The Experimental Silicification of Bacillus subtilis

Aaron Chase Tenesch
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THE EXPERIMENTAL SILICIFICATION OF *Bacillus subtilis*

By

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B.S. Geology, University of Montana, Missoula, MT, 2005

Thesis

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Silica, particularly the stable phase of silica, quartz is chemically and physically resistant to weathering. Microorganisms are commonly incorporated into silica deposits formed by silica precipitation from geothermal fluids that are over saturated with respect to silica. Silica deposits have provided the oldest evidence of life identified on Earth in the form of fossil microorganisms. The first signs of life found beyond Earth may occur as microfossils in silica deposits.

Experiments were conducted on the gram positive bacterium *Bacillus subtilis* to test the effects of silica concentration, dissolved Fe, and dissolved Al upon silicification processes. Results from initial experiments demonstrated that *Bacillus subtilis*, a common soil bacterium, persists in better condition in the presence of Fe and that silica interacts with cellular surfaces in the presence and absence of Fe. The initial experiments were conducted by combining a suspension of bacterial cells with stock solutions in a 1/1 ratio. Results show a decline in silica concentration although the initial concentration was below calculated saturation. The pH of experimental solutions also declined during the experiment. These results led to the development of a different experimental design.

The final experimental design involved introducing small volumes of bacteria to volumes of experimental solution at a 1/125 ratio. The experimental parameters were expanded to include three concentrations of silica intended to represent concentrations well below saturation, approximately at saturation, and 2.5 X saturation. All experiments were conducted in the presence of iron (Fe) and aluminum (Al) (separately) and with no additives (except silica). pH values declined but reached a state of stability within 7 days. The lowest concentration of silica used in the presence of Fe produced the best-silicified bacteria based on SEM/EDS results. The results demonstrate that bacteria do maintain better cellular fidelity and persist for longer time periods in the presence of Fe under all experimental conditions. Al is shown to be important in facilitating a smooth coating on cells, this may produce silicified microfossils that are more resistant to diagenetic and weathering processes. A combination of Fe and Al in silica-rich solutions may produce robust microfossils that can persist through lithogenesis.
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1.0 INTRODUCTION

1.1 Introduction

Any opportunity to analyze samples of extra-terrestrial (likely martian) deposits for signs of life will be rare and costly. Such opportunities must be optimized by selecting sample sites that have the greatest likelihood of exhibiting signs of life such as microfossils or chemical/mineralogical biosignatures. Optimal sample sites can be selected by focusing on analogs to earth deposits that preserve detectable signs of life, particularly ancient deposits. Microfossils are often found in silica deposits. Silica deposits are chemically and physically resistant to weathering processes and have provided evidence of ancient microbial life from Archean (Awramik et al. 1992; Cady 2001; Binet et al. 2008) through Phanerozoic times (Duhig et al. 1992a; Duhig et al. 1992b; Walter et al. 1996; Westall 1997; Guirdry & Chafetz 2003a; Binet et al. 2008; Kaur et al. 2008). The first evidence of life discovered beyond Earth may be identified in a silica deposit.

This study is intended to provide observations about what silica concentrations best silicify microorganisms (undersaturated, at or near saturation, or oversaturated), identify the contributions of other elements important to silicification processes, and provide some understanding of what types of microorganisms are most likely to be silicified into a microfossil that will persist through geologic time. This intent to more completely understand silicification processes will be accomplished by comparing the silicification of a single species of bacteria under conditions of under, near, and oversaturation of dissolved silica in controlled laboratory experiments. These experiments will also involve observing the effects Fe and Al have upon silicification
processes (the importance of Fe and Al to silicification is identified later in this introduction). Changes in solute concentrations and pH will also be measured to promote an understanding of the changes bacterial silicification induces in the surrounding environment. These specific goals lead to the overarching goal of enhancing our ability to identify microfossils in ancient or extra-terrestrial environments by narrowing the definition of sample sites that are likely to yield such fossil evidence. The hypothesis these experiments test is that higher concentrations (near or oversaturated) of dissolved silica will more quickly encrust bacteria but lower concentrations (below saturation) will produce a better silicified microfossil because the dissolved silica may be incorporated inside the cell. The secondary hypothesis is that silicification will be enhanced by the addition of Fe by enhancing encrustation and/or inhibiting autolysis.

1.2 Silica Deposits

Silicification processes are poorly understood. Macroscopic observations of morphological features can not be interpreted as direct evidence of microorganisms. Stromatolites are a morphological feature often found in silica deposits that can indicate the presence of microorganisms. However, McLoughlin et al. (2008) demonstrates that many different shapes of stromatolites can be formed subaerially through non-biogenic processes. Stromatolites can be considered a macroscopic indicator that microfossils or other forms of life may be present but is not a definitive indicator of life. Microfossils or other chemical signatures must be identified to conclusively prove the presence of ancient or extra-terrestrial life. Testing the conditions that lead to siliceous microfossil preservation is a key step in locating extra-terrestrial microfossils or other signs of life.
Siliceous microfossils have been identified in various ancient deposits on Earth. Some research has focused on Cambrian sea-floor silica deposits formed at hydrothermal vents. Duhig et al. (1992a, b) present morphological evidence of microbiota deposited in the Cambrian-Ordovician Mount Windsor volcanic belt in northern Australia, a silica and iron oxide dominated deposit of submarine hydrothermal origin. The morphological evidence of microorganisms presented in Duhig et al. (1992a, b) are displayed by light and electron microscope images that show bacterial filaments and mats preserved in silica and iron oxide minerals. Duhig et al. (1992b) suggests that these deposits are the result of crystallization of silica iron oxyhydroxide gels that form as a result of mixing hydrothermal and sea waters. The range of iron oxide reported varies between 4 and 20 wt% with the remaining balance occurring as silica (minus up to 1 wt% for other major elements). The evidence of microorganisms preserved in iron oxides amongst silica-dominated environments indicate that there is an extreme bias for coating and preservation by iron in the depositional environments examined by Duhig et al. (1992a, b). Another deposit extensively studied to identify microbial remains in siliceous deposits is the early Archean Warrawoona Group of the Pilbara Craton in Western Australia, another hydrothermal/sea-floor deposit (Awramik et al. 1983; Gilkson et al. 2008; VanKranendonk 2008). Gilkson et al. (2008) also considers the early Archean Barberton Greenstone Belt of South Africa, both deposits were sampled and examined for microbiological evidence preserved in silica using chemical methods and electron microscopy analyses. The results of Gilkson et al. (2008) also involve a comparison to experimental silicification conducted at elevated temperatures and demonstrates that the
experimental cultures break down above 100° C and produce carbon compounds that can not be distinguished from inorganic compounds, suggesting that morphological evidence (that can be displayed through electron microscopy or other visual-revealing techniques) of microbiota may be necessary to positively identify the presence of microorganisms.

There are ancient siliceous deposits that are considered to represent chemical evidence of the presence of microorganisms. Banded iron-formations (BIF) are massive siliceous deposits of thinly-laminated chemical sedimentary rocks precipitated in marine environments. They contain alternating layers of reduced or oxidized iron depositional facies, resulting in a banded appearance. BIF present silica and iron deposition of widely debated geochemical conditions and sources. The factor that is generally agreed upon in the literature is that microorganisms changed the Fe oxidation state by inorganic reactions using photosynthesis-produced O$_2$ (Cloud 1973; Nealson & Myers 1990; Awramik 1992) or chemolithoautotrophic iron oxidation (photoferrotrophy) (Konhauser et al. 2002). The bands with increased and oxidized Fe precipitation in BIF is attributed to the oxidation of dissolved Fe species to insoluble iron oxides while silica precipitation is attributed to oversaturation. The silica is likely from detrital or hydrothermal sources (Cloud 1973 Klein & Beukes 1989; Isley 1995; Isley & Abbott 1999; Barley et al. 1997; Konhauser et al. 2002; Pecoits et al. 2009. Cloud (1973) points out that biogenic silica-precipitating species of organisms had not evolved in Precambrian time. Therefore detrital silica would accumulate in marine environments (from detrital sources) until precipitation occurred due to oversaturation. Current research focuses on geothermal sources (largely spreading-centers) for dissolved silica and iron in banded iron formations (Klein &
Beukes 1989; Isley 1995; Isley & Abbott 1999; Barley et al. 1997; Konhauser et al. 2002; Pecoits et al. 2009) that is introduced onto continental shelves during upwelling events (Klein & Beukes 1989; Isley 1995; Isley & Abbott 1999; Barley et al. 1997). Although this is a more complicated scenario than direct chemical precipitation of silica in hot spring environments, the commonality of geothermal sources for high concentrations of silica resulting in large-scale precipitation can not be ignored.

There is also evidence of microbial preservation in silica deposited in ancient subaerial hot spring environments. Walter et al. (1996) compares Devonian through Carboniferous sinters from the Drummond Basin in Australia with more contemporary deposits found in hot spring deposits of Yellowstone National Park (a hot spot). Walter et al. (1996) describes thirteen microfacies found in the Drummond Basin sinters that are comparable to current depositional facies found in Yellowstone National Park. These facies are reported to represent very high temperature pool environments through high to moderate temperature splash zones to cooler waters. The moderate to low temperature zones are generally shown to contain morphological evidence of microorganisms suggested to be sheaths of cyanobacteria (Archea). More recent sinter deposits of approximately 6,280 to 11,500 years in age from the Steamboat Springs deposits in Nevada, U.S.A. are examined in Lynne et al. (2008). Lynne et al. (2008) demonstrates that microbial fossil structures persist through the diagenetic changes that have occurred in that system. The important comparison of these more recent deposits to the extremely ancient deposits discussed earlier is not simply the presence of microorganisms but the ability to distinguish depositional facies. The more recent deposits display textural
details that can lead to important interpretations such as Lynne et al. (2008) reporting microbial remains in sinters identified as low temperature (< 35°C) to moderate temperature (35-60 °C). The ability to make these observations is showcased in many different studies (Walter et al 1996; Guirdry & Chafetz 2003; Hinman & Walter 2005; Lynne et al. 2008) and are usually based on the facies models introduced in Walter (1976). These facies represent different conditions upon deposition and can help to characterize the types of hot spring environments that are likely to preserve microbiota and promote their persistence in the geologic record. The initial silicification of microorganisms may be just as important as the diagenetic changes that do (or do not) occur.

1.3 The Behavior of Silica

Silicification is a general term referring to any form of fossilization involving the permineralization, entombment, or replacement of biogenic material by silica. Silicification, particularly permineralization, provides micro-fossils of exquisite fidelity that are resistant to weathering, sometimes preserving internal details of cells (Kyle et al. 2007). Silica deposits offer a chance to observe microorganisms fossilized in ancient or extreme environments (Awramik et al. 1983; Duhig et al. 1992a; Duhig et al. 1992b; Cady & Farmer 1996; Hinman & Lindstrom 1996; Walter et al. 1996; Westall 1997; Jones et al. 2000; Cady 2001; Jones et al. 2001; McKenzie et al. 2001; Guirdry & Chafetz 2003a; Mountain et al. 2003; Guidry & Chafetz 2003a; Guidry & Chafetz 2003b; Benning & Mountain 2004; Lynn & Campbell 2004; Fernandez-Turiel et al. 2005; Amores & Warren 2007; Kyle & Schroeder 2007; Kyle et al. 2007; Binet et al. 2008;
Handley et al. 2008; Kaur et al. 2008; Lau et al. 2008). The precise chemical environments that produce the most effective preservation of microfossils in silica deposits are undefined. Comparing the extent and habits of microfossil preservation that occur under specific conditions provides the first steps in defining "superior" or "effective" silicifying environments. Changes in solute concentrations, pH, and the morphological habits of silicification must be observed in well-constrained laboratory experiments to develop and understanding of how these factors combine to form silica deposits that preserve physical evidence of microbiological life.

Silica (SiO$_2$) is the dominant constituent of the Earth's crust and is present to some extent in most or possibly all aqueous environments. On the Earth's surface, silica is dissolved via hydrolysis into monomeric silicic acid represented by the following equation: $\text{SiO}_2 + 2 \text{H}_2\text{O} \leftrightarrow \text{Si(OH)}_4$. Silicic acid with a neutral charge is the dominant species that occurs in natural environments below pH 9 (Iler 1979). In contemporary environments river water typically contains less than 15 mg/L silica while shallow ground water often has up to 60 mg/L (Iler 1979, Bridge & Demicco 2008). These values are above equilibrium with respect to quartz (6 mg/L) and well below equilibrium with amorphous silica (100 – 150 mg/L) (Iler 1979) and reflect dissolution of other silicate species such as feldspars. Rivers transport silica to marine environments where silica would accumulate, however biogenic silica precipitation depresses dissolved silica concentrations. Marine silica concentrations are at a minimum along the surface with concentrations of approximately 0.5 mg/L and a generally constant maximum of 10 mg/L below the thermocline (Bridge & Demicco 2008). The concentrations of silica that occur
in these natural environments are stable for long periods of time but biogenic precipitation of silica immobilizes large amounts of silica in marine and freshwater environments.

Modern siliceous sediments occur thru biogenic and chemical precipitation. Marine and freshwater environments deposit biogenic silica: the skeletal remains of diatoms, radiolarian, and sponges (marine). The majority of biogenic silica deposited in marine and freshwater environments is diatom skeletons composed of opal-A. Chemical precipitation of silica occurs in alkaline lakes, soils, and hot springs (Bridge & Demicco 2008). Environments that experience chemical silica precipitation are the environments that are likely to preserve the delicate structures of microorganisms. Hot springs are the most accessible environments in which rapid chemical precipitation of silica currently occurs.

The source water of hot springs usually contains high concentrations of dissolved silica due to the solubility of quartz at elevated temperatures (Figure 1). When geothermal fluids reach the surface degassing and rapid cooling result in silica concentrations that are supersaturated with respect to amorphous silica (White et al. 1965; Fornier 1985). In supersaturated solutions monomeric silica (Si(OH)₄) polymerizes into particles that are considered oligomers up to a dimension of 5 nm, polymerization continues until colloids are formed (polymers greater than 5 nm) (Iler 1979). The effect these different size polymers have upon silicification processes has not been directly explored, although the chemical characteristics of dissolved silica have been
extensively characterized (Alexander 1957; Wirth & Gieskes 1979; Aller 2004; Wolf-Boenisch et al. 2004).

**Figure 1**: Solubility of amorphous silica and quartz under increased temperature recreated from Fornier (1977)

The behavior of silica species in aqueous environments is commonly studied in laboratory and natural environments (White 1956; Alexander 1957; Wirth & Gieskes 1979; McManus et al. 1994; Hinman 1998; Lynne & Campbell 2004; Michalopoulos & Aller 2004; Wolf-Boenisch et al. 2004; Brehm et al. 2005) to name just a few. However, a complete understanding of the behavior of silica has not been gained. Wirth & Gieskes (1979) tested the kinetics of silica dissolution through a range of pH from 5 to 11 with ionic content of 0.01 to 3.5 M Na and Mg (as chloride-salt) at 25 °C. Wirth & Gieskes (1979) demonstrate that the rate constant of silica dissolution can increase by a factor of 360 transisting from a pH of 5 to 11 with a moderate ionic content (0.70 M NaCl). Wirth & Gieskes (1979) also demonstrated the importance of Mg in silica behavior at certain pH values that can be found in natural hot spring environments (White 1956) by
demonstrating that the addition of Mg (maintaining a constant ionic strength in addition of \( \text{Mg}^{2+} \) to NaCl solutions) did not have an effect on silica dissolution at a pH of 8 but produced a decrease of approximately 30% at pH 9 (0.055 M \( \text{Mg}^{2+} \)). Wirth & Gieskes (1979) did not test these results with other cations of similar charge (\( \text{Fe}^{2+} \)).

Hinman (1998) examined silica precipitation and phase transitions in the presence of Na, Mg, K, Al, and Fe at elevated temperatures (140 °C) and initial pH of 8.5 (at 25 °C). The results of Hinman (1998) show that the majority of changes occurred within 4 weeks and that Mg, Fe, and Al are important to the deposition and maturation of silica. Within the experiments presented Al and Fe were shown to increase the precipitation of silica into more ordered initial phases of opal-CT (as opposed to the amorphous opal-A) in the presence of Mg and to increase precipitation of the stable phase quartz in the absence of Mg. This result could indicate that Mg may retard the transition of opal-CT to quartz during diagenesis. Lynne & Campbell (2004) make a detailed examination of the mineralogic phase transitions of silica during low-temperature sinter diagenesis in the Taupo geothermal area of New Zealand. Lynne & Campbell (2004) present a comparison of silica diagenesis based on three different classes of sinter designated for the study: least-disturbed, heat-affected, and weathered sinters ranging in age from "presently silicifying" to sinter of approximately 3500 years of age. X-ray diffraction results presented in Lynne & Campbell (2004) display the transition of initially amorphous (or microcrystalline below the detection limit of X-ray diffraction) opal-A to opal-CT. These transitions are important because they represent the dissolution and reprecipitation of initially unstable silica phases to quartz, the stable silica phase. These
phases can help to distinguish the depositional facies of older deposits and possibly to distinguish some factors of the cationic conditions during deposition of silicified microorganisms. During these transitions, morphological characteristics of silicified microorganisms can be lost completely to dissolution in pore waters or at least obscured by textural changes such as the development of opal-CT bladed lepispheres. Scanning electron microscopy images presented by Lynne & Campbell (2004) demonstrate these changes and the loss of morphologic detail. These depositional tendencies and diagenetic changes need to be considered when searching for deposits that are likely to contain ancient and well-preserved microfossils. Environments that have the ability to precipitate quartz directly or suppress crystal size during opal-CT formation/transition may promote the persistence of observable physical features of microfossils.

1.4 Hot Springs: Silica-Depositing Systems

Silica is the dominant chemical constituent in most hydrothermal fluids and produces siliceous deposits that often contain the silicified remains of microorganisms. Hot springs are the most commonly studied source of hydrothermal fluids. Geothermal vents in the ocean floor may produce silicified microfossils but the logistic difficulties of studying deep ocean features are impractical at best. Subaerial hot spring environments present numerous localities that offer researchers access to a wide range of silicified microfossils throughout the Earth. The siliceous deposits of subaerial hot springs are called sinter.
Hot springs occur on Earth in groups or geothermal fields in areas such as
Yellowstone National Park (White et al. 1956; Brock 1967; Hinman & Lindstrom 1996;
Guidry & Chafetz 2003a; Guidry & Chafetz 2003b; Hinman & Walter 2005; Amores &
Warren 2007; Kyle & Schroeder 2007), Steamboat Springs Nevada, (White et al. 1956;
Walter et al. 1996; Lynne et al. 2008), Kamchatka, Russia (Orleanskiy et al. 1984; Kyle
et al. 2007), the Taupo, Waiotapu, and Whakarewarewa geothermal fields of New
Zealand (Jones et al., 2000; Jones et al. 2001; McKenzie et al., 2001; Mountain et al.
2003; Benning & Mountain 2004; Lynne & Campbell 2004; Handley et al. 2008; Kaur et
al. 2008), El Tatio, Chile (Fernandez-Turiel et al., 2005), and the Daggyai Tso
geothermal field of central Tibet (Lau et al. 2008) (Figure 2).

Figure 2: Map of major geothermal fields around the Earth (Gelennon 2009).

Hot springs span a wide range of chemical composition, the most common types
being acid-sulfate springs or alkaline-chloride springs. White et al. (1956) presents a
compilation of chemical data of hot springs from Steamboat Springs (Nevada, USA) and
Yellowstone National Park (Wyoming, USA). The data presented in White et al. (1956)
demonstrate that most of the Steamboat Springs hot springs have peak temperatures
between approximately 70 and 95 °C with a few springs of lower temperatures (down to 15 °C) and pH values generally between 6.0 and 8.0 with a few outliers reaching 5.0 to 9.0 and one spring flowing at a pH of 2.45. The silica concentrations presented for hot springs of Steamboat springs (White et al. 1956) are from approximately 250 mg/L to 350 mg/L, except for the few springs with temperatures below 50 °C, where silica concentrations are approximately 100 mg/L. The data on hot springs of Yellowstone National Park presented (White et al. 1956) are much more varied chemically, but temperatures range from 84 to 94.8 °C. The pH ranges from 2.0 to 9.0, and silica concentrations range from 240 mg/L up to 500 mg/L. McKenzie et al. (2001) presents chemical data from hot springs of the Taupo Volcanic Zone in New Zealand. The temperatures of the springs presented is between 61 and 94 °C, with a constrained pH range of 7.7 to 8.3 and silica concentrations from 183 to 561 mg/L. Fernandez-Turiel et al. (2005) determined chemical data from the El Tatio hot springs of northern Chile with water temperatures between 70 and 86 °C with pH ranges from 6.41 to 7.64 and silica concentrations between 71.6 mg/L and 219.2 mg/L. These studies present numerous hot spring environments that have the potential to silicify microorganisms mostly in oversaturated conditions. White et al. (1956) conducted analyses upon samples from Steamboat Springs ("spring 21") that had original temperatures of 14 to 84.2 °C and pH values between 6.62 and 8.43 that were cooled for 3 days, all of the samples began with silica concentrations of approximately 300 mg/L that declined over the 3 days to approximately 155 mg/L. If this rapid decline in silica concentrations can be expected from similar (and common) chemical environments of hot springs, it indicates that
microorganisms are subjected to continuous silica precipitation and should be easily encrusted with silica and incorporated into sinter deposits.

1.5 The Interaction of Silica and Bacteria

Many studies have documented the interaction of silica with bacteria in contemporary hot spring environments that result in siliceous microfossil preservation. Encrustation and subsequent entombment will occur in environments supersaturated with respect to silica but this may preserve only the outer cellular membranes or ultimately just a cast of a cell (Jones et al. 2000; Jones et al. 2001; McKenzie et al. 2001; Guidry & Chafetz 2003a; Guidry & Chafetz 2003b; Mountain et al. 2003; Benning & Mountain 2004; Lynn & Campbell 2004; Fernandez-Turiel et al. 2005; Amores & Warren 2007; Kyle & Schroeder 2007; Kyle et al. 2007; Handley et al. 2008; Lau et al. 2008). This type of fossil is hollow and can then be infilled by further silica deposition during diagenesis and may be lost entirely to diagenetic processes. In an attempt to gain a complete understanding of microfossil preservation and persistence, research has focused on different facets of the events and geologic history that produce siliceous microfossils and promote their persistence in the geologic record.

To understand the different facets that affect microfossil preservation and persistence many different events from deposition through diagenesis must be understood. Research has considered the habits of natural systems by identifying the physical morphologies of depositional facies (Braunstein & Lowe 2001; Guidry & Chafetz 2003a; Guidry & Chafetz 2003b; Fernandez-Turiel et al. 2005; Hinman & Walter

Some work has even examined the persistence of more simple organic markers rather than microfossils. Schelbel et al. (2008) tested the perseverance of DNA during silicification, and Binet et al. (2008) explored the imaging of mature organic matter through electron paramagnetic resonance analysis, a technique that may allow the imaging of organic matter without the need for observable morphological features. The methods of Binet et al. (2008) could be used to develop a technique to identifying microorganisms via morphological manifestations that lend themselves to optical or electron microscopy techniques. Observations of silica encrustation on bacterial surfaces, finding chemical traces of bacteria, and defining the pathways that lead to the persistence of detectable siliceous microfossils, is evidence that silica is deposited on bacterial surfaces and produces microfossils. These observations do not define the nature of silica and bacterial surface interactions. Hot spring environments often involve extensive inorganic precipitation of silica via cooling and evaporation. During wide-spread
inorganic silica precipitation, microbiota become encrusted with silica regardless of organic processes. This fact prevents researchers of natural environments from explaining whether bacteria play an active role in silica precipitation or simply fall victim to inorganic precipitation. Silicification experiments conducted on microbiota under conditions of undersaturation with respect to silica can be used to identify organic silica interactions with bacterial surfaces.

Defining the conditions under which signs of life are preserved is difficult in natural environments because they are open environments that cannot be controlled and manipulated. The mechanisms responsible for silicification in hot spring deposits vary throughout a wide range of conditions of pH, silica concentration, and concentrations of other chemical constituents. These relationships are poorly understood and result in different forms of silicification. Many researchers have explored the habits of silica under different conditions in natural environments. Jones et al. (2000) assessed morphological differences in stromatolite formation based upon the aqueous environment (pH, dominant dissolved ions, temperature) and the associated mineral and microbiota assemblages of the stromatolites studied. Guidry & Chafetz (2003b) explore the diagenetic changes that occur in a particular sinter deposit (Deerbone Spring, Yellowstone National Park) and conclude that the porous nature of young sinters result in dissolution and replacement of organic matter, leaving only morphological features of fossils as detectable signs of life, not organic traces. Lynne & Campbell (2004) observed the maturation of silica from a mineralogical standpoint based upon heat, pressure, and weathering. Lynne & Campbell (2004) suggest that polymeric silica deposition produces
a spherical surface morphology (sub micron) and monomeric deposition produces a smooth morphology. Lynne & Campbell use X-ray diffraction analysis to demonstrate the mineral diagenesis that occurs as originally amorphous silica phases progress from opal-A to opal-A/CT to opal-CT to the stable phase of quartz. Amores & Warren (2007) test the silica uptake abilities of microbial mat communities versus abiotic particles under various pH and temperature conditions using colloidal and dissolved silica. McLoughlin et al. (2008) demonstrates silica deposition in terms of stromatolite morphology in abiotic systems of subaerial silica deposition, showing that stromatolite morphology alone can not be considered a sign of biologic activity. All of these, and other studies, demonstrate important factors contributing to the preservation and persistence of microbiota in the geologic record, but natural studies will always involve some unknown factors. In comparison, laboratory experiments may offer a way to define conditions that promote the preservation of signs of life in silica deposits while limiting unknown influences.

Hot springs generally have a constant outflow and silica concentration that changes very little throughout the year (Hinman & Lindstrom 1996). In hot springs, the local chemistry (particularly silica concentration) is largely controlled by inorganic dissolution and subsequent precipitation of lithic constituents driven by temperature/pressure flux (White 1956). Precipitation of silica will continue downstream as hot spring waters flow and cool along the outflow channel away from the source of the hot spring. The continued precipitation of silica along a descending thermal gradient coats cell surfaces and entombs microorganisms under continually changing silica concentrations. Changes in pH can also effect silicification. Amores et al. (2007) reports
enhanced silica immobilization in systems of acidic pH conditions. Undersaturated conditions may occur as hot spring outflow channels change in pH or mix with shallow aquifer and surface runoff water. Identifying the point at which optimal preservation occurs requires a greater understanding of these processes and influencing chemical factors. Once the effect of silica concentration on silicification processes is understood, other factors such as the presence of metals may be considered.

Fe and Al have been shown to potentially influence silicification processes (Urrutia & Beveridge 1994; Fein et al 2002; Phoenix et al 2003, Kyle et al. 2007). Both Fe and Al commonly occur in natural environments exhibiting a wide variety of speciation under natural conditions. These different species exhibit neutral, negative, and multiple positive charges (Figures 3 and 4) under natural conditions. The different species of dissolved Fe and Al will interact in different ways both with dissolved silica and bacteria. The wide range of pH (2-9) found in hot springs (White et al. 1956), will supply both positive and negative species of Fe and Al between pH 4 and 7, with only negative species of Al above pH 7 (Figures 3 & 4). Fe will occur in positively charged species up to a pH of 11. The positively charged species of Fe will be attracted to functional groups on cellular surfaces that are negatively charged due to an elevated pH value such as 8. Al will not supply a positively charged species at an elevated pH and will therefore not be attracted to cellular functional groups. Fe and Al both occur as negatively charged species down to a pH of 4 so both will be attracted to functional groups on cellular surfaces that are positively charged due to low pH conditions (above pH 4). Silica occurs as negatively charged species in the range of pH found in hot
springs (Figure 5). Silicification may be enhanced under high pH conditions because positive Fe species will be attracted to the cellular surface and can therefore supply a positive charge to attract the negatively charged silica species.

**Figures 3 (left) and 4 (right):** Figure 3 is a concentration vs. pH plot that depicts equilibrium concentrations of dissolved Fe species under ideal conditions modified from Snoeyink & Jenkins (1980). Figure 4 is a concentration vs. pH plot that depicts equilibrium concentrations of dissolved Al species under ideal conditions modified from Snoeyink & Jenkins (1980).

**Figure 5:** Figure 5 is a concentration vs. pH plot that depicts equilibrium concentrations of silica species under ideal conditions modified from Feurstenau et al. (2007).
1.6 **Silicification Experiments**

The life and death cycles of microorganisms will affect silica concentrations, other metals concentrations, and pH. These changes will be generally continual, meaning that the conditions supplied downstream in the system will remain constant from one point to the next (not including non-biogenic changes to the system). The effects that bacteria have upon their immediate environment should be considered and limited in experiments concerning the silicification of microorganisms. Limiting these effects will more effectively imitate natural environments. Flow-through experiments can be used to imitate these conditions but flow-through laboratory experiments are not necessarily practical. A method of supplying large volumes of experimental solutions to relatively small volumes of micro-biota may be used as a proxy to flow-through experiments.

Previous silicification experiments have been conducted on many organisms (Francis et al 1978; Ferris et al 1988; Urrutia & Beveridge 1994; Fein et al. 2002; Toporski et al. 2002; Phoenix et al. 2003; Benning et al 2004; Lalonde et al. 2005). Most studies have failed to report or control specific parameters such as changes in pH (Urrutia & Beveridge 1994; Toporski et al. 2002; Benning et al. 2004; Lalonde et al. 2005) and silica concentration (Francis et al 1978; Ferris et al 1988; Urrutia & Beveridge 1994; Toporski et al. 2002) throughout the experiment, or have assumed that pH was only a function of silica concentration (Fein et al. 2002).

Many studies have used the common soil bacterium, *Bacillus subtilis*, chosen for this set of silicification experiments. In previous silicification experiments (Ferris et al
1988; Urrutia & Beveridge 1994; Fein et al. 2002; Phoenix et al. 2003). Ferris et al. (1988) silicified *Bacillus subtilis* with silica concentrations of 300 mg/L, a 1.5 mg/L Fe pre-treatment, pH of 7, and at a temperature of 70° C under ambient pressure. Ferris et al. (1988) demonstrated that Fe was an important factor leading to the formation of microfossils and suggested that even degraded cells of *Bacillus subtilis* promoted silica precipitation. Urrutia & Beveridge (1994) silicified *Bacillus subtilis* with undersaturated silica concentrations of 8.4 mg/L, a 0.15 mg/L Fe pretreatment, a 0.30 mg/L Al addition (not a pretreatment), ambient temperature and pressure conditions, and pH values of 5.5 and 8. Urrutia & Beveridge demonstrate the importance of Fe in preventing autolysis under non-metabolic conditions for *Bacillus subtilis* and suggest that silica is bound to cellular surfaces by a cationic bridging mechanism. Fein et al. (2002) silicified *Bacillus subtilis* in undersaturated silica concentrations of 1.4 mg/L with both a 0.75 mg/L Fe pretreatment at pH of 5.2 and an Al pretreatment at pH 7.3 all under ambient temperature and pressure. Fein et al (2002) suggests that silica is bound to Fe and Al oxides that are electrostatically bound to the bacterial surface, not attributing any silica binding to direct silica-organic interactions. Phoenix et al. (2003) silicified *Bacillus subtilis* under conditions of both undersaturation (60 mg/L) and oversaturation (400 mg/L) with the addition (not pretreated) of Fe in various concentrations from 0.20 to 100 mg/L including a control of 0 mg/L Fe, all experiments were run at a pH of 7 under conditions of descending temperature starting at 90° C declining to 25° C to induce silica precipitation at ambient pressure. Phoenix et al. (2003) concludes that insufficient Fe is bound to the bacterial surface to act as a “salt bridge” for silica sorption. All of these studies
considered *Bacillus subtilis*, but there are many other organisms that can and have been studied using silicification experiments.

Silicification experiments have also been conducted on a variety of other organisms. Westall (1997) silicified a variety of bacteria including one species of gram positive bacteria, *Bacillus laterosporus*, and three species of gram negative bacteria including *Pseudomonas fluorescens*, *Pseudomonas vesicularis*, and *Pseudomonas acidovorans*. All bacteria were silicified in orthosilicate tetraethoxysilane under ambient temperature and pressure conditions. Westall (1997) demonstrates that the gram positive bacteria *Bacillus laterosporus* is more quickly and better coated by silica under the experimental conditions than the gram negative bacteria silicified. Westall (1997) concludes that gram positive bacteria are more likely to be preserved in terrestrial and extraterrestrial geologic records than gram negative bacteria, suggesting that the large amount of peptitoglycan exposed as the outer membrane of gram positive bacteria is more easily silicified because the thick peptitoglycan layer supplies an abundance of cationic binding sites (Beveridge 1984). The outer layer of gram negative bacteria consists of a phospholipid covered lipopolysaccaride layer that covers a thin peptitoglycan layer. Beveridge (1984) states that the polymer, peptitoglycan, is rich in reactive carboxyl and phosphoryl groups that provide nucleation sites for metal cations. These factors suggest that gram positive bacteria such as *Bacillus subtilis* are good candidates for detection in ancient siliceous deposits and are a good choice for silicification experiments. Francis et al. (1978) silicified a great variety of organisms from prokaryote microorganisms to eukaryote microorganisms (even fungi) in a variety
of methods involving immersing samples in ethyl silicate, sometimes with elevated temperatures of 55-60° C under ambient pressure conditions. Francis et al. (1978) demonstrated that some species of eukaryotes (*Vorticella specialis, Tetrahymena pyriformis*, and *Stentor coruleus*) did not silicify at all under their experimental conditions. Francis et al. (1978) suggests that silica does bind directly to cellular surfaces by hydrogen bonding of silicic acid to exposed hydroxyl groups on cellular surfaces. Toporski et al. (2002) silicified *Pseudomonas fluorescens* and *Desulphovibrio indonensis* under supersaturated conditions of 1000 and 5000 mg/L silica using (metasilicate) tetaethylorthosilane with a pH of 8 under ambient temperature and pressure conditions. Benning et al. (2004) silicified *Calthorix specialis* under supersaturated silica concentrations of 300 mg/L at a pH of 7 and temperature of 25° C at ambient pressure. LaLonde et al. (2005) silicified *Sulfurihydrogenibium azorense* in silica concentrations of 90 and 300 mg/L with a pH of 6 at a temperature of 68° C. Lalonde et al. (2005) also silicified heat-killed *sulfurihydrogenibium azorense* under the same conditions in order to compare the silicification of live and dead cells. Lalonde et al. (2005) demonstrate that the species studied does not adsorb silica in undersaturated conditions but can grow exponentially amongst saturated conditions. Lalonde et al. (2005) does conclude that intact cells of *sulfurihydrogenibium azorense* exhibit a low preservation potential.

Herein, silicification experiments conducted using *Bacillus subtilis* and monitoring pH, silica concentration, and the morphology of silicification were completed under conditions that introduce large volumes of experimental solution (15L) to a relatively small volume of bacteria (0.120L). In this study, a range of silica
concentrations and the presence of Fe and Al (separately) have been tested to narrow the range of environments that produce well-preserved siliceous microfossils.

*Bacillus subtilis* is a common gram-positive soil bacteria that has well-defined cell wall characteristics and charge properties (Beveridge & Murray 1980; Urrutia & Beveridge 1994; Fein et al. 1997; Fein et al. 2002). Previous silicification experiments have used *Bacillus subtilis* (Beveridge & Murray 1980; Ferris et al. 1988; Urrutia & Beveridge 1994; Fein et al.; Fein et al. 2002; Phoenix et al. 2003). Gram-positive bacteria have cell walls that lack the outer membrane of polysaccharides indicative to gram-negative cell walls. *Bacillus subtilis* presents a simple cell wall with exposed functional groups that are the likely sites of silicate-bearing interactions (Urrutia & Beveridge 1994; Fein et al. 2002). The lack of an outer polysaccharide membrane may allow silica incorporation inside the cell, promoting the likely hood of silicification. Alternatively, the complete coating of a gram-positive cell in silica may prevent further infilling by silica but may provide a more robust cast of the cell.

The complete project involved two sets of experiments: an initial set and a final set. Chapter 2 describes the methods and results of the initial experiments that gave rise to the modified/final experiments. Chapter 3 is designed to be a stand-alone publication that describes the final or modified set of experiments and has complete introduction, methods, results, and discussion sections; some sections are repeated from the introduction chapter (ch. 1). Chapter 4 is a review of the major points of previous chapters composed into a short summary of the work conducted and the results. There are two appendices: Appendix A presents tabular data of silica concentrations of pH data
that are represented by the graphs in Chapters 2 and 3. Appendix B presents SEM/EDS results that are not presented in Chapter 3.
2.0 LOW VOLUME EXPERIMENTS

2.1 Introduction

The low volume experiments were the initial experiments conducted. The objective was to mimic the methods of some of the experiments presented in Urrutia & Beveridge (1994). The results from the low volume experiments indicated that a different experimental design was needed, a design that would allow small volumes of bacterial suspension to be introduced to large volumes of experimental solution. This chapter describes the methods, results, and conclusions of the low volume (initial) experiments in a publication format.

2.2 Methods

Silicification experiments were conducted using the Bacterium Bacillus subtilis following the general procedure described in Urrutia & Beveridge (1994). Bacillus subtilis was grown in standard Luria Broth (Bacto corp.) to an exponential growth phase with optical density of 0.2 at 600 nm (O.D.\textsubscript{600} = 0.2). 50 mL of cells were pelleted by centrifugation in sterile 50 mL centrifuge tubes (Falcon Co.) at 2100 X g for 15 minutes, washed twice, and resuspended to O.D.\textsubscript{600} of 0.2 with autoclaved Milli-Q filtered water. This suspension of cells was combined in equal volumes with stock solutions (diluting concentrations to 50\% of the stock solution concentration) that all contained 40 mg/L SiO\textsubscript{2} and 0.30 mg/L Fe, 0.60 mg/L Al, or both. Na\textsubscript{2}SiO\textsubscript{3} \textbullet 5 H\textsubscript{2}O was used as a source of SiO\textsubscript{2}. Experiments were run with silica without additives, silica with 0.15 mg/L Fe, silica with 0.3 mg/L Al, and silica with 0.3 mg/L Al and 0.15 mg/L Fe (See table below). All stock solutions were adjusted to an initial pH of 8 using HCl.
*Bacillus subtilis* suspension and stock solutions were combined in 50mL centrifuge tubes (experimental vessels) and placed on a rocker table. Six separate series of solutions were used. Each series involved three experimental vessels of silica (and Al/Fe) and *Bacillus subtilis* solutions, and two control experimental vessels with Milli-Q purified water instead of *Bacillus subtilis* solution added.

Samples were extracted at 10, 20, and 40 days. 1.5 mL aqueous samples were removed from each reaction vessel and sterile filtered using a 0.22 µm pore size polypropylene filters. 1 mL samples were extracted for scanning electron microscopy analysis by transferring the 1 mL of sample to a syringe with a removable 13 mm 0.45 µm pore size Gelman Supor polyethersulfone disc filter. The syringe/detachable filter apparatus was used to fixate with a 2% gluteraldehyde solution in phosphate buffer (pH 8). Samples were then dehydrated in an ethanol series and critical point dried.

Scanning electron microscopy (Hitachi S4700 with Quartz Imaging Xone software for image capture) with electron dispersive spectrometry (Gresham Scientific Instruments SIRIUS 30/SCTW) was used to analyze filtrates. The SEM was set to an accelerating voltage of 20 keV and working distance between 11.9 and 12.5 mm. Samples (filters) were mounted on 15 mm aluminum SEM stubs with carbon paint and coated with 10 nm of C using a Cressington 108A rod coater.

Aqueous samples were analyzed colorimetrically. The molybdate blue method was used for silica analysis (Strickland 1952, Govett 1961, Kolthoff et al. 1969); the
detection limit is 0.01 mg/L. The Ferrozine method was used for iron analysis, (Stookey 1970, Wilson et al. 2000a & b) the detection limit is 0.05 mg/L.

2.3.0 Results

<table>
<thead>
<tr>
<th>Sample Name Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time and [silica]</td>
</tr>
<tr>
<td>Day 10 20mg/L SiO₂</td>
</tr>
<tr>
<td>Day 20 20mg/L SiO₂</td>
</tr>
<tr>
<td>Day 40 20mg/L SiO₂</td>
</tr>
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</table>

Table 1: Sample name matrix for low-volume experiments. The first number designation indicates the day of the experiment on which the sample was extracted (10D-XX-XX indicates the "10-day" sample). The second number indicates the concentration of dissolved silica used (XX-20Si-XX indicates 20 mg/L silica). The third component indicates the presence or absence of other metals (NA indicates "no additives").

Results from these experiments indicate that silica concentrations and pH were not stable throughout the experiment (Figure 6 and 7). These changes occur within the first 10 days. Silica concentrations declined approximately 25% under all of the experimental parameters except in experiments that contained silica and aluminum (xD-Si20-Al samples) that declined approximately 50%. The initial concentration of silica was below saturation and should not have changed by equilibrium-driven precipitation of silica alone. pH values also rapidly declined to a range of roughly 5.75 to 6.5 in the presence of bacteria except in two of three experiments with silica and no additives that only exhibit a decline to approximately 7.6, similar to some of the control parameters. The control samples exhibit more stable pH values, most declined to approximately 7.6 except the silica with Al control (xD-Si20-Al control) that remained at a pH of 8.
Figure 6: Silica concentrations as a function of time in low-volume experiments.

Figure 7: pH values as a function of time in low-volume experiments.
Examination of the filter control sample by SEM/EDS (Figure 8) displays a texture that looks similar to filaments and ectosporues and must be considered when compared to samples the Supor polyethersulfone filter material is stronger than other membrane filters but the texture is not as smooth as other membrane filters. The EDS spectra display C, O, and S.

Figure 8: Electron micrograph (A) and EDS spectra (B) of filter control. (A) captured at a magnification of 4.5 k with a working distance of 9.3 mm and accelerating voltage of 20 keV. The processing of this control in a manner identical to the samples is not verified.

2.3.1 Day 10

*Bacillus subtilis* cells are relatively intact in the 10D-20Si-FeAl sample. A general mass of distinguishable cells and possibly cell fragments are displayed in figure 9C. Figures 9A and 9D display cells that appear to be separating, indicating continued cell division and metabolic activity (white arrows). EDS spectra (9B) collected from cell surfaces detected Si, Al, Fe, Na, K, Ca, Cl, P, and S.
Figure 9: Electron micrographs (A, C, and D) and EDS spectrum (B) of sample from experiments with initial concentrations of 20 mg/L silica, 0.3 mg/L Al, and 0.15 mg/L Fe taken at the 10 day sample point (10D-20SiFeAl). (A) and (B) collected at a magnification of 15 k, working distance of 9.2 mm, and accelerating voltage of 20 keV. (C) collected at a magnification of 5 k, working distance of 9.4 mm and accelerating voltage of 10 keV. (D) collected at a magnification of 25 k, working distance of 9.2 mm, and accelerating voltage of 20 keV. EDS spectrum (B) was collected from the area within the box indicated by the white arrow in (A).

2.3.2 Day 20

Cellular forms are less prevalent in the 20D-Si20-FeAl sample. Many filaments can be seen extending ectospores (Figure 10D (arrow)) and composing woven masses (Figure 10A). EDS spectra indicate the presence of Si, Al, Fe, Na, Mg, Cl, P, and S.
Figure 10: Electron micrographs (A, C, and D) and EDS spectrum (B) of sample from experiments with initial concentrations of 20 mg/L silica, 0.3 mg/L Al, and 0.15 mg/L Fe taken at the 20 day sample point (20D-Si20-FeAl). (A) and (B) were captured at a magnification of 30 k, (C) at 6 k, and (D) at 15 k, all at a working distance of 12.4 mm and accelerating voltage of 20 keV. EDS spectrum (B) was collected from the area displayed by (A).

2.3.3 Day 40

In the 40D-20Si-FeAl sample intact cellular forms are not readily observed but spores and filaments are abundant (Figures 12A, 12C, and 12D). Woven masses of filaments are prevalent and display effective spanning and coverage that could be considered a biofilm (Figure 12A). EDS spectra indicate Si, Al, Fe, Na, Mg, Ca, Cl, P, and S.

The 40D-Si20-NA sample does not display intact cells. Cellular remnants, spores, filaments, and film are not observed at all in some areas of the filter such as displayed in Figure 19C, the textured appearance is similar to masses of filaments with attached spores but is in fact just the texture of the filter (compare to Electron micrograph
of filter control (Figure 8A). Film (Figure 11D) and small clusters (Figure 11A) of primarily spores with some traces of filaments indicate declining metabolic activity in an environment of limited resources. EDS spectra detected Si, Ca, Cl, P, and S.

**Figure 11:** Electron micrographs (A, C, and D) and EDS spectrum (B) of sample from experiments with initial concentrations of 20 mg/L silica, and no added Al or Fe taken at the 40 day sample point (40D-Si20-NA). (A) and (B) were captured at a magnification of 6 k, (C) at 3 k, and (D) at 3 k, all at a working distance of 13.1 mm and accelerating voltage of 20 keV. EDS spectrum (B) was collected from the area displayed by (A).
Figure 12: Electron micrographs (A, C, and D) and EDS spectrum (B) of sample from experiments with initial concentrations of 20 mg/L silica, 0.3 mg/L Al, and 0.15 mg/L Fe taken at the 40 day sample point (40D-20Si-FeAl). (A) and (B) were captured at a magnification of 20 k, (C) at 5 k, and (D) at 10 k, all at a working distance of 12.6 mm and accelerating voltage of 20 keV. EDS spectrum (B) was collected from the area displayed by (A).

2.4 Discussion

The results of these experiments are similar to other studies involving the experimental silicification of *Bacillus subtilis* in the evidence that silicification or the persistence of cells is enhanced in the presence of Fe (Ferris et al. 1988; Urrutia & Beveridge 1994; Fein et al. 2002). The important difference in results of the experiments presented here is that silica was immobilized in experiments that did not contain Fe (Table 1), suggesting that silica can interact directly with the surface of *Bacillus subtilis* cells.
The low pH produced by these experiments could easily inhibit silica deposition on cellular surfaces. Ferris et al. (1988) reports evidence of the release of nucleic acids and proteins, which is the most likely cause for the decline in pH observed in the experiments presented here. At the low pH observed, cell surfaces should be positively charged. A positive charge would not facilitate the adsorption of silicic acid to hydroxyl groups as is proposed in Francis (1978) and would not provide the cationic binding sites that are proposed to be the venue for silica interaction with cell surfaces of gram-positive bacteria (Beveridge 1984; Urrutia & Beveridge 1994; Westall 1997). Ferris et al. (1988) reports a rise in pH from 7 to 8.5 over the first 60 days. This disparity probably arises from differences in the source of silica used or the temperature that the experiments were run at (70° C in Ferris (1988) and ambient in this study). Ferris (1988) used a colloidal silica source and this study used Na$_2$SiO$_3$ ● 5 H$_2$O, but more importantly the Fe concentrations used were an order of magnitude different (1.5 mg/L in Ferris et al. (1988) and 0.15 mg/L in this study). A greater concentration of Fe could result in preventing autolysis (Urrutia & Beveridge 1994) to a greater degree. Inhibitions of autolysis would result in less release of nucleic acids and proteins that could reduce pH values. Results of this study are similar to the results of Ferris et al. (1988) in the fact that Fe is definitely shown to be an important factor in the persistence (deterring autolysis) and silicification of *Bacillus subtilis*.

The experiments presented here show evidence that silica is immobilized in systems with and without Fe present. Fein et al. (2002) also reports silica uptake in all systems involving bacteria. Urrutia & Beveridge (1994) suggest that silica alone with not
interact with the surface of bacterial cells and that cationic salt bridging is necessary for silica sorption. Urrutia & Beveridge (1994) introduced the Fe as a pretreatment of the bacteria prior to initializing silicification experiments which may have enhanced the impairment of autolysins by Fe and would have prevented the interaction of Fe with silica in the experimental solutions, a possible cause for the silica immobilization observed here. Phoenix et al. (2003) displays some chemical evidence supporting the conclusion that insufficient Fe is bound to the cellular surface to act as a "salt bridge" for silica binding (based on disproportional loss of silica and Fe from solutions) which supports the evidence presented by the experiments of this study. Urrutia & Beveridge (1994) also conducted experiments involving the silicification of *Bacillus subtilis* in the presence of Pb, Cd, Cr, Cu, and Zn, results from the experiments all show that the bacteria retained more of these metals in systems that did not involve silica. This result could indicate that there is some direct interaction between silica and the outer cellular membrane of *Bacillus subtilis*, otherwise metal retention might be expected to be equivalent in both systems with and without silica.

### 2.5 Conclusions

Silicification did occur under the specified methods but the reciprocal effect of the bacteria on the experimental vessel can not be ignored. The silicification of *Bacillus subtilis* in undersaturated conditions is demonstrated but was likely inhibited by the decline in pH. The results also demonstrate that *Bacillus subtilis* can persist in the experimental environments supplied, but only for longer times in the presence of Fe as demonstrated in other results. Also, the experimental silica concentrations are not
representative of the silica concentrations that will be encountered in natural hot spring environments. From these results it is evident that a different experimental design is needed, a design that can more effectively mimic natural conditions by preventing the bacteria from extensively changing the experimental environment.
3.0 FINAL EXPERIMENTS

Abstract

Silicification experiments were conducted using the gram-positive bacteria *Bacillus subtilis* under a matrix of conditions involving silica concentrations well below saturation, approximately at saturation, and approximately 2.5 X saturation (initially) with and without the addition of Fe and Al (separately). All experiments were adjusted to an initial pH of 8. Silica concentrations and pH were monitored throughout experiments. Silica was deposited under all experimental conditions including undersaturated conditions. More silica was immobilized in experiments with Fe than in experiments without Fe. Electron microscopy was used to observe the habits of silica deposition under the experimental parameters. All experimental conditions show that cells persist better in the presence of silica and/or Fe and Al than in experimental controls (no silica, no Fe, or Al). Cells display fewer signs of stress in conditions below silica saturation than under conditions at or above saturation. Results indicate the presence of Fe is imperative to maintaining articulated bacterial forms in a stressed environment while the presence of Al can aid in more effectively coating bacteria with silica. The pH of all experimental conditions declined proportional (inverse) to the silica concentration (greatest silica concentration declined the least). The presence of Fe and/or Al may serve as an initial indicator to the likely hood of finding microfossils or other sings of life in silica deposit.
3.1 Introduction

Any opportunity to analyze samples of extra-terrestrial (likely martian) deposits for signs of life will be rare and costly. Such opportunities must be optimized by selecting sample sites that have the greatest likelihood of exhibiting signs of life such as microfossils or chemical/mineralogical biosignatures. Optimal sample sites can be selected by focusing on analogs to earth deposits that preserve detectable signs of life, particularly in ancient deposits. Microfossils are often found in silica deposits. Silica deposits are chemically and physically resistant to weathering processes and have provided evidence of ancient microbial life from Archean (Awramik et al. 1983; Cady 2001; Binet et al. 2008) through Phanerozoic times (Duhig et al. 1992a; Duhig et al. 1992b; Walter et al. 1996; Westall 1997; Guirdry & Chafetz 2003a; Binet et al. 2008; Kaur et al. 2008). The first evidence of life discovered beyond Earth may be identified in a silica deposit. However, silicification processes are poorly understood. Testing the conditions that lead to microfossil silicification is a key step in locating extra-terrestrial microfossils or other signs of life.

Silicification is used here as a general term referring to any form of fossilization involving the permineralization, entombment, or replacement of biogenic material by silica. Silicification, particularly permineralization, provides micro-fossils of exquisite fidelity (Kyle et al. 2007) that are resistant to weathering. Silica deposits offer a chance to observe microorganisms fossilized in ancient or extreme environments (Awramik et al. 1983; Duhig et al. 1992a; Duhig et al. 1992b; Cady & Farmer 1996; Hinman & Lindstrom 1996; Walter et al. 1996; Westall 1997; Jones et al. 2000; Cady 2001; Jones et
al. 2001; McKenzie et al. 2001; Guirdry & Chafetz 2003a; Mountain et al. 2003; Guidry & Chafetz 2003a; Guidry & Chafetz 2003b; Benning & Mountain 2004; Lynn & Campbell 2004; Fernandez-Turiel et al. 2005; Amores & Warren 2007; Kyle & Schroeder 2007; Kyle et al. 2007; Binet et al. 2008; Handley et al. 2008; Kaur et al. 2008; Lau et al. 2008). The precise chemical environments that produce the most effective silicification of microfossils are undefined. There are many natural aqueous environments that are rich in silica and support microbiological life forms. Hot springs can be generalized as the most accessible environments that fit these parameters.

(Fernandez-Turiel et al., 2005), and the Daggyai Tso geothermal field of central Tibet (Lau et al. 2008). The silica deposits that form around hot springs are called sinter.


Hot springs generally have a constant outflow and silica concentration that change very little throughout the year (Hinman & Lindstrom 1996). In hot springs, the local chemistry (particularly silica concentration) is largely controlled by inorganic dissolution and subsequent precipitation of lithic constituents driven by temperature/pressure flux (White 1956). Silica concentrations are controlled by the solubility of quartz at elevated temperatures. Precipitation of silica will continue downstream as hot spring waters flow and cool along the outflow channel away from the source of the hot spring. Continued precipitation of silica along a descending thermal gradient coats cell surfaces and entombs microorganisms while silica concentrations decrease. However, the decline in temperature and continued evaporation may result in increased oversaturation. Undersaturated conditions may occur as hot spring outflow channels change pH or mix with shallow aquifer and surface runoff water. These natural variations will produce a range of silica concentrations which are highest at the source and descend along the
outflow channel of any hot spring, resulting in the preservation of microorganisms under a variety of conditions in a small area. Evaluating the potential for microfossil preservation along these gradients requires a greater understanding of these processes. Once the effects of silica concentration on silicification processes are understood, other factors such as the concentration of metals can be considered.

Fe and Al have been shown to potentially influence silicification processes in laboratory experiments and natural environments (Urrutia & Beveridge 1994; Fein et al 2002; Phoenix et al 2003, Kyle et al. 2007). The presence and concentration of certain metals such as Fe or Al may be used to indicate if a particular silica deposit is likely to contain detectable microorganisms because of the effect these metals have on silicification. The results of Lynn & Campbell demonstrate that morphological details of microfossils can be degraded or lost to diagenetic processes. Wirth & Gieskes (1979) provide evidence that Mg decreases the solubility of silica above pH 9 which may decrease diagenetic overprinting. Hinman (1998) provides evidence that Al and Fe can promote silica deposition directly into more ordered phases; Opal-CT in the presence of Mg and quartz in the absence of Mg. These are very important factors to consider when searching for microfossils, silica precipitating directly into a stable phase (quartz) could circumvent the diagenetic overprinting that can obscure morphologic detail.

Defining the conditions under which signs of life are preserved is difficult in natural environments because they are open systems that cannot be controlled and manipulated. The mechanisms responsible for silicification in hot spring deposits vary
throughout a wide range of conditions of pH, silica concentration, and concentrations of other chemical constituents (White et al. 1956; Brock 1967; Jones et al. 2000; McKenzie et al. 2001; Mountain et al. 2003; Guidry & Chafetz 2003a; Fernandez-Turiel et al. 2005; Kyle & Schroeder 2007; Kyle et al. 2007; Lau et al. 2008). These relationships are poorly understood and result in different forms of silicification. Laboratory experiments offer a way to define conditions that may promote the preservation of signs of life in silica deposits.

Previous silicification experiments have been conducted on many organisms (Francis et al 1978; Ferris et al 1988; Urrutia & Beveridge 1994; Fein et al. 2002; Toporski et al. 2002; Phoenix et al. 2003; Benning et al 2004; Lalonde et al. 2005). Most studies did not report or control specific parameters such as changes in pH (Urrutia & Beveridge 1994; Toporski et al. 2002; Benning et al. 2004; Lalonde et al. 2005) and silica concentration (Francis et al 1978; Ferris et al 1988; Urrutia & Beveridge 1994; Toporski et al. 2002) throughout the experiment, or have assumed that pH was only a function of silica concentration (Fein et al. 2002).

Many studies have used the bacteria chosen for this set of silicification experiments, Bacillus subtilis, in silicification experiments (Ferris et al 1988; Urrutia & Beveridge 1994; Fein et al. 2002; Phoenix et al. 2003). Ferris et al. (1988) silicified Bacillus subtilis with silica concentrations of 300 mg/L, a 1.5 mg/L Fe pre-treatment, pH of 7, and at a temperature of 70° C under ambient pressure. Ferris et al. (1988) demonstrated that Fe was an important factor leading to the formation of microfossils and
suggested that even degraded cells of *Bacillus subtilis* promoted silica precipitation. Urrutia & Beveridge (1994) silicified *Bacillus subtilis* in silica concentrations of 8.4 mg/L, a 0.15 mg/L Fe pretreatment, a 0.30 mg/L Al addition (not a pretreatment), pH values of 5.5 and 8, and ambient temperature and pressure conditions. Urrutia & Beveridge demonstrate the importance of Fe in preventing autolysis under non-metabolic conditions for *Bacillus subtilis* and suggest that silica is bound to cellular surfaces by a cationic bridging mechanism. Fein et al. (2002) silicified *Bacillus subtilis* in silica concentrations of 1.4 mg/L with both a 0.75 mg/L Fe pretreatment at pH of 5.2 and an Al pretreatment at pH 7.3 all under ambient temperature and pressure. Fein et al. (2002) suggests that silica is bound to Fe and Al oxides that are electrostatically bound to the bacterial surface, not attributing any silica binding to direct silica-organic interactions, similar to the conclusions of Urrutia & Beveridge (1994). Phoenix et al. (2003) silicified *Bacillus subtilis* under conditions of both undersaturation (60 mg/L) and oversaturation (400 mg/L) with the addition (not pretreated) of Fe in various concentrations from 0.20 to 100 mg/L including a control of 0 mg/L Fe. All experiments were run at a pH of 7 under conditions of descending temperature starting at 90° C declining to 25° C to induce silica precipitation at ambient pressure. Phoenix et al. (2003) finds that insufficient Fe is bound to the bacterial surface to account for the volume of silica immobilized if the “salt bridge” for silica sorption is the only mechanism employed for silica immobilization. This finding is contradictory to the conclusions of Urrutia & Beveridge (1994) and Fein et al. (2002).
Silicification experiments have also been conducted on a variety of other organisms. Westall (1997) silicified several species of Bacteria including one species of gram positive bacteria, *Bacillus laterosporus*, and three species of gram negative bacteria including *Pseudomonas fluorescens*, *Pseudomonas vesicularis*, and *Pseudomonas acidovorans*. All bacteria were silicified in orthosilicate tetraethoxysilane under ambient temperature and pressure conditions. Westall (1997) demonstrates that the gram positive bacteria *Bacillus laterosporus* is more quickly and better coated by silica under the experimental conditions than the gram negative bacteria silicified. Westall (1997) concludes that gram positive bacteria are more likely to be preserved in terrestrial and extraterrestrial geologic records than gram negative bacteria because. The outer membrane of gram-positive bacteria is more easily silicified because of the large amount of peptitoglycan exposed as the outer membrane. The thick peptitoglycan outer layer of gram positive bacteria supplies and abundance of cationic binding sites (Beveridge 1984) that in turn provide sites for silica sorption (Urrutia & Beveridge (1994). The outer layer of gram negative bacteria consist of a phospholipid-covered lipopolysaccharide layer covering a thin peptitoglycan layer that does not supply the same functional groups of the peptitoglycan layer exposed on gram positive bacteria. Beveridge (1984) states that the polymer peptitoglycan is rich in reactive carboxyl and phosphoryl groups that provide nucleation sites for metal cations. These factors suggest that gram positive bacteria will more easily become encrusted with silica and are a good choice for silicification experiments.
Silicification experiments offer a way to compare the extent and habits of microfossil preservation under controlled conditions that are similar to natural environments. An understanding of these factors provides the first steps in defining "superior" or "effective" silicifying environments and subsequent deposits that are likely to contain ancient terrestrial or extra-terrestrial microfossils. Changes in solute concentrations and pH must be observed during silicification experiments to develop an understanding of how these factors, and silicification processes, are affected by the life and death cycles of microorganisms.

The life and death cycles of microorganisms will affect silica concentrations, other metals concentrations, and pH. The effects that bacteria have upon their immediate environment should be considered and limited in experiments concerning the silicification of microorganisms. Limiting these effects will more effectively imitate natural environments. Flow-through experiments can be used to imitate these conditions but flow-through laboratory experiments are not necessarily practical or economical to sustain over an extended time period. A method of supplying large volumes of experimental solutions to relatively small volumes of micro-biota may be used as a proxy to flow-through experiments.

Herein, silicification experiments conducted using *Bacillus subtilis* that include monitoring pH, silica concentration, and the morphology of silicification is reported. These experiments address problems overlooked by other studies by using large volumes of experimental solution (15L) to a relatively small volume of bacteria (0.120L). A range
of silica concentrations and the presence of Fe and Al (separately) have been tested to help identify environments that produce well-preserved siliceous microfossils. *Bacillus subtilis* is a common gram-positive soil bacterium that has well-defined cell wall characteristics and charge properties (Beveridge & Murray 1980; Urrutia & Beveridge 1994; Fein et al. 1997; Fein et al. 2002). Gram-positive bacteria have cell walls that lack the outer membrane of polysaccharides indicative of gram-negative cell walls. Therefore, *Bacillus subtilis* presents a simple cell wall of peptitoglycan with exposed functional groups that are the likely sites of silicate-bearing interactions (Urrutia & Beveridge 1994; Fein et al. 2002). The lack of an outer polysaccharide membrane may allow silica incorporation inside the cell, promoting the likelihood of silicification.

### 3.2 Methods

Silicification experiments were conducted using the gram-positive bacterium, *Bacillus subtilis*. The experiment was designed to mimic a flow-through environment by supplying a large volume of experimental solution (15 L) to a relatively small volume of bacteria (0.120 L initially, declining 0.030 L per sample point) resulting in a ratio of 125/1. The large volume of experimental solution relative to volume of bacteria help to limit the effect the bacteria can have upon their environment.

**Experimental design**

The high volume experimental design consisted of isolating bacterial suspension in "bags" made from dialysis tubing (described below). The tubing was tied closed at each end using Teflon tape and then placed inside 15 L reservoirs (carboys) of
experimental solution. Three concentrations of silica (20, 160, and 400 mg/L SiO₂) and a control (0 mg/L SiO₂) were used. Sigma ACS reagent grade Na₂SiO₃ ⋅ 5 H₂O was used as a source of silica. Further, each experiment was performed in the presence of either Fe (0.15 mg/L) or Al (0.3 mg/L), and in the absence of both. Fe and Al were added in the form of commercial Aqua Solutions ICP standards in 3.5 % HNO₃ solution. This matrix represented 12 separate experimental or control conditions. All experiments and controls were adjusted to an initial pH of 8 using Fisher Scientific trace metal grade HCL diluted to a concentration of 1.2 M. All experiments were run under ambient temperature and pressure conditions. For each sample point (3 days, 7 days, 14 days, and 29 or 33 days), three bags were removed and sampled along with one sample from the reservoir solution.

**Bacterial culturing**

*Bacillus subtilis* was grown to an optical density of 0.2 measured at 600 nm (OD₆₀₀ = 0.2) in Bacto Corp. Luria Broth. Autoclaved, 500-mL Erlenmeyer flasks with stir bars for agitation/aeration were used as culture vessels. Bacterial cells were pelleted by centrifugation (2100 X g for 15 minutes), washed twice by resuspension in autoclaved Milli-Q filtered water, and finally resuspended in sterile-filtered experimental solution before placement inside the dialysis bags.

**Bacteria sequestering**

The dialysis bags consisted of approximately 12 cm of 42-mm wide Spectrum Labs Spectra Por 3RC dialysis tubing with a molecular weight cut-off of 3500 amu tied off at each end using Teflon plumbing tape that had been twisted into a string (autoclaved
in water before use). One end of the dialysis tubing was sealed before and the other after introducing 0.01 L of bacterial suspension with a sterile pipette. To tie off the end of the tubing, a simple square knot was first used, the end of the tubing was then folded against itself in the direction opposite the square knot, allowing a second square knot to pull the folded tubing together against the first knot of tubing/Teflon. A third square knot was then tied opposite the second to once again cinch the folded tubing together, preventing leakage. The exposed ends of the dialysis tubing were rinsed with approximately 100 mL of sterile Milli-Q filtered water before being placed in the 15 L carboys of experimental solution.

**Sampling**

Samples were extracted at 3, 7, 14, and 28 to 33 days. Each sample was extracted from the dialysis tubing bags by cutting one end off with a sterile razor blade and emptying the contents directly into a sterile syringe attached to a 13-mm filter holder with a 13 mm filter (0.45 µm pore size Gelman Supor polyethersulfone disc filter). Each dialysis tube/bag was manually agitated before removing the sample to help suspend bacteria and/or precipitates. A .004 L aliquot of the filtered solution was collected in 5-mL snap-cap culture tubes for aqueous analysis. The syringe filter holder was then used to fix the sample in 25% Electron Microscopy Sciences gluteraldehyde solution buffered and diluted to 2.5% in sterile filtered (0.22 µm Whatman GD/X polypropylene filter (housing and filter)) experimental solution, similar to the procedure used for *Bacillus laterosporus* by Westall (1997). Samples were then dehydrated in an ethanol series and dried by the critical point method (CPD). Some samples (14 and 33 day samples with Al
added (14D-Sixxx-Al & 33D-Sixxx-Al) were prepared via hexamethydisiloxane (Electron Microscopy Science) evaporation instead of CPD. A control was prepared by processing, mounting, and coating a clean filter in the same manner as the samples.

Analysis

Aqueous samples were analyzed colorimetrically. The molybdate blue method was used for silica analysis (Strickland 1952, Govett 1961, Kolthoff et al. 1969); the detection limit is 0.01 mg/L silicon (results were converted to silica). The Ferrozine method was used for iron analysis, (Stookey 1970, Wilson et al. 2000a, Wilson et al 2000b) the detection limit is 0.1 mg/L.

Scanning electron microscopy (Hitachi S4700 with Quartz Imaging Xone software for image capture) with electron dispersive spectrometry (Gresham Scientific Instruments SIRIUS 30/SCTW EDS unit with Quartz One software) was used to analyze the morphology and chemistry of filtered samples. Filters of dehydrated sample were adhered to aluminum SEM stubs with carbon tape. The edges of the filter were then sealed with carbon paint to ensure grounding (SEM stubs, carbon tape, and carbon pain all Electron Microscopy Sciences brand). Samples were coated with 10 nm of carbon using a Cressington 108A carbon coater and then coated with ~ 10 nm of Pt/Pd using a Pelco Model 3 sputter coater. Electron micrographs for visual analysis were collected with an accelerating voltage of 5 keV and working distance from 5.3 to 5.6 mm. EDS analysis was conducted at an accelerating voltage of 30 keV and working distance of 11.7 to 12.2 mm. Some images captured during EDS analysis are also displayed.

3.3.0 Results
Sample Name Matrix

<table>
<thead>
<tr>
<th>Time and [silica]</th>
<th>No Additives (NA)</th>
<th>0.15 mg/L Fe (Fe)</th>
<th>0.3 mg/L Al (Al)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3 0 mg/L SiO₂</td>
<td>3D-0Si-NA</td>
<td>3D-0Si-Fe</td>
<td>3D-0Si-Al</td>
</tr>
<tr>
<td>Day 3 20 mg/L SiO₂</td>
<td>3D-20Si-NA</td>
<td>3D-20Si-Fe</td>
<td>3D-20Si-Al</td>
</tr>
<tr>
<td>Day 3 160 mg/L SiO₂</td>
<td>3D-160Si-NA</td>
<td>3D-160Si-Fe</td>
<td>3D-160Si-Al</td>
</tr>
<tr>
<td>Day 3 400 mg/L SiO₂</td>
<td>3D-400Si-NA</td>
<td>3D-400Si-Fe</td>
<td>3D-400Si-Al</td>
</tr>
<tr>
<td>Day 7 0 mg/L SiO₂</td>
<td>7D-0Si-NA</td>
<td>7D-0Si-Fe</td>
<td>7D-0Si-Al</td>
</tr>
<tr>
<td>Day 7 20 mg/L SiO₂</td>
<td>7D-20Si-NA</td>
<td>7D-20Si-Fe</td>
<td>7D-20Si-Al</td>
</tr>
<tr>
<td>Day 7 160 mg/L SiO₂</td>
<td>7D-160Si-NA</td>
<td>7D-160Si-Fe</td>
<td>7D-160Si-Al</td>
</tr>
<tr>
<td>Day 7 400 mg/L SiO₂</td>
<td>7D-400Si-NA</td>
<td>7D-400Si-Fe</td>
<td>7D-400Si-Al</td>
</tr>
<tr>
<td>Day 15 0 mg/L SiO₂</td>
<td>15D-0Si-NA</td>
<td>15D-0Si-Fe</td>
<td>15D-0Si-Al</td>
</tr>
<tr>
<td>Day 15 20 mg/L SiO₂</td>
<td>15D-20Si-NA</td>
<td>15D-20Si-Fe</td>
<td>15D-20Si-Al</td>
</tr>
<tr>
<td>Day 15 160 mg/L SiO₂</td>
<td>15D-160Si-NA</td>
<td>15D-160Si-Fe</td>
<td>15D-160Si-Al</td>
</tr>
<tr>
<td>Day 15 400 mg/L SiO₂</td>
<td>15D-400Si-NA</td>
<td>15D-400Si-Fe</td>
<td>15D-400Si-Al</td>
</tr>
<tr>
<td>Day 29-33 0 mg/L SiO₂</td>
<td>29D-0Si-NA</td>
<td>33D-0Si-Fe</td>
<td>33D-0Si-Al</td>
</tr>
<tr>
<td>Day 29-33 20 mg/L SiO₂</td>
<td>29D-20Si-NA</td>
<td>33D-20Si-Fe</td>
<td>33D-20Si-Al</td>
</tr>
<tr>
<td>Day 29-33 160 mg/L SiO₂</td>
<td>29D-160Si-NA</td>
<td>33D-160Si-Fe</td>
<td>33D-160Si-Al</td>
</tr>
<tr>
<td>Day 29-33 400 mg/L SiO₂</td>
<td>29D-400Si-NA</td>
<td>33D-400Si-Fe</td>
<td>33D-400Si-Al</td>
</tr>
</tbody>
</table>

**Table 2:** Sample name matrix for low-volume experiments. The first number designation indicates the day of the experiment on which the sample was extracted (3D-XX-XX indicates the "3-day" sample). The second number indicates the concentration of dissolved silica used (XX-20Si-XX indicates 20 mg/L silica). The third component indicates the presence or absence of other metals (NA indicates "no additives").

**Aqueous Analysis**

The concentration of dissolved silica in all of the 400-mg/L experiments decreased. Silica concentrations in the silica-only and silica plus Al experiments (400 and 160 mg/L) remained stable through the life of the experiment around 150 to 160 mg/L (figures 13 and 15) while the silica plus Fe parameters (400 and 160 mg/L) decreased to between 100 and 120 mg/L and remained stable at that concentration (Figure 14). All silica concentrations declined prior to these meta-stable concentrations prior to the 14-day sample point. All experiments with silica concentrations of 20 and 0 mg/L remained stable during the life of the experiments (figures 13,14, and 15).
pH values declined in all experiments between the 3-day and 7-day sample points and remained stable thereafter (figures 16, 17, and 18), the pH decline was greatest in control experiments (0mg/L silica). The next greatest pH decline was in experiments with 20 mg/L silica, then 160 mg/L silica, and least in experiments with initial silica concentrations of 400 mg/L (figures 16, 17, and 18). The pH of control experiments (0 mg/L silica) using NA, Fe, and Al declined to approximately 6.5, 5.8, and 6.6 respectively (figures 16, 17, and 18). The pH of experiments with 20 mg/L silica and NA, Fe, and Al declined to approximately 6.8, 6.0, and 6.7 respectively (figures 16, 17, and 18). The pH of experiments with 160 mg/L silica and NA, Fe, and Al declined to approximately 7.1, 6.0, and 7.2 respectively (figures 16, 17, and 18). The pattern in pH decline was ubiquitous regardless of whether metals were added, although experiments with Fe showed a greater decline in pH. Iron concentrations decreased from the starting value, 0.15 mg/L, to below detection (0.1 mg/L) prior to the first sample point.
Figure 13: Dissolved silica concentrations as a function of time for experiments without additives.

Figure 14: Dissolved silica concentrations as a function of time for experiments in the presence of dissolved iron.
Figure 15: Dissolved silica concentrations as a function of time for experiments in the presence of dissolved aluminum.

Figure 16: pH values as a function of time for experiments without additives.
Figure 17: pH values as a function of time for experiments with dissolved iron.

Figure 18: pH values as a function of time for experiments with dissolved aluminum.
3.3.1 SEM/EDS Results From First Sample Point

The filter control shows globular nodules projecting up from the sample surface that could be mistaken as biological constituents (Figure 19A), although the type of filter used is stronger than traditional membrane filters the texture exhibited is the drawback of a Supor polyethersulfone filter. EDS spectra show the presence of O, C, and S in this control sample (Figure 19B).

![Figure 19: Electron micrograph and EDS spectra of filter control. (A) captured at a magnification of 10 K with a working distance of 5.5 mm and accelerating voltage of 5 keV. Scale 3 µm. (B) collected from the area displayed by (A) at a working distance of 12 mm and accelerating voltage of 30 keV. The processing of this control has not been verified as identical to the samples.]

Sample 3D-0Si-Fe (Figures 21A and 21B) shows an abundance of cells. Cells are numerous, rod-shaped, and well-inflated. Sample 3D-20Si-Fe (Figure 21) contains intact cells with coatings and colloidal silica spheres of approximately 0.15 µm in diameter. Filaments can be seen spanning the space between bacterial cells.

Sample 3D-160Si-Al displays cellular shapes and filaments that are faintly visible but whole cells are not commonly observed (Figures 22A, 22C, and 22D. Matted, filamentous films are observed at higher magnifications (Figures 22C and 22D). EDS spectra (Figure 22B) indicate the presence of Si, O, and S.
Figure 20: Electron micrographs of 3D-0Si-Fe sample. (A) captured at a magnification of 5 K. Scale 6 µm. (B) captured at 10 k. Scale 3 µm. (A and B) captured with a working distance of 5.5 mm and accelerating voltage of 5 keV.

Figure 21: Electron micrographs of 3D-20Si-Fe sample. (A) captured at magnification of 5 K. Scale 6 µm. (B) captured at 10 K. Scale 3 µm. (A and B) captured at a working distance of 5.6 mm and accelerating voltage of 5 keV.

Figure 22: Electron micrographs and EDS spectrum of 3D-Si160-Al sample. (A) captured at a magnification of 5 K. Scale 6 µm. (C) 20 K. Scale 1.50 µm. (D) 50 K. Scale 0.60 µm. (A, C, and D) captured at a working distance of 5.4 mm and accelerating voltage of 5 keV. (B) was collected from the area displayed by (A) at a working distance of 12 mm and accelerating voltage of 30 keV.
3.3.2 SEM/EDS Results From Final Sample Point

33D-20Si-Fe samples are the only samples that display signs of silicification or even intact cells in the presence of silica. Intact cells are rare in the 33D-20Si-Fe sample but some small cells can be observed with filaments attached (Figures 23A, 23C (arrow)). The cells and associated filaments exhibit a spherical texture (Figure 23D) to the coatings similar to the texture observed in other experiments with dissolved Fe. EDS spectra (Figure 23B) indicate the presence of Si, Fe, O, and S.

![Figure 23: Electron micrographs and EDS spectrum of 33D-20Si-Fe sample. (A) was captured at a magnification of 5 K. Scale 6 µm. (C) and (D) 20 K. Scale 1.50 µm. (A, C, and D) captured at a working distance of 5.3 mm and accelerating voltage of 5 keV. (B) was collected from the area displayed by (A) at a working distance of 12 mm and accelerating voltage of 30 keV.]

3.4 Discussion

The experiments presented in this study differ from any previous silicification experiments primarily in their design. This design supplies large volumes of experimental solution to small volumes of bacteria at least partially diminishes the ability of the bacteria to affect their artificial environment. These factors result in stable silica
concentrations (once silica concentrations settle to "stable" levels) and the silicification of bacteria with and without the addition of other metals, although cellular integrity and longevity is better maintained in experiments that include the addition of Fe.

*Bacillus subtilis* was effectively silicified under the experimental parameters of this study. It displayed silica interaction with all experiments and encrustation in experiments with higher concentrations of silica (160 and 400 mg/L). *Bacillus subtilis* was used in these silicification experiments because it is a common and robust bacterium that has been consistently shown to easily become coated with silica under a variety of conditions, particularly in the presence of Fe (Ferris et al 1988; Urrutia & Beveridge 1994; Fein et al. 2002; Phoenix et al. 2003). The gram positive nature of *Bacillus subtilis* provides a thick peptitoglycan outer membrane that is easily silicified due to the exposed carboxyl and phosphoryl functional groups (Beveridge 1984). Some researchers (Westall 1997) assume that the easily coated nature of gram positive bacteria will promote detection of these types of bacteria in the geologic record (terrestrial and extraterrestrial). Gram negative bacteria have been shown to allow silicification of internal constituents such as cytoplasm and organelles (Francis et al. 1978; Westall 1997; Toporski et al. 2002). This could result in a more solid microfossil that can persist through geologic time.

All EDS spectra of experiments involving silica indicate the presence of Si. This result implies a direct interaction of silica with the cellular surface even in undersaturated conditions. Other researchers that used undersaturated silica concentrations (Urrutia &
Beveridge 1994; Fein et al. 2002) have suggested that silica is bound to cellular surfaces of *Bacillus subtilis* only through cationic or "salt bridging" mechanisms. They propose that Fe or Al oxides are electrostatically bound to the cell surface and subsequent sorption of silica to Fe or Al oxides occurs. The silica concentrations used in this study are greater (~2 X Urrutia & Beveridge (1994) and ~ 14 X Fein et al. (2002)) and the experiments with Fe and Al that the no-additive experiments are compared to were conducted differently in this experiment. Urrutia & Beveridge (1994) and Fein et al. (2002) introduced Fe and Al as a pre-treatment to cells that were then rinsed and finally introduced to silica solutions. This pre-treatment sets the stage for the "cationic bridging" conclusion and does not accurately reflect natural conditions. Experiments involving Fe and Al in this experiment did not use pretreatments, Fe and Al were introduced as a constituent of the silica-rich solutions. Phoenix et al. (2003) presents some chemical evidence that insufficient Fe is bound to the cellular surface of *Bacillus subtilis* to account for silica uptake by an exclusive "salt bridge" mechanism (based on disproportional loss of silica and Fe from solutions) the remaining possibility is that some silica uptake must have occurred as a result of direct interaction of silica with the bacterial surface. Urrutia & Beveridge (1994) also conducted experiments involving the silicification of *Bacillus subtilis* in the presence of Pb, Cd, Cr, Cu, and Zn, results from the experiments all show that the bacteria retained more of these metals in systems that did not involve silica. This result indicates that there is some direct interaction between silica and the outer cellular membrane of *Bacillus subtilis*, otherwise metal retention might be expected to be equivalent in systems that do and do not have silica present. The other major difference in the design of this study from Urrutia & Beveridge (1994) and
Fein et al. (2002) is the experimental design that allows small volumes of bacteria to be introduced into large volumes of experimental solution resulting in a 1/125 ratio (bacterial suspension/experimental solution) as opposed to the 1/1 ratio of other studies. This difference could also explain some of the differences in observations between this study, Urrutia & Beveridge (1994), and Fein et al. (2002).

The concentration of dissolved silica stabilized at a lower concentration in experiments that included dissolved iron (160 and 400 mg/L silica plots: Tables 8, 9, and 10). This result is similar to the results of Ferris et al. (1988), Urrutia & Beveridge (1994), and Fein et al. (2002) concerning silica concentration but can not be interpreted as an enhancement of the silicification of bacterial cells, only as silica precipitation. The volume of experimental solution provided in this study would not yield these observations if silica sorption onto cell surfaces was the only mechanism that effected the concentration of dissolved silica. Also, the control experiments exhibit similar silica concentrations as the bacterial experiments indicating that the interaction of silica with Fe was controlling silica saturation, rather than the interaction of silica with the bacteria.

Scanning electron micrographs of samples from experiments with silica and Al (Figure 22) display a smooth coating compared to a rough or spherical texture exhibited by experiments with silica and Fe (Figures 7 and 9). This observation is also made by Urrutia & Beveridge (1994). Similar observations can be made from the electron micrographs presented in Ferris et al. (1988), Fein et al. (2002), and Phoenix et al. (2003). A smooth coating could promote microfossil resistance to diagenetic processes
by reducing or eliminating pore space within the surface of the fossil, particularly if the presence of Al caused quartz to be precipitated directly from solution (Hinman 1998).

The pattern of pH changes observed in the experiments presented here shows that the silica concentration has a buffering effect under these conditions. The initial concentration of silica and decline in pH has an inverse relationship (Tables 11, 12, and 13), i.e. the highest silica concentration (400 mg/L) exhibited the least decline in pH, controls experienced the greatest decline in pH. Ferris et al. (1988) reports a rise in pH from 7 to 8.5 over the first 60 days of experiments. This result is not corroborated in this study where experiments with Fe and silica concentrations similar to Ferris et al. (1988) declined in pH from 8 to approximately 7.5 over the course of approximately 30 days. This disparity could arise from the source of silica used (colloidal silica in Ferris (1988) and Na₂SiO₃ • 5 H₂O in this study) but more importantly the Fe concentrations used were an order of magnitude different (1.5 mg/L in Ferris et al. (1988) and 0.15 mg/L in this study) and there was a great difference in the temperature used (70° C in Ferris et al. (1988) and ambient in this study). The greater concentration of Fe could result in more effectively preventing autolysis (Urrutia & Beveridge 1994). Inhibition of autolysis would result in less release of nucleic acids and proteins that could reduce pH values, Ferris et al. (1988) does report some evidence of the release of nucleic acids and proteins. The results of this study are similar to the results of Ferris et al. (1988) in the fact that Fe is definitely shown to be an important factor in the persistence (deterring autolysis) and silicification of Bacillus subtilis. But the pH decreases more in these experiments that involve Fe compared to the experiments that do not (compare Figure 12 to Figures 11 and
13) suggesting that the pH decrease observed here is not solely due to autolysis, otherwise the pH decrease would be least in the presence of Fe because Fe is shown to inhibit autolysis by this study, Ferris et al. (1988), Urrutia & Beveridge (1994), and Fein et al. (2002).

3.5 Conclusion

Direct observation of the effects of silica concentration, Fe, and Al upon silicification processes have been made to help facilitate the selection of natural sample sites that have the highest likelihood of containing silicified remains of microorganisms. *Bacillus subtilis* is shown to interact with silica regardless of the presence of Fe and Al and to become covered in a robust crust of silica when introduced to silica concentrations similar to those found in hot spring environments. These results indicate that gram-positive bacteria such as *Bacillus subtilis* are good candidates for long-term preservation in the terrestrial and extra-terrestrial geologic record.

Fe and possibly Al are shown to be imperative to the silicification process. Samples without Fe or Al display some encrustation with increasing silica concentration but cellular fidelity is poor and intact cells generally show signs of extreme stress. Samples with iron in them show much better cellular structure indicating that the presence of iron allows some of the bacteria to persist in better condition than in other samples. The presence of aluminum provides a smooth coating that could more effectively sequester silicified cells from the open environment. If precipitation can
occur in more ordered phases such as opal-CT or quartz then diagenetic processes will be less likely to obscure morphological evidence of microfossils.

Much more experimental work needs to be conducted to understand the long-term preservation potential of different microorganisms in different environments. The preference in long-term preservation between gram positive bacteria that externally silicify quickly and robustly with the more slowly internally silicified gram negative bacteria needs to be tested. This can be done with long-term diagenetic experiments that consider the effects of Al, Fe, and possibly Mg upon silica deposition and diagenesis with attention to the perseverance of morphological detail. A complete understanding of these factors will allow the selection of preferred sample sites for the detection of ancient or extra terrestrial life.
4.0 SUMMARY

Results from the low-volume experiments indicate that silica concentrations and pH were not stable throughout the experiment. These changes occur within the first 10 days. Results indicate that the experiment is fundamentally flawed due to the intent to emulate natural environments, this flaw was a driving factor in developing a different experimental design.

The high-volume experiments provide more insight into silicification processes. Silica concentrations in the silica-only and silica plus Al experiments (400 and 160 mg/L) decreased/remained around 150 to 160 mg/L while the silica plus Fe parameters (400 and 160 mg/L) decreased to between 100 and 120 mg/L by the 14-day sample point. Results indicate that the saturation of silica is lower in the presence of Fe. The control experiments exhibit similar silica concentrations as the bacterial experiments indicating that the interaction of silica with Fe was controlling silica saturation, not interaction with bacteria. All experiments with 20 and 0 mg/L silica remained stable at the initial concentration throughout the experiments indicating that the intent of the high-volume experiments was accomplished. This result indicates that the experiments with higher silica concentrations, similar to hot spring fluids, are a proxy to natural silica-rich environments.

. pH values declined in all experiments between the 3-day and 7-day sample points and remained stable thereafter. The pH decline was greatest in control experiments that
lacked silica. The next greatest pH decline was in experiments with 20 mg/L silica, then 160 mg/L silica, and least in experiments with initial silica concentrations of 400 mg/L. Electron micrographs and EDS spectra provide evidence of silica encrustation in cells exposed to silica concentrations of 160 and 400 mg/L. In all experiments using 20 mg/L silica (with Fe, Al, and no additives) the presence of Si associated with cells or cellular remnants is identified by EDS. The association of Si with cells in experiments without Fe or Al and silica concentrations well below saturation indicates that the cellular surface of *Bacillus subtilis* does in fact interact directly with silica.

Cells, filaments, and spores are more abundant in experiments conducted with silica than without and greater yet with increasing silica concentration. When equivalent silica concentrations are compared, the addition of Al appears to enhance cellular fidelity and survival while the presence of Fe produces even better cellular fidelity. Experiments with 400 mg/L silica and Fe are the only experiments to contain intact cells and filaments after 29-33 days.

These results indicate that environments rich in silica that have both Fe and Al present have a high potential to effectively preserve gram-positive bacteria because the peptitoglycan membrane of gram-negative bacteria is completely and robustly encrusted in silica. However, a preference in fossil longevity through diagenesis is not defined in this study. Silica diagenesis can obscure or destroy morphological and chemical traces of microorganisms. Silica diagenesis may be limited by the conditions present during silicification and sinter deposition. Mg may inhibit the phase transition of Opal-CT to
quartz but more importantly quartz may be precipitated directly in the presence of Fe or 
Al and the absence of Mg (Hinman 1998). Microorganisms that do not have the thick 
peptitoglycan membrane of gram positive bacteria have been shown to silicify internally 
(Westall 1997; Toporski et al. 2002; Benning et al. 2004; Lalonde et al. 2005). Internally 
silicified microorganisms may not be more resistant to weathering and diagenetic 
processes. Different organisms need to be silicified in laboratory experiments and then 
subjected to diagenetic experiments that consider the precipitation of more ordered 
phases of silica (opal-CT or quartz as opposed to opal-A) in the presence of both Fe for 
cellular fidelity and Al for effective coating and elimination of pore space.
References


http://www.uweb.ucsb.edu/~glennon/geysers/geysermap.gif acquired 5/14/2009 19:00

*Analytica Chimica Acta*, 25, p. 69-80


Van Kranendonk M.J., Philippot P., Lepot K., Bodorkos S., Pirajno F., (2008) Geological setting of the Earth's oldest fossils in the ca. 3.5 Ga Dresser
Formation, Pilbara Craton, Western Australia. *Precambrian Research* 167, 1-2, p. 93-124


## Appendix A

### Aqueous Data

#### Low-Volume (Initial) Experiments

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## High-volume (Final) Experiments

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Appendix B

Scanning Electron Microscopy and Electron Dispersive Spectrometry

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File name: Si0_day3_36

File name: Si0_day3_34
File name: Si0_day3_28

File name: Si0_day3_27
File name: Si0_day3_22

File name: Si0_day3_21
File name: Si0_day3_20

File name: Si0_day3_18
File name: Si0_day3_02

File name: Si0_day3_01

3D-0Si-Fe
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File name: Si0_Fe_day3_EDS03
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File name: Si0_Fe_day3_08
File name: Si0_Al_day3_EDS03

File name: Si0_Al_day3_EDS02
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File name: Si20_day3_15
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File name: Si20_day3_13
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File name: Si20_AI_day3_08
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File name: Si160_Fe_day3_EDS01
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File name: Si400_Fe_day3_EDS04

File name: Si400_Fe_day3_EDS03
File name: Si400_Fe_day3_12

File name: Si400_Fe_day3_11
File name: Si400_Al_day3_EDS09

File name: Si400_Al_day3_EDS07
File name: Si400_Al_day3_EDS01

File name: Si400_Al_day3_09
File name: Si400_A1_day3_02

File name: Si400_A1_day3_01

33D-0Si-Fe
File name: Si0_Fe_day33_EDS03

File name: Si0_Fe_day33_EDS01

33D-20Si-Fe
File name: Si20_Fe_day33_EDS04

File name: Si20_Fe_day33_EDS03
File name: Si20_Fe_day33_01

29D-160Si-NA

File name: Si160_day29_03_
File name: Si160_day29_03

File name: Si160_day29_02