralized with iron in the laboratory and then quantified on the GC and GC-MS to determine the degradation rate. The fate of the lipids upon recrystallization to more ordered forms can be determined by heating the samples to 50, 100, and 150°C and then extracted and analyzed via GC and GC-MS to determine how the structures changed. To determine how the lipids are associated with the iron oxides before and after heating, they can be examined using the HRTEM to determine whether they're
being incorporated into the structure of the minerals.

\textit{A1.2 Cyanobacterial Photoferrotrypho}

As previously noted, the stimulation of $^{14}\text{C}$-bicarbonate uptake in the presence of light and Fe$^{2+}$ (Pierson et al., 1999) and the observed deposition of ferrihydrite in the periplasmic space of \textit{Cyanothece minervae} suggest that it is a candidate for cyanobacterial photoferrotrypho. Although the mechanism of ferrihydrite deposition in the periplasmic space is not known, a working hypothesis is that Fe$^{2+}$ diffuses into the cell through the outer membrane porin channels, which only allows the passage of small solutes such as ions (Hoiczyk and Hansel, 2000). Once inside the periplasmic space, the cyanobacterium could use the Fe$^{2+}$ as an electron donor for photosynthesis.

Demonstration of photoferrotrophy in \textit{C. minervae} relies most heavily on isolation of this organism. $^{14}\text{C}$ uptake experiments on newly isolated cultures can be conducted to test for Fe$^{2+}$ stimulation of photosynthesis and to determine which photosystem is functioning. For example, the herbicide DCMU can be used to inhibit photosystem II. If a stimulation of $^{14}\text{C}$ uptake in the light with Fe$^{2+}$ is observed and it is sensitive to DCMU, then that suggests photosystem II may be functioning in a photoferrotrophic manner.

Pierson and Olson (1989) have postulated that iron-dependent photosynthesis using one photosystem may have been an important step in the evolution of oxygenic photosynthesis in ancestral cyanobacteria (which presently utilizes both PSI and PSII). These hypothesized ancestral photoferrotrypho cyanobacteria could have oxidized
$\text{Fe}^{2+}$ directly during the Precambrian and, like the purple bacteria, mediated iron transformations in the absence of oxygen.