Merging genomics, transcriptomics and geochemistry to assess nitrogen cycling in terrestrial hot springs

BY

SARA THERESA LOIACONO
B.S., Purdue University, 2003

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Earth and Environmental Sciences
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

Defense Committee:

D'Arcy Meyer-Dombard, Chair and Advisor
Fabien Kenig
Neil Sturchio

This thesis is dedicated to my husband, Matthew, for his unwavering belief in this “science machine” he married, and to my parents who taught me to work hard and dream big.

ACKNOWLEDGEMENTS

I would like to thank my advisor, D'Arcy Meyer-Dombard, who allowed me the freedom to pursue this project, taught me the importance of collaborating with other scientists, and offered me countless opportunities to present this work at national and local meetings - for this I am ever grateful. I would also like to thank my committee members, Fabien Kenig and Neil Sturchio, for the advice and encouragement offered to me throughout the course of this project. Many thanks are in order for my colleagues at Arizona State University, especially for "Uncle" Everett Shock, who not only provided invaluable geochemical data but also served as a mentor and a cheerleader for this work, and without whom this Yellowstone field research would not have been possible. Additionally, thank you to Jeff Havig for the geochemical and isotope data that was vital in supporting the genetic data of this project. I would also like to thank Amisha Poret-Peterson, Hilairy Hartnett, Eric Boyd, Trinity Hamilton, José de la Torre, Stefan Green, and Wesley Swingley for their many insightful discussions and help with data interpretation. I wish to thank the Yellowstone National Park Research Office and numerous field assistants for facilitating with sample collection. Specific thanks are owed to several UIC undergraduate students (former and current) who supported this research both in the field and in the lab: Jo Carbone, Marci Burton, Justin Meyers, Lauren Brewer, Varvara Vassilev, Michelle Abrams, Maya Kucharczyk, and Eric Staley. Thank you to Lindsay MacKenzie for her much needed pep talks, and to my fellow graduate students at UIC for helping to keep me "sane" during this entire process. Finally, thank you to the agencies that funded this project: the NASA Astrobiology institute via the ASU "Follow the Elements" grant (# NASA 08-NAI5-0018) and the Illinois Space Grant Consortium Graduate (ISGC) via a Graduate Fellowship.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

AOA	Ammonia-Oxidizing Archaea
AOB	Ammonia-Oxidizing Bacteria
AOM	Ammonia-Oxidizing Microorganisms
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DNRA	Dissimilatory Nitrate Reduction to Ammonium
LGB	Lower Geyser Basin
LUCA	Last Universal Common Ancestor
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SBCs	Streamer Biofilm Communities
YNP	Yellowstone National Park

SUMMARY

A multi-faceted study was conducted to evaluate nitrogen cycling in Mound Spring and "Bison Pool," two geochemically similar, alkaline hot springs of the Lower Geyser Basin in Yellowstone National Park. Downstream geochemical trends are supported by results of genomic and transcriptomic studies of Mound Spring and "Bison Pool" chemotrophic and phototrophic microbial communities. Nitrogen stable isotope trends of "Bison Pool" and Mound Spring biomass corroborate genomic and transcriptomic data and reflect topographically-driven differences in exogenous nitrogen input between the two hot springs.

Results indicate that both chemotrophic and phototrophic communities at Mound Spring are genetically capable of fixing nitrogen, and *nifH* gene transcripts suggest downstream variability in *nifH* expression that may be a result of downstream changes in fluid chemistry and microbial community variation between sampling sites. Results of gene surveys targeting functional nitrification and denitrification genes indicate a marked difference between these processes at Mound Spring and "Bison Pool." Nitrification potential is present only in the chemotrophic streamer biofilm communities at "Bison Pool," yet the absence of *amoA* transcripts suggests that *in situ* nitrification was not occurring at the time of sampling. Genetic evidence of nitrification is absent from all Mound Spring chemotrophic and phototrophic communities sampled, and denitrification gene markers are relatively few. In contrast to Mound Spring, the microbial communities of "Bison Pool" appear to have widespread genetic capability of most denitrification processes, and the extensive presence of *nirS* gene sequences and transcripts suggests that nitrite reduction is an important metabolic process in the "Bison Pool" ecosystem.

1. INTRODUCTION

1.1 Why study the hot springs at Yellowstone National Park?

Hydrothermal systems are arguably the best modern examples of early life-supporting environments on Earth. Studies of biodiversity, metabolic strategies, and functional ecology in modern hydrothermal systems have the potential to provide insight into the metabolism and evolution of primitive life, with origins at least 3.2 Ga (Rasmussen, 2000). It has been proposed that LUCA, the Last Universal Common Ancestor of modern life, originated in alkaline, hydrothermal vent environments in Archean oceans (Lane *et al*., 2010; Russell *et al*., 1993). Modern hydrothermal vent environments have been shown to abiotically synthesize several organic molecules necessary for cellular structures and function, such as amino acids (e.g. Huber and Wächtershäuser, 2003; Amend and Shock, 2000, 1998; Marshall, 1994; Hennet *et al*., 1992) and long-chain hydrophobic hydrocarbons (potential membrane precursors) (Konn *et al*., 2009; Cody, 2004; McCollom *et al*., 1999). Furthermore, abiotic formation of nucleotides and monosaccharides (ribose and deoxyribose), biomolecules that make up nucleic acids (RNA and DNA), is thermodynamically favorable under hydrothermal conditions (LaRowe and Regnier, 2008). Alkaline hydrothermal vents were likely prolific on the early Earth ocean floor (Russell and Arndt, 2005; Martin and Russell, 2003), and modern alkaline hydrothermal systems, such as Lost City, have been shown to produce geochemical proton gradients (Shock *et al*., 1998), which are used by microorganisms to generate adenoside triphosphate (ATP), a source of energy for microbial metabolisms. Additionally, the widespread occurrence of the deepest lineage of the 16S rRNA tree of life, *Aquificales*, in terrestrial hot springs (reviewed in Reysenbach *et al*., 2005)

further supports the idea that extant life in hydrothermal systems may provide a modern analogue to Archean life.

In addition to evidence for ancient hydrothermal systems on Earth, data from recent NASA missions suggest ancient hydrothermal activity on Mars as well as postulated hydrothermal activity on other solar bodies. Perhaps most compelling is evidence from the Mars Exploration Rover Spirit, which discovered silica-rich outcrops and soils adjacent to the Home Plate volcanic plateau in Gusev Crater. The silica near Home Plate is consistent with that formed through silica-sinter formation, a process that occurs in alkaline hydrothermal systems on Earth, conceivably indicative of ancient hydrothermal activity in that locale (e.g. Ruff *et al*., 2011; Arvidson *et al*., 2008; Squyres *et al*., 2008).

Studies of modern analogue hydrothermal systems have taken place over a range of geographical locales, such various seafloor locations near the Mid-Atlantic Ridge and terrestrial environments such as Iceland, Russia, China, and the United States. The most-studied of these modern hydrothermal environments are those of Yellowstone National Park (YNP), Wyoming, USA (Figure 1). The park is home to the most diverse system of hydrothermal features in the world (National Park Service, 2010), with thermal features displaying a range of temperatures, pH, and elemental/ion concentrations both within and between hot springs (e.g. Shock *et al*., 2010, 2005; Fournier, 2005; Estep, 1984). The geochemical and microbial diversity present makes YNP an ideal place for studying the functional ecology and metabolic processes of prokaryotic organisms, and studies of the microbial diversity of the thermal features in the park have been taking place since the early 1900s (Setchell, 1903). The discovery of thermophilic *Thermus aquaticus* in the Lower Geyser Basin (LGB) of YNP (Brock and Freeze, 1969) initiated

advances in molecular biology, most notably the development of polymerase chain reaction (PCR) (Mullis *et al*., 1992), a method for *in vitro* amplification of nucleic acids that revolutionized the study of microbial diversity. Since then, considerable work on the microbial diversity of hot springs at YNP has been conducted (Boomer *et al*., 2009; Hall *et al*., 2008; Meyer-Dombard *et al*., 2005; Fouke *et al*., 2003; Hugenholtz *et al*., 1998; Barns *et al*., 1996, 1994; and others), with studies using 16S rRNA analysis to investigate microbial diversity on both a temporal and spatial scale. Many of these studies have also evaluated the relationship between geochemistry and microbiology within hot spring environments (Swingley *et al*., 2012; Shock *et al*., 2010, 2005; Inskeep and McDermott, 2005; Spear *et al*., 2005). However, microbial metabolic processes and biogeochemical cycling in the thermal features of the park are currently less constrained.

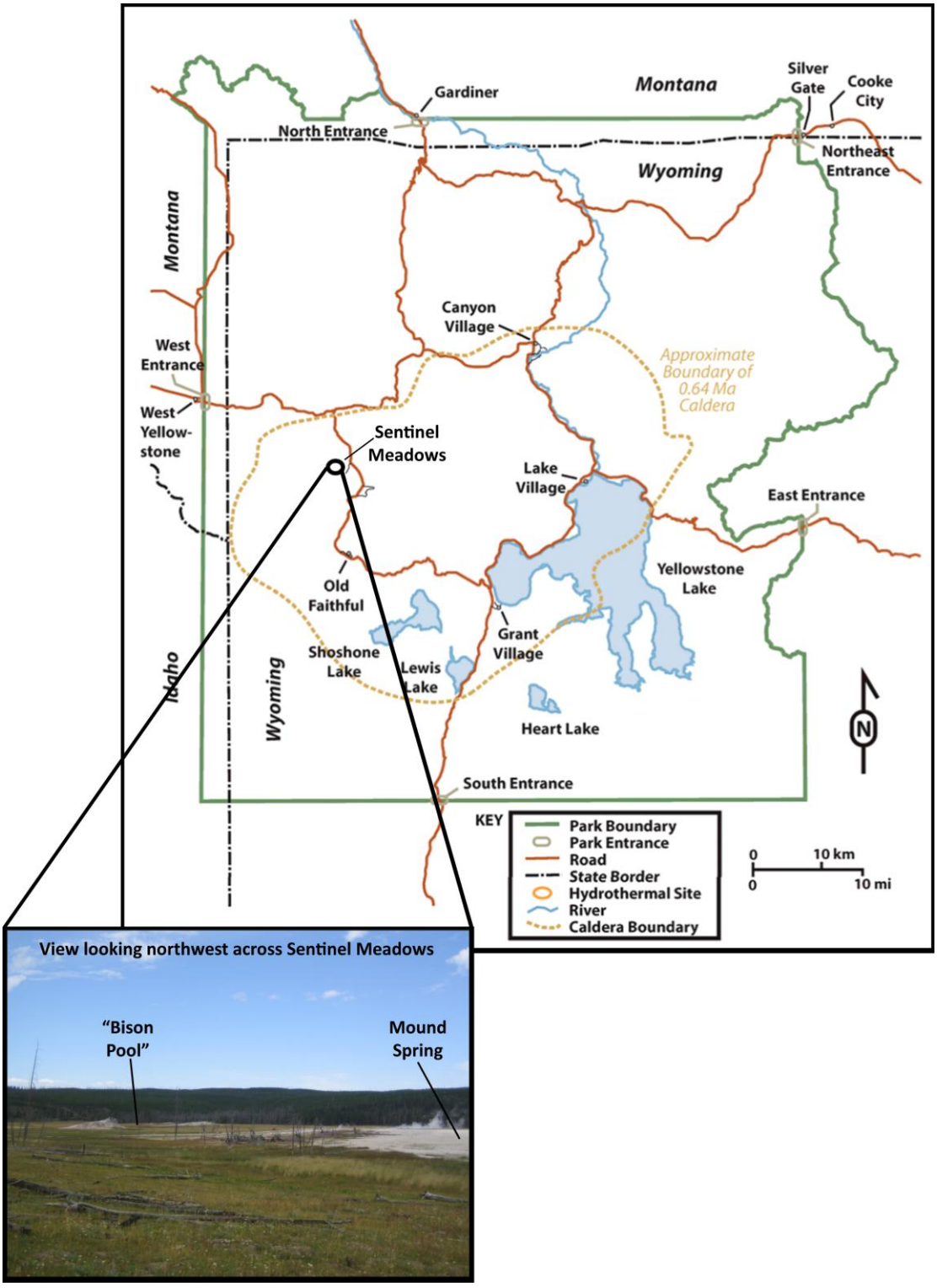


Figure 1. Map of Yellowstone National Park. Inset image shows Sentinel Meadows in the Lower Geyser Basin (LGB) of YNP. The locations of the two hot springs sampled in this study, Mound Spring and "Bison Pool," are indicated. (Image extensively modified from Havig *et al.*, 2011.)

1.2 Previous "gene hunting" work in hydrothermal areas

Recent work in YNP and other hydrothermal areas has focused on "gene hunting": screening thermal sediment and biofilm samples for the presence of genes utilized in specific metabolic processes. Genes involved in carbon cycling (e.g. *aclB*, *cbbM*), sulfur cycling (e.g. *soxEF1*, *dsrAB*), and nitrogen cycling (e.g. *nifH*, *amoA*, *nirK/S, narG*) have been the focus of many studies (Swingley *et al*., 2012; Dodsworth *et al*., 2011b; Havig *et al*., 2011; Hamilton *et al.*, 2011a, b; Zhang *et al*., 2008; Hall *et al*., 2008; Steunou *et al*., 2008, 2006; and others). Although research has evaluated and confirmed the presence of many of these genes in various thermophilic microbial communities, the existence of a gene in the DNA of an organism does not prove that it is actively used in the ecosystem, and few researchers have done work to confirm the utilization (expression) of the genes discovered in thermal samples (Loiacono *et al*., 2012; Jiang *et al*., 2010; Zhang *et al*., 2008; Steunou *et al*., 2008; Botero *et al*., 2005).

To verify gene expression, genetic analysis must focus on extraction of messenger RNA (mRNA), which is transcribed from DNA. mRNA carries the genetic code for synthesis of functional proteins; thus, the presence of mRNA in an environmental sample provides evidence for *in situ* gene use. Preliminary work is limited, but has been successful in extracting, amplifying, and analyzing mRNA from thermal biofilm and sediment samples, and the expression of various target genes on a spatial and diel scale has been documented in the hot springs of YNP and other global terrestrial thermal features (Jiang *et al*., 2010; Zhang *et al*., 2008; Steunou *et al*., 2008; Botero *et al*., 2005). This study utilizes and adapts previously demonstrated mRNA extraction methods to examine and compare nitrogen cycling in geochemically similar alkaline thermal features of the LGB of YNP.

1.3 The nitrogen cycle

Nitrogen occurs in all living organisms and is essential for life, as it is a critical element for the synthesis of amino acids, proteins, nucleic acids, and other forms of biomass. Nitrogen is also an important limiting agent of primary productivity in both terrestrial and marine ecosystems (Vitousek and Howarth, 1991). The nitrogen cycle involves various redox transformations of nitrogenous species, beginning with the reduction of atmospheric N_2 to NH_3, which is typically followed by the oxidation of NH_3 to NO_3^- and the subsequent reduction of NO_3^- back to N_2 (Figure 2). Diverse groups of microorganisms obtain energy through enzymatic mediation of these processes, and several diagnostic genes, displayed in Table I, are necessary to encode the enzymes that catalyze these processes.

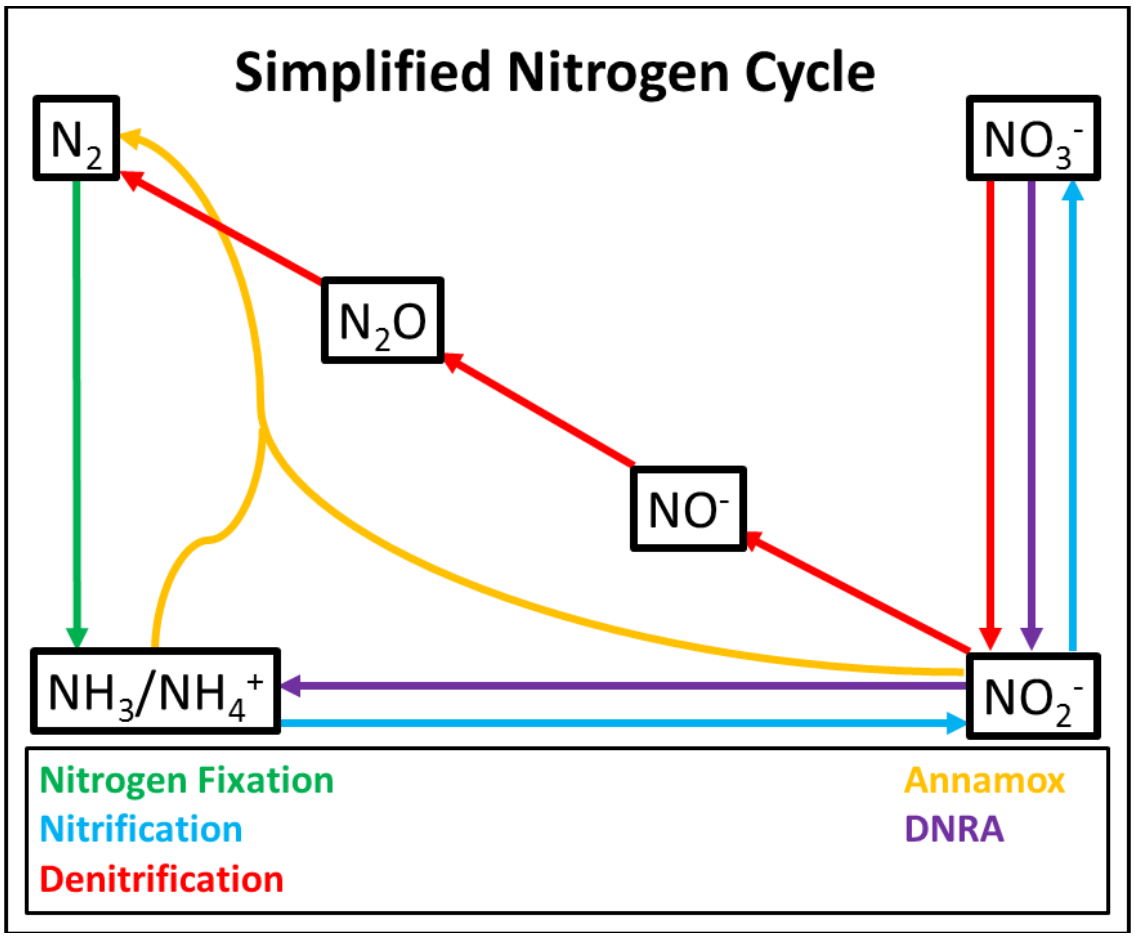


Figure 2. Simplified nitrogen cycle. Arrows indicate redox transformations of nitrogen species associated with each nitrogen cycle process. Arrows are color-coded according to the process they represent: green = nitrogen fixation; blue = nitrification; red = denitrification; yellow = annamox; purple = DNRA (dissimilatory nitrate reduction to ammonium).

TABLE I: FUNCTIONAL GENE PRIMERS AND CONDITIONS USED IN THIS STUDY

Process	Gene	Primer Names	Approx. Size of Gene Fragment	Primer Concentration	Sequence (5' – 3')[a]	EDGE Lab Amplification Conditions	Reference
Nitrogen Fixation	*nifH*	nifH1F nifH1R	400 bp	100 pmol/µL	GGHAARGGHGGHATHGGNAARTC GGCATNGCRAANCCVCCRCANAC	94°C for 2 min; 35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C; 72°C for 10 min	Mehta *et al.* (2003)
Nitrification (bacterial)	*amoA*	amoAF[b] amoAR[b]	491 bp	40 pmol/µL	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	94°C for 5 min; 42 cycles of 60 s at 94°C, 90 s at 55°C, 90 s at 72°C; 60°C for 90 s; 72°C for 10 min	Rotthauwe *et al.* (1997)
Nitrification (bacterial)	*amoA*	301F 302R	674 bp	50 pmol/µL	GACTGGGACTTCTGGCTGGACTGGAA TTTGATCCCCTCTGGAAAGCCTTCTTC	95°C for 5 min; 35 cycles of 1 min at 94°C, 1 min at 55°C, 4 min at 72°C; 72°C for 7 min	Norton *et al.* (2002)
Nitrification (archaeal)	*amoA*	arch-amoAF arch-amoAR	635 bp	10 pmol/µL	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	95°C for 5 min; 30 cycles of 45 s at 94°C, 1 min at 53°C, 1 min at 72°C; 72°C for 15 min	Francis *et al.* (2005)
Denitrification	*narG*	narG1960F narG2650R	650 bp	100 pmol/µL	CCGGGTTTCCCCATTCGG TGCGGCTGGATCTCCTT	94°C for 2 min; 35 cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C; 72°C for 7 min	Philippot *et al.* (2002)
Denitrification	*nirK*	Flacu R3Cu	472 bp	50 pmol/µL	ATCATGGTSCTGCCGCG GCCTCGATCAGRTTGTGGTT	94°C for 2 min; 35 cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C; 72°C for 7 min	Hallin and Lindgren (1999)
Denitrification	*nirS*	Cd3AF R3cd	425 bp	50 pmol/µL	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	94°C for 2 min; 35 cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C; 72°C for 7 min	Throbäck *et al.* (2004)
Denitrification	*norB*	cnorB2F cnorB6R	389 bp	50 pmol/µL	GACAAGNNNTACTGGTGGT GAANCCCCANACNCCNGC	95°C for 3 min; 40 cycles of 30 s at 95°C, 40 s at 57°C, 1 min at 72°C; 72°C for 5 min	Braker and Tiedje (2003)
Denitrification	*nosZ*	nosZ-F nosZ1622R	453 bp	100 pmol/µL	CGYTGTTCMTCGACAGCCAG CGCRASGGCAASAAGGTSCG	94°C for 2 min; 35 cycles of 30 s at 94°C, 1 min at 53°C, 1 min at 72°C; 72°C for 10 min	Throbäck *et al.* (2004)

[a] H = A, C, or T; R = A or G; N = A, C, G, or T; V = G, C, or A; K = G or T; S = G or C; Y = T or C; M = A or C.

[b] Primers are named amoA-1F and amoA-2R in Rotthauwe *et al.* (1997).

The most abundant form of nitrogen on Earth is atmospheric N_2, which is not readily accessible for biosynthesis. Consequently, nitrogen must be "fixed," or reduced from N_2 to NH_3, rendering it biologically available. This occurs through enzymatic mediation of redox reactions by diazotrophic (nitrogen-fixing) bacteria or archaea. The enzyme involved in nitrogen fixation, nitrogenase, is comprised of two parts: an iron-containing dinitrogenase reductase, encoded by the *nifH* gene, and a molybdenum-iron dinitrogenase that is encoded by *nifD* and *nifK* (Brigle *et al*., 1985; Bishop *et al*., 1982). Of the genes involved in nitrogen fixation, *nifH* is the most highly conserved (Normand and Bousquet, 1989), and thus often targeted in molecular microbial ecology studies.

Nitrification, the two-step oxidation of NH_3 to NO_2^- and NO_3^-, is the next step in the nitrogen cycle and is mediated by aerobic bacteria and archaea. This process is an important sink for fixed nitrogen, which can be lost by conversion to gaseous nitrogen species through the process of denitrification (Junier *et al*., 2010). Of all known ammonia-oxidizing microorganisms (AOM), none, to date, are known to fully complete the oxidation of NH_3 to NO_3^-; rather, the process is completed by two distinct groups of microorganisms, the first , which oxidizes NH_3 to NO_2^-, and the second group, which completes the oxidation of NO_2^- to NO_3^-. Biological ammonia oxidation to nitrite is catalyzed by enzymes encoded by the ammonia monooxygenase operon (*amo*), which contains at least three subunit genes: *amoA*, *amoB*, and *amoC* (Norton *et al*., 2002). The *amoA* subunit contains the putative active enzyme site and is thus the focus of most *amo* genetic studies (Norton *et al*., 2002). The second step of nitrification, oxidation of NO_2^- to NO_3^-, may also be an important process in hydrothermal environments; however, few studies on this redox process in high-temperature environments have been conducted, to date

(reviewed in Dodsworth *et al*., 2011a). Alternatively, the nitrification process may be bypassed and NH_4^+ oxidized to N_2 using NO_2^- as an electron acceptor in the annamox reaction (Figure 2), a process mediated by anaerobic bacteria (reviewed in Kuenen, 2008; Mulder *et al*., 1995).

Microbially-mediated anaerobic reduction of NO_3^- to N_2, denitrification, is the final stage of the nitrogen cycle and is the primary biological process responsible for returning nitrogen to the atmosphere. This step-wise reduction of nitrate to nitrogen gas ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) typically occurs via a microbial suite rather than a single organism (Burr *et al*., 2005). The completion of this process involves the sequential use of four enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, which are encoded by the *nar* (or *nap*), *nir*, *nor*, and *nos* gene complexes, respectively (Myrold, 1998). The *nar* gene complex, specifically the *narG* subunit, which contains the active site, has been the most widely studied of the nitrate reductase genes (Philippot, 2002), and is, consequently, the target of most microbial ecology studies of this primary step in the denitrification process. However, nitrate reductase catalyzes both dissimilatory nitrate reduction and nitrate respiration, so the presence of *narG* gene markers in a sample is not strictly indicative of denitrifiers. The next step in the denitrification process, the reduction of NO_2^- to NO using nitrite reductase, is the primary diagnostic step that distinguishes denitrifiers from nitrate-respirers, and, thus, the genes involved in this step, *nirK* and *nirS*, are often targeted when looking for evidence of denitrification in an environmental sample (Priemé *et al*., 2002). Although evolutionally unrelated and structurally different, both *nirK* and *nirS* have the same putative functions, and therefore, either can be used when searching for the presence of *nir* genes in a sample (Philippot, 2002). However, due to the high genetic diversity of denitrifiers, variations in PCR

amplification efficiency of currently designed *nirK* and *nirS* primers have been observed when used on environmental samples (Braker *et al*., 2000). Therefore, a combination of *nirK* and *nirS* PCR-directed screens are often employed in environmental "gene hunting" studies. The *norB* gene, encoding nitric oxide reductase, is used as the diagnostic gene for identifying organisms capable of mediating the NO to N_2O conversion (Braker and Tiedje, 2003); however, a lack of studies on this gene exists relative to the others in the denitrification suite. The reduction of N_2O is the last step of denitrification, which is catalyzed by nitrous oxide reductase and encoded by the *nosZ* gene. This gene is not ubiquitous amongst denitrifiers, therefore, its presence is only diagnostic of microbial populations capable of nitrous oxide reduction (Zumft, 1997). As an alternative to denitrification, nitrate and nitrite can also be converted through dissimilatory nitrate reduction to ammonium (DNRA), which involves the microbially-mediated conversion of nitrate and nitrite to ammonium (Figure 2). However, since this process does not return nitrogen to the atmosphere, no bulk loss of fixed nitrogen results, leaving nitrogen available in the ecosystem in a biosynthesis-ready form.

Although the global importance of nitrogen cycling is evident, until recently, there has been a paucity of data available in regards to nitrogen cycling in hydrothermal environments. Recent work in "extreme" environments (reviewed in the following sections) has revealed new information on the biodiversity and distribution of microorganisms involved in nitrogen cycling; however, many gaps still exist in our knowledge of the limits and microbial diversity associated with high-temperature nitrogen cycling. Furthermore, cultivation efforts within the last decade have led to the revision of putative thermal limits for multiple steps in the nitrogen cycle.

1.3.1 Nitrogen fixation in hydrothermal systems

Various genomic studies have indicated the presence of *nifH* genes across a range of environments, such as diverse soil ecosystems (Burr *et al*., 2005; Poly *et al*., 2001; Rosado *et al*., 1998), the open ocean (reviewed in Zehr *et al*., 2003; Zehr and McReynolds, 1989), and both marine and terrestrial (non-marine) hydrothermal systems (Hamilton *et al*., 2011a; Hall *et al*., 2008; Mehta *et al*., 2003), despite the oxygen-sensitive nature of nitrogenase (Gallon, 1981). Several studies of pure cultures have demonstrated regulation of nitrogen fixation in response to the availability of fixed nitrogen (total NH_3, NO_3^- and NO_2^-) (Vaughn and Burgess, 1989; Daesch and Mortenson, 1972; Shah *et al*., 1972), and observations from environmental studies suggest that *nifH* diversity may be constrained by other environmental factors, such as geographical distribution and fluid conductivity (Hamilton *et al*., 2011a; Moisander *et al*., 2007). In a recent survey of the hot springs at YNP, *nifH* was detected in 89% of the 64 sediment and biofilm samples collected, spanning a range of geographical locales and geochemical parameters (temperatures of 16°C to 89°C; pH of 1.90 to 9.78; highly variable metabolically important geochemical species). One of these samples, sediment collected from the outflow of an acidic hot spring at 89°C, represents the highest-temperature for *nifH* detection in a terrestrial hydrothermal environment, to date (Hamilton *et al*., 2011a) (Table VII, Appendix A).

The widespread presence of *nifH* genes suggests the genetic potential for nitrogen fixation in a variety of ecosystems; however, *in situ* nitrogen fixation cannot be assumed by indirect evidence, such as the presence of the *nif* gene complex, alone. Instead, other methods, such as gene expression studies and isotopic tracers, can be employed to obtain direct evidence of nitrogen fixation. Methanogenic archaea isolated from marine hydrothermal vent samples

display the capability for biological nitrogen fixation at 92°C in culture (Mehta and Baross, 2006), setting the current upper temperature limit for this process (Table VII, Appendix A). Recent work by Hamilton and colleagues (2011b) has also confirmed active nitrogen fixation by bacterial diazotrophs in acidic, terrestrial hydrothermal sediments through the use of acetylene reduction assays and isotope tracer experiments, with results indicating nitrogen fixation at 82°C (Table VII, Appendix A). A few transcription studies targeting *nifH* mRNA have also successfully identified *in situ* nitrogen fixation, with active *nifH* expression documented in coastal microbial mats (Severin and Stal, 2010), soils (Lotta *et al*., 2009; Bürgmann *et al*., 2003), freshwater planktonic communities (Zani *et al*., 2000), and photosynthetic mat communities of terrestrial hydrothermal systems (Steunou *et al*., 2008, 2006). However, in terrestrial high-temperature environments, *in situ* transcription of the *nifH* gene in chemosynthetic microbial communities has yet to be demonstrated.

1.3.2 Nitrification in hydrothermal systems

Nitrification occurs in a variety of ecosystems, and, until recently, all known AOM were thought to belong to two distinct groups of bacteria. Ammonia-oxidizing bacteria (AOB), which complete the oxidation of ammonia to nitrite, are characteristically slow-growing, with growth limited by the amount of total ammonia available (Bollmann *et al*., 2002), thus making their relative contribution to nitrogen cycling in oligotrophic (low NH_3) environments questionable. Investigations of this potential niche vacancy led to the discovery of chemolithoautotrophic ammonia-oxidizing archaea (AOA), which are capable of catabolizing in oligotrophic environments and may be the dominant AOM in many natural environments (Di *et a*l., 2010; Schleper, 2010; Offre *et al*., 2009; Tourna *et al*., 2008). Information on the first known AOA, a

marine crenarchaeon, *Nitrosopumilus maritimus*, was published in 2005 (Könneke *et al*.), and since then, various researchers have investigated the role of AOA in global nitrogen cycling, including numerous studies of AOA in "extreme" environments, ranging from sub-glacial ecosystems (Boyd *et al*., 2011) to hydrothermal environments.

Evidence of AOA has been discovered in a variety of high-temperature ecosystems, such as marine hydrothermal vents (Wang *et al*., 2009), high-temperature subsurface environments (Spear *et al*., 2007), and geographically diverse terrestrial hot springs, including the Great Basin, USA (Dodsworth *et al*., 2011a, b; Zhang *et al*., 2008), China, (Jiang *et al*., 2010; Zhang *et al*., 2008), Kamchatka, Russia (Reigstad *et al*., 2008; Zhang *et al*., 2008), Iceland (Reigstad *et al*., 2008), and YNP (de la Torre *et al*., 2008; Pearson *et al*., 2008). These studies led to an influx of information concerning high-temperature nitrification and expanded previous upper-temperature limits for the process (Table VII, Appendix A). Isolates of the thermophilic archaeon, "*Candidatus Nitrosocaldus yellowstonii*," extracted from a YNP alkaline hot spring demonstrated the ability to oxidize ammonia at temperatures up to 74°C (de la Torre *et al*., 2008). Archaeal *amoA* genes have been recovered from environmental samples at temperatures up to 97°C (Reigstad *et al*., 2008), and transcripts of archaeal *amoA* have been amplified from hot spring biofilms and sediments collected at temperatures as high as 94°C (Jiang *et al*., 2010), corroborating the importance of AOA in high-temperature ecosystems. In fact, archaeal *amoA* genes recovered from Great Basin hot spring sediments indicate a greater abundance of *amoA* genes in hydrothermal sediment than in other non-thermal sediments (Dodsworth *et al*., 2011b). Whereas numerous lines of evidence support archaeal nitrification in hydrothermal systems, evidence of high-temperature AOB is lacking. Isolation of a moderately

thermophilic nitrifying bacteria from a hot spring of the Baikal Rift Zone, Russia led to the discovery of bacterial oxidation of NO_2^- to NO_3^- at up to 58°C (Lebedeva *et al*., 2011), and amplification of bacterial *amoA* cDNA from a microbial mat collected in a Japanese subsurface gold mine alludes to the genetic potential for bacterial ammonia-oxidation at temperatures of up to 62°C (Hirayama *et al*., 2005) (Table VII, Appendix A). However, to the author's knowledge, no evidence currently exists for *in situ* thermophilic bacterial nitrification.

1.3.3 Denitrification in hydrothermal systems

Knowledge of denitrification process and DNRA at high temperatures has been modified and extended multiple times over the past two decades, predominantly through the cultivation of isolated strains of thermophilic nitrate-reducing microorganisms (Mishima *et al*., 2009; *Poli et al*., 2009; Cava *et al*., 2008; Pires *et al*., 2005; Nakagawa *et al*., 2004; Miroshnichenko *et al*., 2003; Takei *et al*., 2003; Gotz *et al*., 2002; Huber *et al*., 2002; Hafenbradl *et al*., 1996; and others). Culture-based methods have also set the current upper temperature limits for microbial nitrate reduction (Table VII, Appendix A). The extraction and cultivation of a hyperthermophilic archaeon, *Pyrobaculum aerophilum*, from an Italian hot spring established the current upper temperature limit for nitrate reduction (NO_3^- to N_2O or N_2) by a terrestrial thermophile as 100°C (Völkl *et al*., 1993), well above the 70°C previously recognized (Hollocher and Kristjansoon, 1992). Several years later, the same research group isolated a marine thermophilic archaeon, *Pyrolobus fumarii*, from a Mid-Atlantic Ridge vent sample, extending the overall upper limit for nitrate reduction to 113˚C (Blöchl *et al*., 1997).

Despite the abundance of culture-based data regarding denitrification and DNRA, knowledge of these processes in natural thermal environments is limited. Evidence for nitrate

reduction in terrestrial hydrothermal systems has recently been published based upon the results of acetylene block and $^{15}NO_3^-$ tracer assays in Great Basin hot spring sediments (Dodsworth *et al.*, 2011b; Hedlund *et al.*, 2011) (Table VII, Appendix A). These seminal reports also highlight the importance of NO_3^- reduction in high-temperature terrestrial systems, with experimental results indicating *narG* gene copies and *in situ* rates of denitrification comparable to (and sometimes greater than) those found in non-thermal soils and aquatic environments (Dodsworth *et al.*, 2011b). Despite these results, it is important to note that the denitrification rates measured in this study may not be typical of all terrestrial thermal ecosystems. The Great Basin results are substantially higher than those observed in previous studies of thermal soil at YNP (Burr *et al.*, 2005), thus, emphasizing the importance of local geochemical parameters in denitrification and DNRA processes. Furthermore, since the acetylene block technique used to measure denitrification rates inhibits the reduction of N_2O to N_2, the activity of nitrous oxide reductase and concomitant completion of the full denitrification process cannot be determined by this method. Perhaps this is partially the reason for the lack of data regarding the microbial N_2O to N_2 transformation in natural thermal environments. Although some thermophilic organisms are known to completely reduce NO_3^- to N_2 (Cava *et al.*, 2008), several microorganisms with incomplete denitrification pathways (truncating at N_2O) are known (Zumft and Kroneck, 2007), and Hedlund *et al.* (2011) postulated that incomplete denitrification may actually be dominant at higher temperatures, evidenced by N_2O emissions observed in both natural Great Basin hot springs and cultured environmental representatives. Additional studies are necessary to further constrain the genetic potential and *in situ* activity of both individual and complete denitrification processes in natural hydrothermal ecosystems.

1.4 Purpose of this study

This study uses genomic, transcriptomic, geochemical, and isotopic analytical techniques to comparatively assess microbial nitrogen cycling in two geochemically similar hot springs in the LGB of YNP. The presence and expression of various putative nitrogen cycle genes are evaluated to identify nitrogen fixation, nitrification, nitrate reduction, and denitrification potential and *in situ* expression in both chemotrophic and phototrophic communities. Results allow comparison of nitrogen cycle processes between the two hot springs, which are verified with genetic and isotopic data and explained within the context of topographically- and microbially-driven variations in nutrient availability. The results of some of this work have been published in *Environmental Microbiology*, Volume 14, pp. 1272-1283, under the title "Evidence for high-temperature *in situ nifH* transcription in an alkaline hot spring of Lower Geyser Basin, Yellowstone National Park." A full reference for the article is listed below.

Loiacono S.T., Meyer-Dombard, D.R., Havig, J.R., Poret-Peterson, A.T., Hartnett, H.E., and Shock, E.L. **(2012)**. Evidence for high-temperature *in situ nifH* transcription in an alkaline hot spring of Lower Geyser Basin, Yellowstone National Park. *Environ Microbiol* **14**: 1272-1283.

2. EXPERIMENTAL PROCEDURES

2.1 Sample collection

Samples for nucleic acid extraction were collected in August, 2010 from various locations in the outflow channels of Mound Spring and "Bison Pool," two alkaline hot springs located in Sentinel Meadows, LGB, YNP (44° 33' 53.4"N, 110° 51' 36.2"W and 44° 34' 10.7"N, 110° 51' 54.7"W, respectively). Mound Spring is visibly devoid of streamer-biofilm communities (SBCs) that are found in some of the chemosynthetic regions of other LGB hydrothermal features (Meyer-Dombard *et al*., 2011), so only sediment and photosynthetic mat communities were sampled from Mound Spring sites. (See Figure 3 and Figure 12, Appendix C for examples of each type of biomass sampled.) Biomass samples (SBCs and/or sediment) were collected from three to four locations in the chemosynthetic zone of both Mound Spring and "Bison Pool" outflow channels using flame-sterilized metal scoops and forceps. Photosynthetic mat samples were collected at one location within the outflow channel of each hot spring using sterile forceps. Detailed sample locations can be found in Figures 4 and 5.

Samples for DNA analysis were stored in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI, USA), frozen on dry ice in the field, and stored at -20°C upon return to the laboratory. Samples for RNA analysis were collected, immediately placed in sterile 2 mL Nalgene® cryogenic vials (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 1 mL of RNAlater RNA Stabilization Reagent (QIAGEN, Valencia, CA, USA), briefly shaken to ensure complete submersion of the sample in the stabilization fluid, and then immediately frozen on dry ice in the field. Samples for RNA analysis were transferred to a dry shipper at the end of the day,

where they remained at liquid N_2 temperatures for transport to the laboratory. RNA samples were stored in the lab at -80°C until subsequent RNA extraction and analysis.

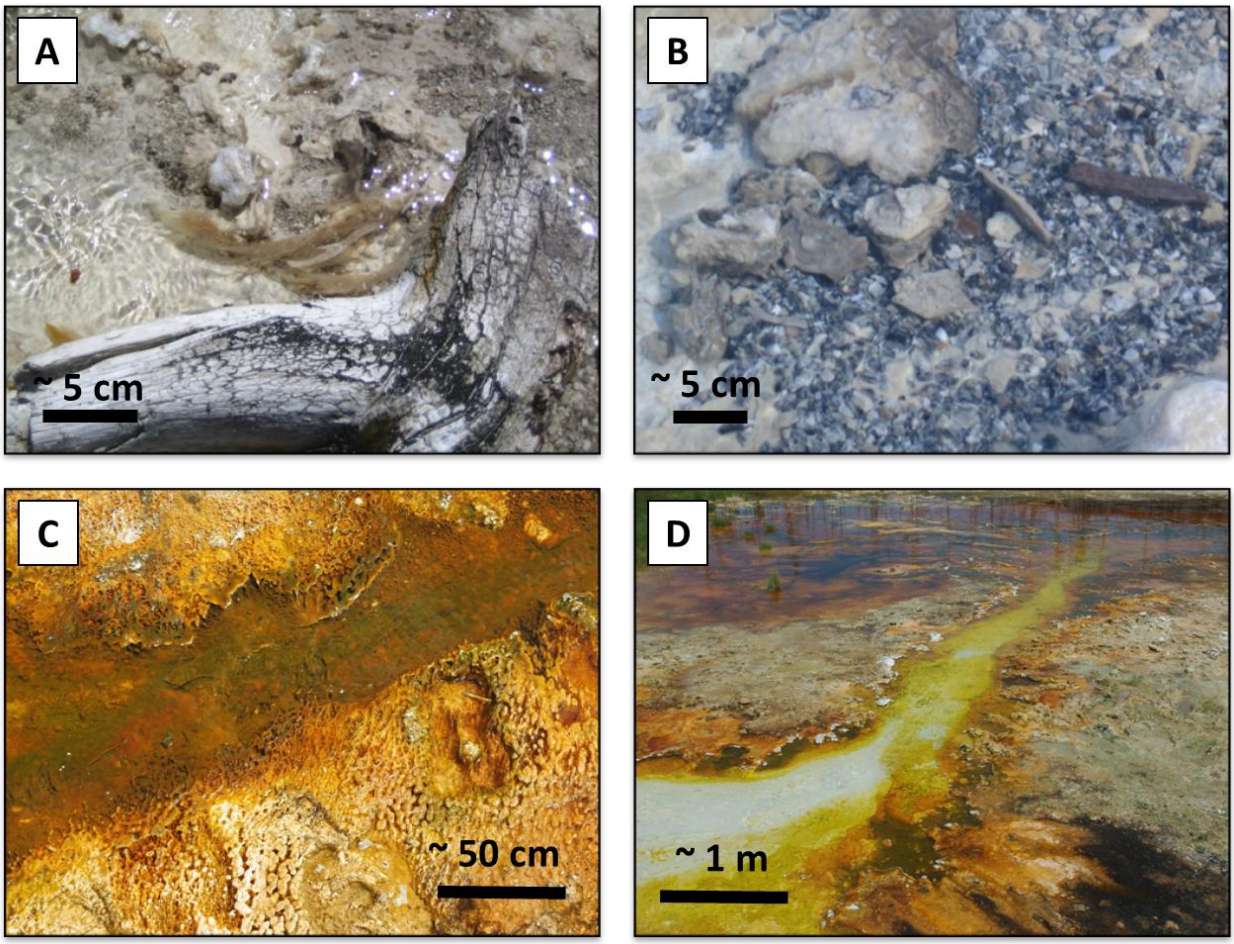


Figure 3: Examples of biomass samples types collected from Mound Spring and "Bison Pool." Images A and B (A: Streamer Biofilm Communities (SBCs); B: Sediment) represent those collected in the chemosynthetic portions of the outflow channels. Image C represents a photosynthetic mat sample. Image D shows the visual delineation between the chemosynthetic zone (to the left; upstream) and the photosynthetic zone (to the right; downstream), a transitional ecotone that is termed the photosynthetic "fringe." (See Figure 12, Appendix C for additional images.)

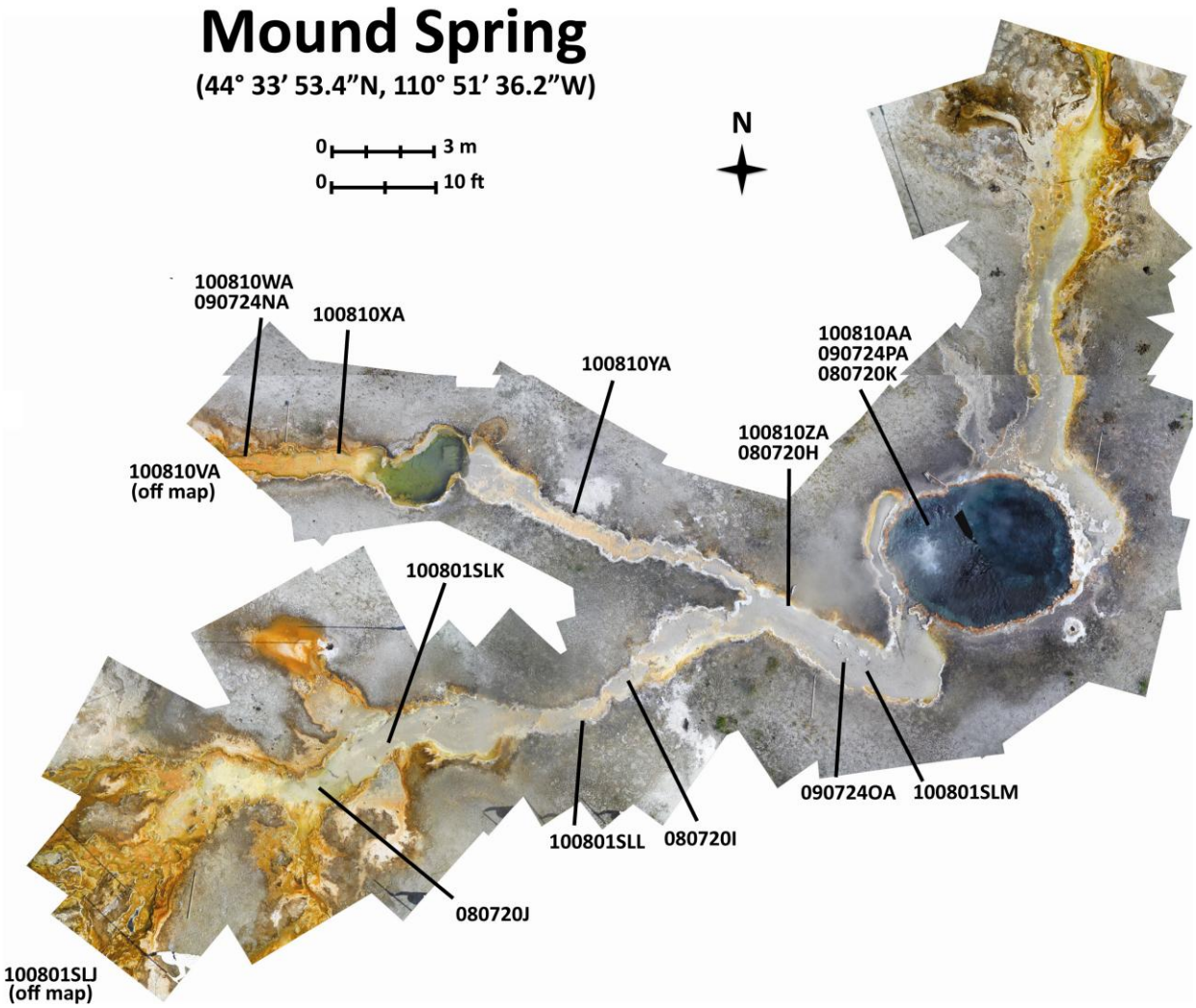


Figure 4: Map of Mound Spring source and outflow channels showing the locations of the geochemical and biomass samples listed in Table II. Photo mosaic was generated using images taken in July 2011 (on the same day as the images used in the "Bison Pool" map (Figure 5)). Photosynthetic zones can be visually identified as the orange areas on the map. Note the deep blue color of the source pool and lack of vegetation present immediately surrounding the hot spring. Sample IDs follow the format "YYMMDD" followed by the sample letter ID.

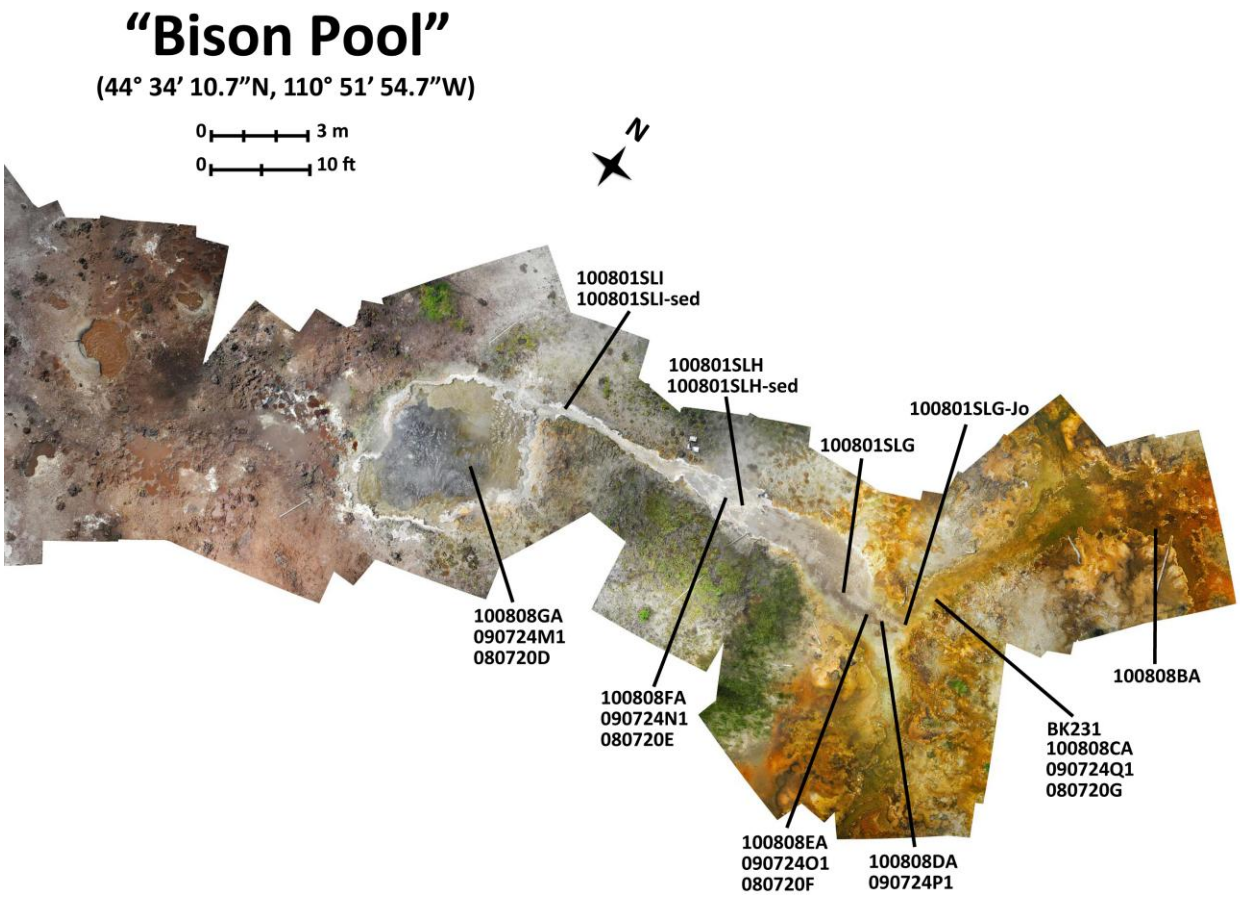


Figure 5. Map of "Bison Pool" source and outflow channels showing the locations of the geochemical and biomass samples listed in Table II. Photo mosaic was generated using images taken in July 2011, shortly after a precipitation event, so the source pool, which is usually blue in color, displays an atypical brown tint, likely as a result of organic influx during the event. (See Figure 14A-E, Appendix D.) (Note the brown, organic-rich area to the west of the "Bison Pool" source.) Photosynthetic zones can be visually identified as the orange areas on the map. Vegetation appears green. Sample IDs follow the format "YYMMDD" followed by the sample letter ID.

Biofilms and sediments for isotope analysis were collected in replicate and combined to obtain representative bulk samples and minimize the influence of any local anomalies potentially biasing the sample in terms of maturity, concentration, community structure, or other physical variations. Sampling areas typically covered approximately 25 cm x 25 cm. Samples were collected using 150 mm long, milled teflon tweezers that were prepared by acid washing (soaking in a 10% solution of trace-element-clean Omni*Trace*® nitric acid (EMD Chemicals, Inc., Gibbstown, NJ, USA) for three days) followed by triple rinsing in 18.2 MΩ·cm deionized (DI) water (Barnstead NANOpure® DIamond™ UV ultrapure water system, Thermo Fisher Scientific Inc., Waltham, MA, USA) prior to the trip. The tweezers were transported to the field in clean plastic bags. In the field, tweezers were rinsed with local hot spring water followed by DI water between collections from different hot spring sites and rinsed with hot spring sample site water between collection of samples from the same site. Samples were collected while wearing vinyl gloves and were placed in plastic containers that had been acid washed and triple-rinsed with DI water.

2.2 DNA extraction and PCR

Bulk environmental DNA was extracted using a mechanical bead-beating protocol optimized for efficient extraction of high quality DNA from siliceous alkaline hydrothermal systems (Meyer-Dombard *et al*., 2005) using Q-BIOgene's FastDNA Spin Kit for Soil (now MP Biomedicals, LLC, Solon, OH, USA) or the Mo BIO PowerBiofilm DNA Isolation Kit (Mo BIO Laboratories, Inc., Carlsbad, CA, USA). Individual layers of photosynthetic mat samples were not separated prior to extraction. Extracted DNA was amplified with the primer pairs in Table I, targeting bacterial and archaeal functional nitrogen cycle genes. The reactions were performed

in 20 µL total volume, containing 2.25 mM $MgCl_2$, 2 µL GeneAmp 10X PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 100 µM dNTPs (Sigma-Aldrich, Inc., Saint Louis, MO, USA), 0.5 µL each primer, 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA), and 1 µL template DNA. Alternatively, 20 µL PCR reactions were performed using 10 µL DreamTaq™ Green PCR Master Mix (2X) (Fermentas Inc., Glen Burnie, MD, USA), 7 µL nuclease-free water, 0.5 µL each primer, and 1 µL template DNA. Amplification was performed using a Gene Amp PCR system (Applied Biosystems, Carlsbad, CA, USA), with cycling conditions as reported in Table I. PCR products were run on a 1.5% agarose gel to screen for the presence of amplicons of the desired size.

2.3 RNA extraction and RT-PCR

Environmental RNA was extracted from sediment and microbial mat samples using the PowerBiofilm RNA Isolation Kit (Mo BIO Laboratories, Inc., Carlsbad, CA, USA), DNased on-column, and eluted with 50 µL nuclease-free water. Extracted RNA was purified, when necessary, using Microcon centrifugal filter devices (Millipore, Billerica, MA, USA) or the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Purified RNA concentration and purity was determined via NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA), which indicated post-purification concentration values ranging from approximately 2 to 110 ng/µL to for each sample. RNA (1-2 µL) was then reverse transcribed in multiple 20 µL reactions using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and resultant cDNA was pooled and purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA). Suitability of cDNA for functional gene PCR was verified by PCR amplification of bacterial 16S rRNA genes using the universal primer set 27F-

1492R (Lane, 1991) and conditions as previously described (Meyer-Dombard *et al*., 2005). It should be noted that, although initial RNA concentrations of different samples varied by an order of magnitude, reverse transcription of RNA throughout the full range of concentrations produced amplifiable cDNA. cDNA (1-2 µL) was amplified via PCR targeting functional nitrogen cycle genes using the primers and conditions previously described for DNA amplification, but with 25 ng/µL BSA added to each 20 µL reaction if DreamTaq™ Green PCR Master Mix (2X) (Fermentas Inc., Glen Burnie, MD, USA) was not used. Non-reverse-transcribed RNA controls were also subjected to PCR with functional gene and/or 16S rRNA primers to screen for DNA contamination.

2.4 Molecular cloning and phylogenetic analysis

PCR products were purified using a QIAquick kit (QIAGEN, Valencia, CA, USA), and were cloned into *E. coli* hosts using the TOPO TA Cloning kit with the pCR 2.1 vector (Invitrogen Corporation, Carlsbad, CA, USA). Multi-band PCR products were run on a sterile 1.5% agarose gel, and amplicons of the appropriate size were excised prior to kit purification. Plasmid DNA from *nifH* clones was extracted and purified using a DirectPrep 96 Miniprep kit (QIAGEN, Valencia, CA, USA). Plasmids were sequenced by Polymorphic DNA Technologies (Alameda, CA, USA) and the UIC DNA Services Facility (Research Resources Center, University of Illinois at Chicago, IL, USA) using standard M13 primers. For all other (non-*nifH*) functional genes, clones were picked into 50 µL nuclease-free water, and 1 µL of the bulk solution was used as a template for M13 PCR. M13 PCR reactions were performed using the reaction mix described for functional gene PCR, but with 0.5 µL of each 20 µM M13 primer and the following cycling conditions: 95°C for 5 min.; 30 cycles of 30 s at 94°C, 30 s at 52°C, 60 s at 72°C; 72°C for 10 min.

M13 PCR products were screened on a 1.5% agarose gel for a band of the appropriate size (functional gene fragment length + 201 bp from the vector). PCR products were then sent to the UIC DNA Services Facility where they were cleaned using Agencourt AMPure® beads (Beckman Coulter, Inc., Indianapolis, IN, USA) and sequenced with M13 primers. Between 6 and 32 clones were sequenced for each DNA and cDNA sample. (See Figure 16, Appendix E for a simplified molecular methods flowchart.)

Full length sequences from all samples were preliminarily assigned an OTU (operational taxonomic unit) based on results of a nucleotide BLAST (Basic Local Alignment Search Tool) search against the NCBI (National Center for Biotechnology Information) database (Altschul *et al*., 1997). Sequences were edited with CodonCode Aligner (Codon-Code Corporation, Dedham, MA, USA), and compared with other sequences in GenBank. Sequences from "Bison Pool" clones were also compared with previous "Bison Pool" metagenomic data using a Local BLAST in BioEdit (Ibis Biosciences, Carlsbad, CA, USA). Sample sequences for *nifH* and archaeal *amoA* genes were aligned using BioEdit, and then imported into PAUP (Sinauer Associates, Inc. Publishers, Sunderland, MA, USA) where phylogenetic trees and distance matrices were constructed and evaluated in order to identify unique phylotypes in the clone library. For all other functional genes, unique phylotypes were determined using similarity matrices constructed in BioEdit. Representative sequences for each *nifH* and archaeal *amoA* sample were chosen using a 97% and 99% nucleotide similarity cutoff, respectively (based on PAUP distance matrices). In each resultant group of highly-similar sequences, the sequence of the highest quality was chosen as the representative for the group. Representative sequences were aligned with reference sequences using ClustalW (Larkin *et al*., 2007), and then imported into

BioEdit where sequences were manually adjusted as needed. All reference sequences for phylogenetic reconstructions were taken from the NCBI database. Maximum parsimony and neighbor joining trees using reference and representative sequences were constructed using PAUP. After evaluation of various arrangements, a *nifH* gene from a termite gut bacterial symbiont (AB011954) and a partial archaeal *amoA* gene from an uncultured crenarchaeote extracted from a subsurface thermal spring in the Austrian Central Alps (AM260489) were selected as the outgroups and used to root all subsequent *nifH* and archaeal *amoA* trees, respectively. Alignments and subsequent phylogenetic representations were constructed for both nucleotide and translated (amino acid) sequences, resulting in similar tree topology using both alignment methods. Phylogenetic arrangements for the remaining functional genes (*narG* and *nirS*) were not constructed. Potential chimeric sequences could not be checked with typical methods such as Bellerophon (Huber *et al*., 2004) due to short sequence length; therefore, phylogenetic reconstruction using only the front or back halves of the alignment was used to check for changes in tree topology. *nifH* sequences were submitted to the GenBank database under accession numbers JN859510-JN859532. Names for *nifH* clones follow the format 'YNP_MS_X_NIFY', where YNP = Yellowstone National Park, MS = Mound Spring, X = abbreviated sample name, NIF = nitrogen fixation, and Y = nucleic acid designation (D = DNA, C = cDNA). Archaeal *amoA* clonal names follow the format 'YNP_BP_X_AMOY', where YNP = Yellowstone National Park, BP = "Bison Pool," X = abbreviated sample name, AMO = ammonia oxidation, and Y = nucleic acid designation (D = DNA, C = cDNA).

2.5 Geochemical analysis

Temperature and pH were measured *in situ* at the time of sample collection using hand-held meters calibrated in the field (YSI 30 and Orion 290A plus meters, which operate up to 100°C). Relevant redox-sensitive chemical species (dissolved oxygen, nitrate, nitrite, total ammonia, and ferrous iron) as well as dissolved silica were measured on site as previously described (Shock *et al*., 2010; 2005) using portable spectrophotometers (HACH model 2700 and 2800) and reagents (Hach Company, Loveland, CO, USA). Spectrophotometric measurements were made in the field shortly after sample collection to ensure measurements were environmentally relevant. (See Figure 13, Appendix C for geochemical sampling images.) The accuracy of field-based spectrophotometric tests may be affected by various potential sources of error (interfering substances, human error, etc.), the sum of which produce an estimated error of ±5-10% (Shock *et al*., 2010). Additional analyses by ion chromatography were conducted in the Shock laboratory (Arizona State University) as described previously (Meyer-Dombard *et al*., 2011; Shock *et al*., 2010).

2.6 Stable isotope analysis

Stable isotope analysis of Mound Spring and "Bison Pool" biofilm and sediment were conducted at Arizona State University by the GEOPIG laboratory. Biofilm and sediment samples were dried at approximately 90°C for three days and then ground with an agate mortar and pestle until uniformly powdered. They were then weighed, placed into tin capsules, sealed, and analyzed using a Costech Model ECS 4010 Elemental Analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) coupled to a Thermo Delta^{plus} Advantage Isotope Ratio Mass Spectrometer (EA IR-MS) (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data were

standardized using three glycine working standards with a range of isotopic compositions (low: $\delta^{15}N$ = 1.35‰, mid: $\delta^{15}N$ = 27.9‰, and high: $\delta^{15}N$ = 51.8‰) that spanned the expected isotopic variations. The glycine working standards were characterized using USGS40 and USGS41 isotopic reference materials. Linearity checks were performed using NIST 2710 (Montana Soil). Standard deviations of $\delta^{15}N$ values of Mound Spring and "Bison Pool" biomass and contextual samples ranged from ±0.05-0.35‰.

3. PHYSICAL AND GEOCHEMICAL PARAMETERS OF SAMPLE LOCATIONS

3.1 Physical Characteristics of Mound Spring and "Bison Pool"

Both Mound Spring and "Bison Pool" are located within the Sentinel Meadows Group of the LGB, with the "Bison Pool" source pool situated approximately 672 meters northwest of the Mound Spring source. The Mound Spring source is rimmed with a siliceous sinter shelf (Figure 14F, Appendix D) and sits atop a low, wide, sinter mound (Figure 1, inset and Figure 15, Appendix D). Its thermal fluids flow away from the source pool to the south in an approximately 1.5 m wide outflow channel. A few meters from the source, this channel curves west and narrows slightly (approximately one meter in width), then splits into two separate outflow channels, flowing northwest and west (Figure 4). Mixing of meteoric waters with thermal fluids at Mound Spring likely occurs during precipitation events, especially due to gravitational direction of precipitation into the outflow channels as a result of the sloped topography of the hot spring. Additionally, the sloped nature of Mound Spring, coupled with the channel-occupying photosynthetic mats, contributes to overflow of hot spring fluids in the photosynthetic zone onto the surrounding sinter mound. Little vegetation grows on the sinter mound surrounding Mound Spring, so it is largely isolated from exogenous inputs of organic meadow material, such as those from plants and soil (Figure 4).

In contrast, "Bison Pool" is positioned topographically level with the surrounding meadow, consequentially allowing a greater opportunity for organic meadow input and precipitation to infiltrate its source and outflow. As the name suggests, bison also frequent this hot spring, providing additional sources of allochthonous organic input that are rarer at Mound Spring, such as bison excrement and fur (for evidence, see the brown area to the west of the

"Bison Pool" source in Figure 5 and also Figure 14A-E, Appendix D). Like Mound Spring, the "Bison Pool" source is rimmed with sinter; however, unlike Mound Spring, the outflow channel there is narrower and rimmed with sinter for approximately seven meters before widening downstream. This narrow, rimmed nature of the upstream portion of the outflow channel results in more rapid fluid flow in that location as well (Swingley *et al.,* 2012). SBCs cling to the underside of the sinter lip in the upstream, chemosynthetic portions of the "Bison Pool" outflow channel. Farther downstream, these SBCs also attach to and form around solid organic particles in the outflow channel, such as sticks/twigs, bison excrement, and insects (Figure 3A and Figure 12A-C, Appendix C). The "Bison Pool" fluids outflow away from the source to the east, with the channel splitting to the northeast and southeast near the advent of the photosynthetic "fringe" (Figure 5).

Both hot springs are boiling at the source, and fluid temperatures decrease down the outflow channel due to cooling and evaporative effects. The photosynthetic "fringe," which marks the transition from strictly chemosynthetic microbial communities to photosynthetic communities, is visually discernible at both Mound Spring and "Bison Pool" by the advent of orange and red pigment-producing photosynthetic microorganisms on the downstream (photosynthetic) side of the "fringe" (Figures 3D, 4, and 5). Although the exact temperature of the advent of photosynthesis depends on a variety of geochemical variables (Cox *et al*., 2011), obvious photosynthetic growth typically begins at approximately 63°C to 66°C at both Mound Spring and "Bison Pool."

3.2 Geochemistry of Mound Spring and "Bison Pool"

Thermal features in the Sentinel Meadows Group of the LGB are likely sourced by fluids derived from underground reservoirs within layers of permeable rhyolitic flows bounded by relatively impermeable volcanic tuff (Fournier, 2005). Hydrothermal fluid compositions in the basin are governed primarily by water-rock interactions, with little vapor influence, resulting in predominantly alkaline hot springs in the LGB and characteristically high chloride and silica concentrations and low sulfate and sulfide concentrations in Mound Spring, "Bison Pool" (Table II) and most other LGB hot springs (Meyer-Dombard *et al*., 2011, 2005). Results of fluid geochemical analyses at each Mound Spring and "Bison Pool" sampling location are displayed in Table II, along with measurements from previous years for comparison. (See Figures 4 and 5 for corresponding sampling locations.) It should be noted that the accuracy of field-based spectrophotometric tests may be affected by various potential sources of error (see section 2.5), but regardless, overall trends in downstream geochemical parameters remain pertinent, as measurements at all locations within the outflow channel should be affected relatively equally by any uncertainties in measurement.

TABLE II: SELECT GEOCHEMICAL MEASUREMENTS OF FLUID AT MOUND SPRING AND "BISON POOL"[a]

Year	Sample ID (Mound Spring)	Distance from source (m)	pH	Temp (°C)	Cl^- (mmol/kg)	SO_4^{2-} (mmol/kg)	NO_3^- (µmol/kg)	NO_2^- (µmol/kg)	ΣNH_3 (µmol/kg)	Fe^{2+} (µmol/kg)	ΣS^{2-} (µmol/kg)	SiO_2 (mmol/kg)	DO[b] (µmol/kg)
2010	100810VA	26.5	8.95	59.4	7.60	0.18	22.6	0.28	1.67	bdl	5.61	9.68	25.0
2010	100810WA	22.5	8.88	67.3	5.60	0.13	33.9	0.37	1.11	bdl	9.20	10.9	43.8
2010	100810XA	19.5	8.87	67.5	10.5	0.24	8.10	0.35	bdl	bdl	10.9	13.7	37.5
2010	100810YA	13	8.83	71.9	10.1	0.21	51.6	0.37	2.78	0.18	29.5	8.04	21.9
2010	100810ZA	5	8.62	86.0	8.00	0.16	51.6	0.33	bdl	bdl	40.4	10.7	15.8
2010	100810AA	0	8.48	93.9	7.10	0.14	71.0	0.20	3.33	bdl	49.1	9.52	bdl
2009	090724NA	22.5	8.76	66.6	8.10	0.19	96.8	2.76	bdl	bdl	6.99	4.95	90.6
2009	090724OA	4.5	8.31	87.6	7.40	0.15	208	1.22	bdl	0.36	27.3	6.86	21.9
2009	090724PA	0	8.28	93.3	7.40	0.14	153	0.98	1.67	bdl	20.3	6.97	nd
2008	080720J	19	8.84	69.1	7.60	0.19	165	nd	nd	nd	12.7	6.52	nd
2008	080720I	10.5	8.65	77.4	7.40	0.20	290	nd	nd	nd	18.0	6.54	nd
2008	080720H	5	8.65	81.7	7.30	0.19	276	nd	nd	nd	23.4	6.11	nd
2008	080720K	0	8.45	93.6	7.30	0.20	294	nd	nd	nd	22.0	6.28	nd

Year	Sample ID (Mound Spring)	Distance from source (m)	pH	Temp (°C)	Type[c]	Zone[d]	NO_3^- (µmol/kg)	NO_2^- (µmol/kg)	ΣNH_3 (µmol/kg)	Fe^{2+} (µmol/kg)	ΣS^{2-} (µmol/kg)	SiO_2 (mmol/kg)	DO (µmol/kg)
2010	100801SLJ	29	8.92	57.2	Photomat	P	14.5	0.70	bdl	nd	nd	nd	40.6
2010	100801SLK	17	8.78	68.1	Sediment	C	27.4	nd	nd	nd	nd	nd	nd
2010	100801SLL	11.5	8.71	72.7	Sediment	C	46.8	0.15	2.78	nd	nd	nd	21.9
2010	100801SLM	3.5	8.60	80.2	Sediment	C	77.4	0.11	bdl	nd	nd	nd	2.88

[a] Cl^- and SO_4^{2-} were determined using ion chromatography. All other measurements were determined spectrophotometrically in the field. (bdl = below detection limit; nd = not determined).

[b] DO = dissolved oxygen.

[c] Photomat = photosynthetic mat.

[d] P = photosynthetic; C = chemosynthetic.

TABLE II: SELECT GEOCHEMICAL MEASUREMENTS OF FLUID AT MOUND SPRING AND "BISON POOL"[a] (continued)

Year	Sample ID ("Bison Pool")	Distance from source (m)	pH	Temp (°C)	Cl^- (mmol/kg)	SO_4^{2-} (mmol/kg)	NO_3^- (µmol/kg)	NO_2^- (µmol/kg)	ΣNH_3 (µmol/kg)	Fe^{2+} (µmol/kg)	ΣS^{2-} (µmol/kg)	SiO_2 (mmol/kg)	DO[b] (µmol/kg)
2010	100808BA	22.5	8.57	55.6	6.8	0.19	8.10	0.67	0.56	bdl	bdl	9.2	144
2010	100808CA	14	8.29	63.6	6.7	0.18	9.70	0.43	0.56	bdl	0.03	11.2	125
2010	100808DA	12.5	8.17	69.5	6.6	0.18	12.9	0.61	1.11	bdl	0.19	9.5	81.3
2010	100808EA	12	8.04	74.6	15.3	0.38	8.10	0.61	1.11	bdl	0.56	10.9	81.3
2010	100808FA	6.5	7.94	77.3	17.5	0.43	9.70	0.70	bdl	bdl	0.72	9.4	56.3
2010	100808GA	0	7.50	91.9	6.2	0.16	16.1	0.39	1.11	bdl	7.20	8.2	11.0
2009	090724Q1	14	8.06	66.2	6.2	0.16	91.9	1.61	0.56	0.90	0.47	5.8	93.8
2009	090724P1	12.5	7.96	70.1	7.4	0.19	30.6	0.54	bdl	0.18	0.72	7.5	56.3
2009	090724O1	12	7.82	75.7	6.3	0.15	29.0	1.06	bdl	bdl	1.06	6.0	81.3
2009	090724N1	6.5	7.64	80.4	6.6	0.16	387	1.13	bdl	0.36	2.18	4.4	18.8
2009	090724M1	0	7.27	93.4	6.5	0.15	48.4	1.89	3.89	0.18	6.99	5.8	68.8
2008	080720G	14	8.05	72.3	6.5	0.16	69.3	nd	nd	nd	0.31	5.8	nd
2008	080720F	12	7.99	75.0	6.6	0.17	45.2	nd	nd	nd	0.22	5.6	nd
2008	080720E	6.5	7.75	83.1	6.4	0.20	82.2	nd	nd	nd	2.59	5.8	nd
2008	080720D	0	7.46	93.7	6.4	0.20	71.0	nd	nd	nd	5.27	5.9	nd

Year	Sample ID ("Bison Pool")	Distance from source (m)	pH	Temp (°C)	Type[c]	Zone[d]	NO_3^- (µmol/kg)	NO_2^- (µmol/kg)	ΣNH_3 (µmol/kg)	Fe^{2+} (µmol/kg)	ΣS^{2-} (µmol/kg)	SiO_2 (mmol/kg)	DO (µmol/kg)
2010	100808CA	14	8.29	63.6	Photomat	P	9.70	0.43	0.56	Bdl	0.03	11.2	125
2010	BK231	14	8.29	63.6	Photomat	P	9.70	0.43	0.56	Bdl	0.03	11.2	125
2010	100801SLG-Jo	13.5	8.02	62.2	Sediment	C	21.0	0.26	bdl	nd	nd	nd	81.3
2010	100801SLG	11	7.96	64.3	SBC	C	nd	nd	nd	nd	nd	nd	nd
2010	100801SLH-sed	7	7.86	73.1	Sediment	C	14.5	0.30	bdl	nd	nd	nd	47.7
2010	100801SLH	7	7.86	73.1	SBC	C	14.5	0.30	bdl	nd	nd	nd	47.7
2010	100801SLI-sed	1	7.39	87.8	Sediment	C	32.3	0.11	1.67	nd	nd	nd	19.8
2010	100801SLI	1	7.39	87.8	SBC	C	32.3	0.11	1.67	nd	nd	nd	19.8

[a] Cl^- and SO_4^{2-} were determined using ion chromatography. All other measurements were determined spectrophotometrically in the field. (bdl = below detection limit; nd = not determined).

[b] DO = dissolved oxygen.

[c] Photomat = photosynthetic mat.

[d] P = photosynthetic; C = chemosynthetic.

Temperature and chemistry vary along the downstream profile, and data indicate increases in dissolved oxygen and nitrite, as well as decreases in nitrate with increasing distance from the source (decreasing temperature) at both Mound Spring and "Bison Pool" (Table II, Figure 6). Nitrate is the most abundant inorganic nitrogen species in both Mound Spring and "Bison Pool" fluid, yet it is present at low concentrations (Table II), suggesting that these hot springs may be nitrogen-limited systems. Furthermore, nitrate concentrations of Mound Spring fluids are typically higher than those at "Bison Pool" (Figure 6), perhaps highlighting an important difference in availability of nitrogen species between the two thermal features. Total ammonia concentrations throughout the outflow channel of both hot springs are typically near or below the detection limit (1.11 µmol/kg) for field spectrophotometric methods, but overall appear to decrease down the outflow channel. These results are consistent with previous Mound Spring and "Bison Pool" data and with data obtained from other Sentinel Meadows Group thermal features (Meyer-Dombard *et al*., 2011; Shock *et al*., 2010).

Increased dissolved oxygen content of Mound Spring and "Bison Pool" fluids as a function of increased distance from the source can be attributed primarily to atmospheric mixing, as a result of both increased exposure time and areas of turbulent flow in the outflow channel. In the photosynthetic zone, photosynthesis by microbial mat communities also contributes additional dissolved oxygen. A downstream increase in constituent nitrogen species would be expected in both hot springs as a result of evaporative processes; however, this trend is not observed. The presence and relative amounts of geochemical species is controlled not only by physical processes (evaporation, cooling, mixing, etc.) but also by disequilibria-fueled redox reactions, which provide energy to microorganisms through mediation of these redox

processes. Consumption of fixed nitrogen sources (NO_3^-, NO_2^-, NH_3/NH_4^+) through microbial metabolisms may partially account for the apparent decrease in overall inorganic nitrogen species with distance. Therefore, in locations farther from the source, it is plausible that biological nitrogen fixation may be an important process to compensate for this loss of bioavailable nitrogen. A downstream increase in nitrite concentration would be expected based on evaporative trends; however, nitrite concentrations remain relatively constant (or perhaps slightly increase) down the outflow channel of both hot springs. Perhaps this reflects limits in measurement precision, as values are typically close to those of field blanks.

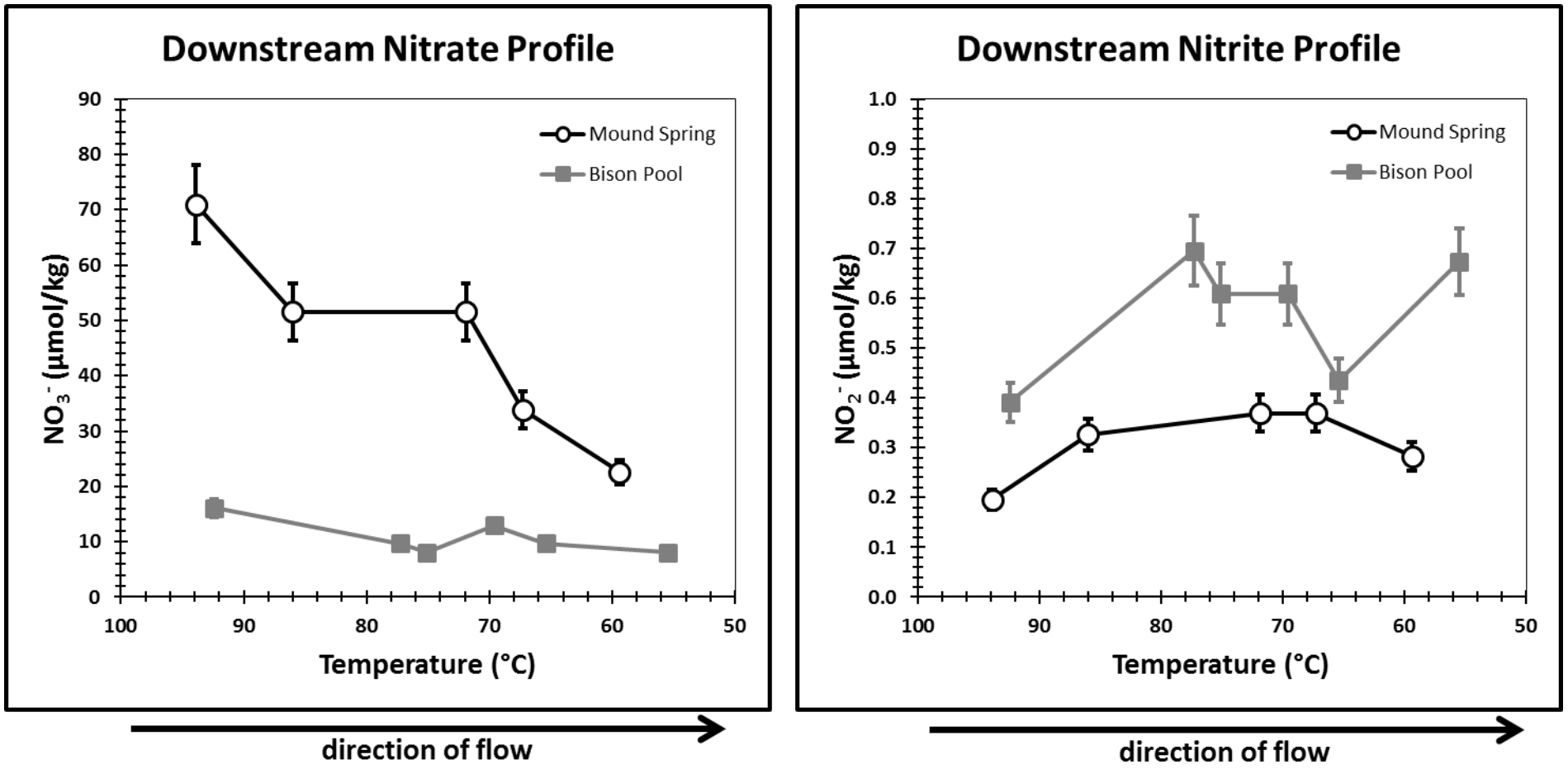


Figure 6. Downstream nitrate and nitrite profile of "Bison Pool" and Mound Spring thermal fluids. Values plotted are from 2010. Data points represent fluid measurements corresponding with 2010 biomass samples (see Table II). When not visible, error bars are smaller than data points.

4. NITROGEN FIXATION AT MOUND SPRING AND "BISON POOL"

4.1 Introduction

Here, the presence, expression, and diversity of prokaryotic *nifH* genes in sediments and biofilms of Mound Spring and "Bison Pool" are evaluated, providing a comparison of nitrogen fixation between two geochemically similar and geographically proximal hot springs at YNP. Based on the known ability of many photosynthetic organisms to fix nitrogen (e.g. Steunou *et al*., 2008, 2006), *nifH* is hypothesized to be both present and expressed in the photosynthetic microbial communities at Mound Spring and "Bison Pool." To date, few studies have addressed nitrogen fixation in strictly chemosynthetic portions of terrestrial hydrothermal systems (Hamilton *et al*., 2011a, b); therefore, the data presented here expound the limited data available in the literature in regards to this important high-temperature nitrogen-cycling process. Previous metagenomic studies at "Bison Pool" revealed the absence of *nifH* in the chemosynthetic communities of that hot spring (Swingley *et al*., 2012; Havig *et al*., 2011). However, microbial communities are incredibly dynamic and, therefore, it is plausible that community structure and diversity have changed in the five-year time span between collection of the metagenomic samples (in 2005) and collection of the samples for this study (in 2010). Furthermore, genetic data collected from chemosynthetic sediments at Mound Spring in previous years (Meyer-Dombard, unpublished data) indicate the potential for nitrogen fixation in the chemosynthetic communities, a stark contrast to the microbial communities at "Bison Pool." The nucleic acid based data presented here are supported by results of analysis of nitrogen isotope ratios from the same sediments and biofilms. To the author's knowledge, this

is the first study of *nifH* expression in chemosynthetic zones of a terrestrial hydrothermal system.

4.2 Presence and expression of *nifH* genes

Nucleic acids (DNA and mRNA) extracted from Mound Spring and "Bison Pool" chemosynthetic and photosynthetic biomass samples were subjected to (RT-)PCR-directed screens to search for the presence and *in situ* expression of *nifH*. No *nifH* DNA or cDNA amplicons were detected in chemosynthetic samples from "Bison Pool," (Table II, sediment and SBC sample types) but *nifH* was identified in both DNA and cDNA from "Bison Pool" photosynthetic mat samples (BK231 and 100808CA). PCR amplification of *nifH* genes from DNA extracted from all four biomass samples at Mound Spring (100801SLM, 100801SLL, 100801SLK, and 100801SLJ) (Tables II and III) yielded products of the expected size (approximately 400 bp). *nifH* amplicons from cDNA were also detected in all samples, with the exception of 100801SLM, which was collected at the highest temperature (80.2°C). 16S rRNA from 100801SLM cDNA was amplifiable using a universal bacterial primer set; however, despite repeated attempts, *nifH* was never successfully amplified from 100801SLM cDNA. cDNA amplification attempts of both 16S rRNA and *nifH* from another high-temperature Mound Spring sample (collected at 88°C during the same field season) produced identical negative results (not shown). PCR amplification of *nifH* from DNase-treated RNA yielded negative results, confirming that *nifH* cDNA-derived amplicons represent transcripts and not DNA contamination.

Since no *nifH* gene copies were detected in "Bison Pool" chemosynthetic communities, further analysis focused on Mound Spring *nifH* sequences only. Results of DNA sequencing of Mound Spring clones indicate the genetic capacity for nitrogen fixation in all Mound Spring

samples collected. However, results of cDNA amplification and sequencing show *nifH* expression at the time of sampling in only the three lower temperature samples (57.2°C to 72.7°C) (Table III). Consequently, it appears that the microbial community in some high-temperature Mound Spring samples contains the genetic capacity to fix nitrogen, yet may not express *nifH*, or may do so only transiently. This pattern of *nifH* expression could reflect the high energetic cost associated with biological nitrogen fixation (Hoover, 2000) and the distribution of bioavailable nitrogen along the outflow channel, which becomes more limited downstream (Table II, Figure 6). *nifH* expression is tightly regulated in response to fixed nitrogen limitation, with transcription not occurring when nitrate, ammonium, and other readily-assimilated forms of nitrogen are available (Rabouille *et al*., 2006; Dodsworth *et al*., 2005; Holl and Montoya, 2005; Dixon and Kahn, 2004; Mulholland *et al*., 2001; Halbleib and Ludden, 2000; and others). The absence of evidence for *nifH* expression in the >80°C samples could also be a result of low RNA concentrations or the presence of unidentified inhibitors in the PCR reaction. However, this is unlikely since extracted RNA from all sediment samples was of similar concentrations (2-3 ng/µl) and 16S rRNA amplification of cDNA from all samples yielded positive results, indicating suitability of the extracted nucleic acids for PCR.

Table III. *nifH* CLONE LIBRARY DATA FOR NUCLEIC ACIDS EXTRACTED FROM MOUND SPRING SAMPLE SITES[a]

Sample ID	Nucleic Acid	Type[b]	Zone[c]	Number of Clones Sequenced	Representative Clone IDs	Number of Clones Represented	Closest GenBank match (accession number)	% Similarity[d]	
								nt	aa
100801SLJ	DNA	Photomat	P	13	YNP_MS_SLJ_NIF1D; 2D	1; 2	Roseiflexus sp. RS-1 *nifH* region (CP000686)	96; 95	98; 95
					YNP_MS_SLJ_NIF3D	2	Synechococcus sp. JA-3-3Ab *nifH* region (CP000239)	97	100
					YNP_MS_SLJ_NIF4D	2	Anabaena variabilis ATCC 29413 *chlL* region (CP000117)	80	92
					YNP_MS_SLJ_NIF5D	6	Synechococcus sp. JA-3-3Ab *chlL* region (CP000239)	99	99
	cDNA	Photomat	P	14	YNP_MS_SLJ_NIF1C; 2C	2; 6	Roseiflexus sp. RS-1 *nifH* region (CP000686)	96; 98	98; 100
					YNP_MS_SLJ_NIF3C	1	Synechococcus sp. JA-3-3Ab *nifH* region (CP000239)	95	95
					YNP_MS_SLJ_NIF4C	1	Uncultured organism clone IG6-1 *nifH* gene (HM113538)	96	93
					YNP_MS_SLJ_NIF5C	1	Anabaena variabilis ATCC 29413 *chlL* region (CP000117)	77	78
					YNP_MS_SLJ_NIF6C	3	Synechococcus sp. JA-3-3Ab *chlL* region (CP000239)	98	99
100801SLK	DNA	Sediment	C	22	YNP_MS_SLK_NIF1D	2	Uncultured prokaryote clone N-1-8 *nifH* gene (GU232746)	76	82
					YNP_MS_SLK_NIF2D	19	Uncultured organism clone IG6-1 n*ifH* gene (HM113538)	94	94
					YNP_MS_SLK_NIF3D	1	Synechococcus sp. JA-3-3Ab *chlL* region (CP000239)	83	91
	cDNA	Sediment	C	16	YNP_MS_SLK_NIF1C; 2C	15; 1	Uncultured organism clone IG6-1 n*ifH* gene (HM113538)	94; 94	94; 94
100801SLL	DNA	Sediment	C	10	YNP_MS_SLL_NIF1D; 2D	5; 5	Uncultured organism clone IG6-1 n*ifH* gene (HM113538)	93; 93	94; 94
	cDNA	Sediment	C	15	YNP_MS_SLL_NIF1C; 2C	11; 4	Uncultured organism clone IG6-1 n*ifH* gene (HM113538)	94; 94	94; 94
100801SLM	DNA	Sediment	C	6	YNP_MS_SLM_NIF1D; 2D	3; 2	Uncultured organism clone IG6-1 n*ifH* gene (HM113538)	94; 94	94; 94
					YNP_MS_SLM_NIF3D	1	Rhodobacter sphaeroides ATCC 17025 *bchL* region (CP000661)	95	88
	cDNA	Sediment	C	n/a	n/a	n/a	n/a	n/a	n/a

[a] Table modified from Loiacono *et al.*, 2012.

[b] Photomat = photosynthetic mat.

[c] P = photosynthetic; C = chemosynthetic.

[d] Percent similarity is the maximum identity of the nucleotide (nt) or amino acid (aa) sequence to the sequence of its closest GenBank match.

4.3 Phylogenetic analysis and diversity of *nifH* DNA and cDNA sequences

Representative clones for each Mound Spring sample are listed in Table III along with their closest GenBank match based on *nifH* nucleotide and amino acid sequence comparison of sample clones to the database. Of the combined 27 DNA and cDNA clones sequenced from the photosynthetic mat sample (100801SLJ), over 40% are most closely related to *nifH* from *Roseiflexus* sp. RS-1 (nucleotide sequence identity of 95-98%). Three clones are closely related to *Synechococcus* sp. JA-3-3Ab *nifH* sequences (nucleotide sequence identity of 95-96%) (Table III). One cDNA clone (YNP_MS_SLJ_NIF4C) appears to be related to an environmental *nifH* sequence obtained from a photosynthetic mat sample collected from Imperial Geyser, a nearby thermal feature within the LGB of YNP (Hamilton *et al*., 2011a). Overall, phylogenetic reconstruction using aligned amino acid sequences places all true-*nifH* photosynthetic mat clones within three distinct clades (Figure 7: Clusters 1, 3, and 4). The remaining clones from photosynthetic samples contain sequences similar to *nifH* homologs, the majority of which most closely match the *chlL* gene of two heterocystous cyanobacteria species, indicating amplification of a *nifH* homolog involved in light-independent chlorophyll synthesis.

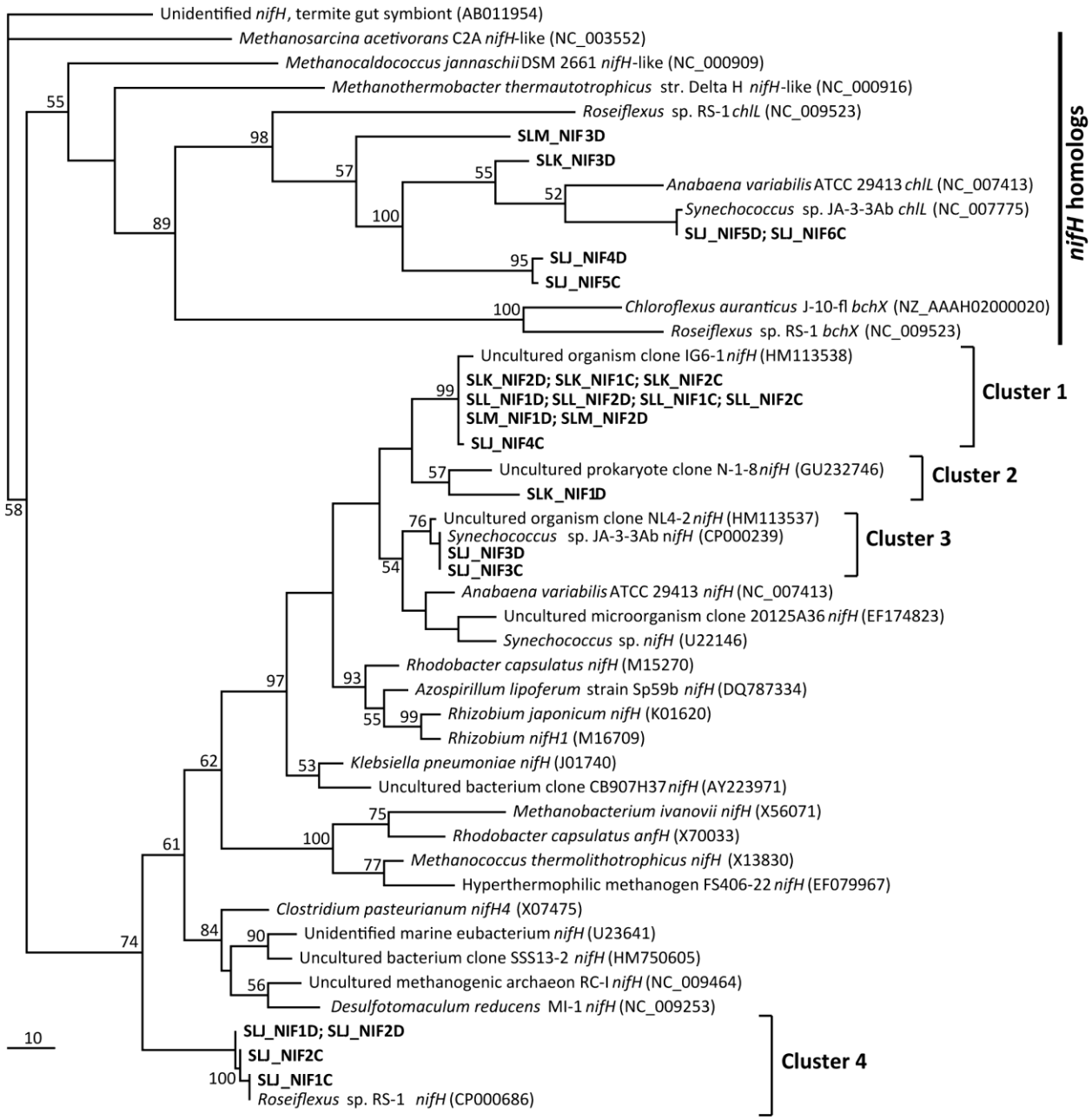


Figure 7. Maximum parsimony phylogenetic inference of *nifH* sequences obtained from environmental clones at Mound Spring. The phylogenetic reconstruction shown was generated using translated (amino acid) sequences. Representative clones from this study (as reported in Table III) are listed in bold. Clone names have been shortened to fit the tree and exclude "YNP_MS_." This maximum parsimony arrangement was constructed using 1000 random addition replicates. Bootstrap values indicate 100 parsimony replicates; values less than 50 are not displayed. The tree is rooted with *nifH* from a diazotrophic termite gut symbiont (AB011954). The scale bar indicates 10 substitutions. Figured modified from Loiacono *et al.*, 2012.

DNA and cDNA clones from chemosynthetic sediment samples (100801SLK, 100801SLL, and 100801SLM) represent less diverse *nifH* sequences than those from photosynthetic mat samples (Table III, Figure 7). In over 94% of the combined DNA and cDNA clone libraries for all sediment samples, *nifH* nucleotide sequences are between 86% and 94% identical to the environmental *nifH* sequence (IG6-1) obtained by Hamilton and colleagues (2011a) from Imperial Geyser. These sequences appear to be novel, clustering to form a unique monophyletic clade (Figure 7: Cluster 1). Of the remaining clones from the Mound Spring chemosynthetic zone samples, two are distantly related (maximum nucleotide identity of approximately 76%) to *nifH* from an uncultured prokaryotic deep-sea hydrothermal vent clone (GU232746). These sequences, represented by clone SLK_NIF1D on Figure 6, cluster independently from the rest of the Mound Spring chemosynthetic samples and also appear to represent novel *nifH* sequences. Overall, all but two chemosynthetic clones phlogenetically cluster within one of two distinct clades, which contain exclusively uncultured organisms from hydrothermal environments (Figure 7: Clusters 1 and 2). These clades are also part of a more deeply-rooted clade containing methanogen *nifH* sequences, suggesting that the Mound Spring chemosynthetic clones may represent archaeal, rather than bacterial *nifH* genes.

4.4 ^{15}N isotope ratios of sediments and biofilms

Results of nitrogen stable isotope analysis of Mound Spring biomass are displayed in Table IV and plotted in Figure 8 along with values obtained from "Bison Pool" (Havig *et al*., 2011). In all sample sets, an initial increase in $\delta^{15}N$ values is observed in the downstream direction (direction of decreasing temperature), as indicated. This change, however, at Mound Spring is less pronounced, exhibiting a somewhat flat trend of negative $\delta^{15}N$ values in the

chemosynthetic portion of the outflow channel, followed by an increase in $\delta^{15}N$ values in the photosynthetic zone downstream. The effects of abiotic processes on the isotopic signature of Mound Spring and "Bison Pool" biomass cannot be ignored, since processes such as downstream ammonia degassing ($NH_{3(aq)}/NH_{3(g)}$) and $NH_{3(aq)}/NH_4^+{}_{(aq)}$ speciation in hot spring fluids have isotope fractionation factors >1 (Holloway *et al*., 2011). However, biotic processes may also be largely responsible for the observed downstream increase in biomass ^{15}N. This increase in $\delta^{15}N$ at "Bison Pool" has been previously interpreted as the result of nitrogen recycling down the outflow channel, with biological nitrification and denitrification removing isotopically light nitrogen from the system and leading to the successive enrichment of biomass ^{15}N along the downstream profile of a nitrogen-limited system (Havig *et al*., 2011). Indeed, studies of microorganisms under controlled culture conditions have shown fractionation of the fixed nitrogen pool as a result of preferential discrimination by microorganisms in favor of isotopically light nitrogen (^{14}N) (Mariotti *et al*., 1981; Delwiche and Steyn, 1970). Geochemical measurements of Mound Spring fluids further support this idea, as downstream decreases in nitrate concentration and increases in nitrite can be plausibly explained by consumption of nitrate and production of nitrite as a result of denitrification and nitrification. However, this hypothesis requires further evaluation with genomic and transcriptomic-based methods in order to identify the presence and expression of genes associated with denitrification and nitrification in the microbial communities at Mound Spring.

TABLE IV. NITROGEN ISOTOPE ANALYTICAL RESULTS FOR MOUND SPRING BIOMASS[a,b]

Year	Sample ID	Distance from source (m)	Temp (°C)	Type[c]	% N (by dw)	SD %N (±)	$\delta^{15}N$ (‰)	SD $\delta^{15}N$ (±)
2010	100810VA	26.5	58.4	Sediment/Photomat	0.811	0.000	-0.32	0.33
2010	100810WA	22.5	67	Sediment/Photomat	0.345	0.001	-0.79	0.11
2010	100810YA	13	71.2	Sediment	0.059	0.001	-2.17	0.11
2010	100810ZA	5	86.2	Sediment	0.019	0.005	-2.02	0.19
2010	100810AA	0	94.1	Sediment	0.042	0.041	-2.35	0.16

[a] Table modified from Loiacono *et al*., 2012.

[b] Isotope data for "Bison Pool" biomass reported in Havig *et al*., 2011.

[b] Photomat = photosynthetic mat.

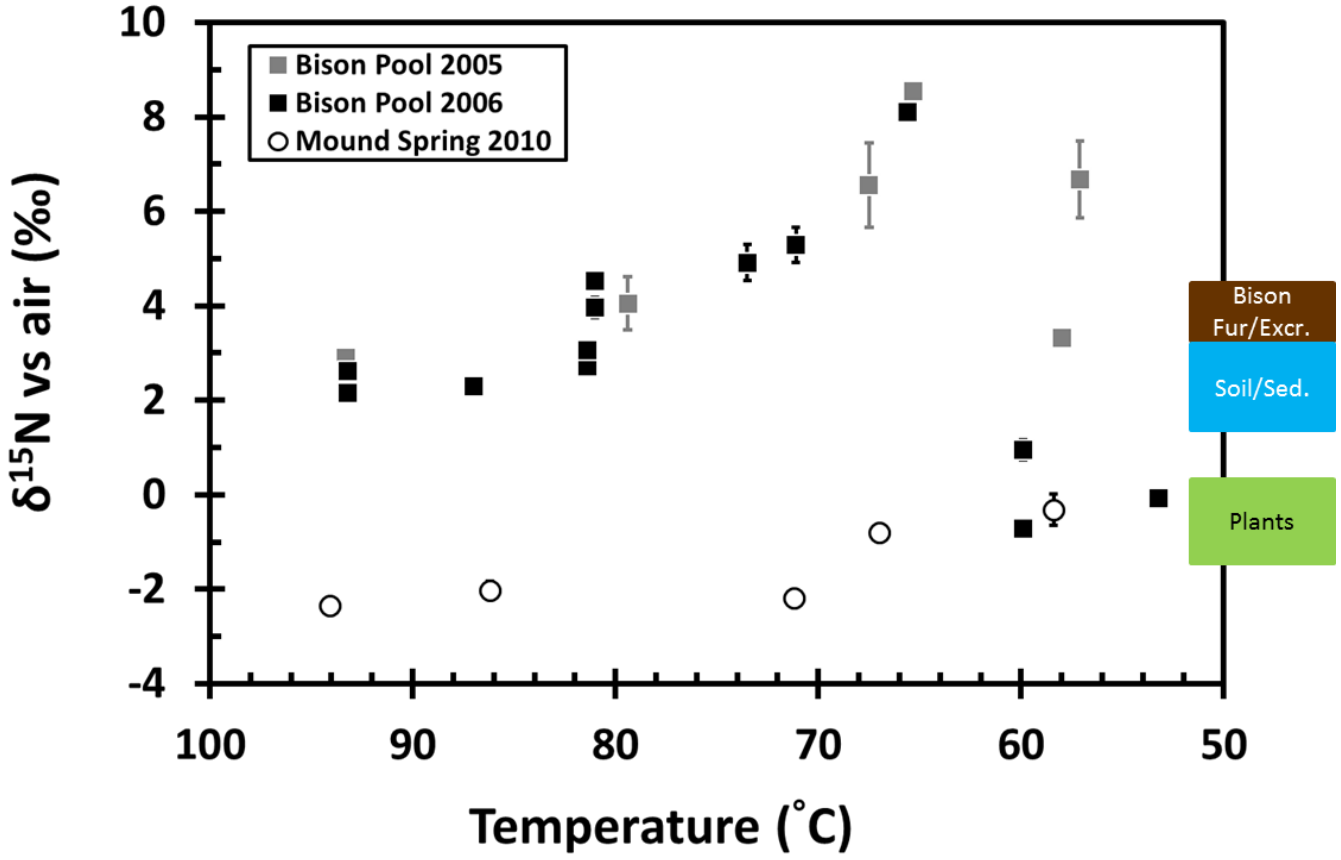


Figure 8. Nitrogen stable isotope values (relative to atmospheric N_2) in biomass from "Bison Pool" and Mound Spring plotted as a function of temperature. Biomass from "Bison Pool" consists of SBCs and photosynthetic mats. Biomass from Mound Spring consists of sediment and photosynthetic mats. Temperature decreases with increasing distance from the hot spring source pool. Nitrogen isotope values for samples collected in 2005 and 2006 are displayed for "Bison Pool"; values for Mound Spring are reported for samples collected in 2010. When not visible, error bars are smaller than data points. $\delta^{15}N$ ranges for select groups of contextual samples (bison fur and excrement; soil and sediment; plants (grasses and sedges)) are represented by boxes on the right side of the plot. Figure modified from Loiacono *et al*., 2012.

In contrast to samples from "Bison Pool," which are enriched in ^{15}N relative to atmospheric N_2, all sediment and mat samples at Mound Spring have $\delta^{15}N$ values that are depleted in ^{15}N relative to air (i.e. negative $\delta^{15}N$). $\delta^{15}N$ values averaging -2.35‰ were obtained for sediment samples collected near the hot spring source, and values increase downstream to a maximum of -0.32‰ in the 58.4°C sediment/photosynthetic mat sample (Table IV, Figure 8). Most contextual samples collected in Sentinel Meadows (bison fur, bison excrement, insects, meadow soil, and sinter soil) have $\delta^{15}N$ values of between 1 and 5‰ (Havig *et al*., 2011) (Table VIII, Appendix B). Only two allochthonous samples (grasses and sedges) have isotopic signatures (0.29‰ and -1.35‰, respectively) closer to those obtained for Mound Spring sediment communities (Table VIII, Appendix B). Thus, grasses and sedges can be considered likely contributors to the overall meadow isotopic composition, which is a plausible external source of light nitrogen to the nitrogen isotopic composition of the source biomass (Havig *et al*., 2011).

Regardless of the sources of available nitrogen, Mound Spring biomass is isotopically much lighter than "Bison Pool" biomass. One explanation for this difference is greater inputs of nitrogen from ^{15}N-enriched sources (i.e. bison fur and excrement) at "Bison Pool." This difference may be a result of slight topographic differences between the two hot springs. The "Bison Pool" source and outflow are topographically level with the meadow, whereas Mound Spring is positioned atop a low, wide sinter mound, isolating it to a greater extent from many allochthonous sources of nitrogen from the surrounding meadow.

^{14}N-enriched inputs can at least partially explain the negative $\delta^{15}N$ compositions of Mound Spring biomass. Yet, it is noteworthy that the increase in downstream $\delta^{15}N$ at Mound Spring is much less pronounced than at "Bison Pool." Assuming the processes of nitrification

and denitrification are also active at Mound Spring, a downstream trend similar to that of "Bison Pool" would be expected. The difference in trend allows several interpretations, one being that nitrification and denitrification processes are less active in Mound Spring than at "Bison Pool." Although this is possible, genetic verification is needed. Alternatively, the damped downstream $\delta^{15}N$ trend at Mound Spring could be explained by the repeated introduction of isotopically light ^{14}N into the system, tempering the effects of downstream enrichment in ^{15}N due to nitrogen recycling.

The process of biological nitrogen fixation is one way that isotopically light nitrogen could enter the Mound Spring system. At "Bison Pool," the rapid decrease in $\delta^{15}N$ was attributed to the onset of biological nitrogen fixation by photosynthetic organisms in the lower temperature portions of the outflow channel, a process restricted by the absence of *nifH* in the chemosynthetic zone microbial communities (Havig *et al.*, 2011). This observation is corroborated with the "Bison Pool" *nifH* genomic and transcriptomic data from this study, as well. The process of biological nitrogen fixation has been shown to discriminate somewhat against the heavier (^{15}N) isotope (Hamilton *et al.*, 2011b; Macko *et al.*, 1987; Delwiche and Steyn, 1970). Therefore, it is plausible that the somewhat flat downstream $\delta^{15}N$ trend and negative $\delta^{15}N$ composition of Mound Spring biomass may be indicative of biological nitrogen fixation in the Mound Spring outflow channel. The presence and expression of *nifH* genes in both chemosynthetic and photosynthetic microbial communities at Mound Spring further validates this hypothesis.

Several potential explanations exist for the variation in nitrogen fixation capacity and *in situ nifH* expression between the chemotrophs at Mound Spring and "Bison Pool." One

hypothesis is that the *nifH* gene is not present in SBCs (which are visibly present at "Bison Pool" but not Mound Spring); however genomic evaluation of chemotrophic sediment communities of "Bison Pool" revealed no evidence of *nifH* either. Furthermore, genomic data from SBCs at Flat Cone, a nearby hot spring in Sentinel Meadows, indicates the existence of *nifH* gene copies in some SBCs (Meyer-Dombard, unpublished data). An alternative, and likely more plausible, supposition for the contrast in nitrogen fixation processes between Mound Spring and "Bison Pool" is that the microbial communities of these hot springs are affected indirectly by the topographical differences between the two thermal features. The implications of this are reflected in both the biomass isotopic signature and microbial genomes of the communities in each hot spring. Since "Bison Pool" is topographically level with the surrounding meadow, it likely receives more inorganic and organic nitrogen input from the meadow than does Mound Spring, which is somewhat more topographically isolated from sources of meadow nitrogen. Bison excrement and fur, sticks, insects, and exogenous grasses are commonly found throughout the "Bison Pool" outflow, providing a source of carbon and nitrogen to fuel microbial metabolisms within the hot spring. At Mound Spring, the presence of these objects in the outflow channels is a much rarer occurrence. This difference in meadow input is amplified during precipitation events. During periods of heavy precipitation, soil, plants, animal waste, and other meadow material washes into "Bison Pool." The results of these precipitation events have been observed firsthand and are visible in Figure 5 and Figure 14C-D, Appendix D. Perhaps "Bison Pool" is a more eutrophic (or periodically eutrophic) system than is Mound Spring. With greater inputs of organic and inorganic nitrogen that can be incorporated into biomass or used as a source of energy, perhaps nitrogen fixation at "Bison Pool" is unnecessary due to more

frequent influx of fixed nitrogen sources. In contrast, Mound Spring is a more oligotrophic system, making nitrogen fixation a necessary process to provide sources of utile nitrogen to the local ecosystem, a difference that is reflected in the genome and transcriptome of the microbial communties at Mound Spring.

4.5 Conclusions

Using DNA-based methods, *nifH* genes were identified in sediment samples collected from locations along the outflow channel of Mound Spring, a silica sinter-depositing, alkaline thermal feature in the Lower Geyser Basin of YNP. The presence of *nifH* at all sampling sites indicates the genetic capacity for nitrogen fixation over a broad range of temperatures (57°C to 80°C). Previous studies have reported the presence and diversity of *nifH* genes in YNP over a wide range of temperatures (16.3°C to 89.0°C) and pH (1.90 to 9.78) (Hamilton *et al*., 2011a,b; Hall *et al*., 2008). The results of this study expand upon those data and add to our current knowledge of *nifH* distribution and diversity by identifying high-temperature activity of *nifH* in alkaline, terrestrial hydrothermal environments (Table VII, Appendix A).

The potential for nitrogen fixation by the microbial communities in Mound Spring sediments is supported by results of nitrogen stable isotope analysis of Mound Spring biomass. Although the potential influence of allochthonous inputs of isotopically light nitrogen sources cannot be ignored, $\delta^{15}N$ values of all samples are depleted relative to most contextual samples, permitting the interpretation that biological metabolic processes are responsible for fractionation and preferential incorporation of the light isotope into biomass (Delwiche and Steyn, 1970). It is plausible that the somewhat flat downstream $\delta^{15}N$ trend and negative $\delta^{15}N$

compositions observed in biomass at Mound Spring are a reflection of the slight fractionation associated with biological nitrogen fixation.

The presence of the *nifH* gene in all Mound Spring samples indicates the genetic capacity for nitrogen fixation in the microbial communities at all sampling sites. Evidence for biological nitrogen fixation in the sediments and photosynthetic mats of Mound Spring was confirmed through amplification of *nifH* sequences from extracted mRNA. Amplified cDNA revealed *in situ nifH* expression at the time of collection in samples obtained from both photosynthetic (mat) and chemosynthetic (sediment) communities. DNA and cDNA *nifH* amplicons form several unique phylogenetic clades, and, thus, appear to represent novel *nifH* sequences in both photosynthetic and chemosynthetic microbial communities. Although *nifH* expression was not confirmed in the highest temperature sample (80.2°C), *in situ* expression was validated in all other samples collected. Variability of expression downstream at Mound Spring may be affected by downstream changes in fluid chemistry and microbial community variation between sampling sites. Collectively, these results may reflect transient or selective expression of *nifH* as a result of the high energetic cost associated with nitrogen fixation, with expression occurring in response to downstream fixed nitrogen limitation. However, further experimentation is needed to validate this hypothesis. Furthermore, it is possible that the contrast in nitrogen fixation between Mound Spring and "Bison Pool" (which only contains *nifH* in photosynthetic communities) may be be a reflection of topographically-imposed nutrient variability between the two hot springs. This is the first report of *in situ nifH* expression in chemosynthetic communities of terrestrial hydrothermal systems, and sets a new upper

temperature limit (72.7°C) for nitrogen fixation in alkaline, terrestrial hydrothermal environments (Table VII, Appendix A).

5. NITRIFICATION AND DENITRIFICATION AT MOUND SPRING AND "BISON POOL"

5.1 Contextual framework

The results of the nitrogen fixation study at Mound Spring and "Bison Pool" led to a comparative analysis of nitrification and denitrification process between the two hot springs. Given the idea that "Bison Pool" is a periodically eutrophic system, whereas Mound Spring is more oligotrophic, differences in nitrification and denitrification are implied. Nitrogen stable isotope trends in "Bison Pool" and Mound Spring biomass corroborate the differences in nitrogen fixation potential and *in situ* activity amongst the two thermal features. Additionally, it was hypothesized that the damped downstream increase in $\delta^{15}N$ at Mound Spring relative to "Bison Pool" (Figure 8) could be attributed not only to the repeated introduction of isotopically light nitrogen through nitrogen fixation but also to less active nitrification and denitrification at Mound Spring, which would result in less downstream enrichment of ^{15}N in Mound Spring biomass. This idea seems plausible given the more oligotrophic nature of Mound Spring. To evaluate these ideas, functional nitrification and denitrification genes and transcripts (Table I) were amplified from Mound Spring and "Bison Pool" sediment and biomass. Results highlight further differences in nitrogen-cycling process between these two geochemically similar hot springs and provide evidence of the capability and possible *in situ* activity of high-temperature nitrification and dentrification.

5.2 Nitrification processes at Mound Spring and "Bison Pool"

5.2.1 Presence and expression of *amoA* genes

Both bacterial and archaeal *amoA* genes were targeted via PCR-directed screens of Mound Spring and "Bison Pool" biomass. Bacterial *amoA* amplicons of the expected size

(approximately 491 bp and 674 bp, dependent upon primer set used) (Table I) were not detected in any Mound Spring samples, but were detected in both DNA and cDNA of all "Bison Pool" SBC samples. However, despite repeated sequencing attempts using DNA- and cDNA-generated PCR products (from both primer sets) and both traditional Sanger sequencing and 454 pyrosequencing, most "Bison Pool" sequences produced positive BLAST matches with known bacterial *amoA* sequences over only very short read lengths (typically corresponding with approximately the primer length). BLAST matches to known bacterial *amoA* sequences over longer read lengths (up to 62 bp) but relatively low percent similarity (approximately 80% or less) were discovered for "Bison Pool" sequences from all SBC sample sites; however, this relatively short base pair match does not provide enough information to be evidence of bacterial nitrification in the SBCs of "Bison Pool." Furthermore, 2005 metagenomic data did not indicate the presence of any *amoA* genes in the microbial communities of "Bison Pool" (Swingley *et al*., 2012). This lack of nitrification potential is not entirely confounding as thermodynamic calculations indicate that little energy is available to be harnessed through ammonia oxidation at all "Bison Pool" locations (7-10 kcal/mol e^- transferred (approximately 29-42 kJ/mol e^-)) (Swingley *et al*., 2012; Shock *et al*., 2010). The theorized biological energy quantum of 20 kJ/mol (Schink, 1997) is based on the requirements of actively growing cells, yet it is likely that the microbial populations of hot springs and other "extreme" environments are functioning only with enough energy to meet a metabolic "bare minimum" rather than enough energy to sustain active growth. In fact, pure cultures of non-thermophilic populations have demonstrated the ability to catabolize at energy levels well below this theoretical level (as little as 10.6 ± 0.7 kJ/mol free energy) (Hoehler *et al*., 2001). Therefore, although many other

metabolic reactions are more energy-yielding than ammonia oxidation at “Bison Pool,” microbially-driven ammonia oxidation cannot be ruled out as a source of autotrophic energy. Perhaps AOB are present at “Bison Pool,” but with *amoA* genes that are too divergent from known AOB *amoA* genes to be captured with traditional primer sets. Alternatively, maybe ammonia oxidation is taking place, but by AOA rather than AOB.

To evaluate ammonia oxidation by AOA in “Bison Pool” and Mound Spring, biomass samples were screened using primers targeting strictly archaeal *amoA* genes (Table I). No genetic evidence of archaeal ammonia oxidation was discovered at Mound Spring. This, along with the lack of amplifiable bacterial *amoA* genes and transcripts, suggests that nitrification is largely absent in the microbial communities of that hot spring. However, archael *amoA* genes were found in two chemosynthetic “Bison Pool” SBC DNA samples: 100801SLI and 100801SLG (Tables V and VI). (See Figure 5 and Table II for sample locations.) Interestingly, *amoA* gene transcripts were not detected in mRNA extracted from these locations, suggesting that although the genetic capacity for ammonia oxidation is present, this nitrification step was either not occurring *in situ* at the time of sample collection or was occurring at such low rates that no transcripts could be detected via RT-PCR amplification. *amoA* genes were not detected in any of the photosynthetic communities sampled.

Given the notion of “Bison Pool” as a periodically eutrophic system, it seems plausible that both AOB and AOA could fill potential niches in the hot spring ecosystem, with AOB thriving during more eutrophic periods and AOA during oligotrophic times. However, the absence of identifiable true bacterial *amoA* gene copies in the environmental samples collected for this study suggests that AOB may be largely or entirely absent from the “Bison Pool”

microbial community. Perhaps this is a result of niche competition, with AOA outcompeting the slow-growing AOB. If AOB are part of the "Bison Pool" community structure, perhaps they are present in low numbers, flourishing during eutrophic periods when meadow carbon and nitrogen infiltrate the hot spring system and then becoming metabolically inactive, or dormant, under the stress of more oligotrophic conditions. The absence of *amoA* gene sequences from any AOM in the 2005 "Bison Pool" metagenome (Swingley *et al*., 2012) but presence of AOA in the 2010 "Bison Bool" SBCs from this study is evidence of this dynamic nature of hot spring microbial communities.

TABLE V. MOUND SPRING AND "BISON POOL" CLONE LIBRARY FOR FUNCTIONAL NITRIFICATION AND DENITRIFICATION GENES

Sample ID	Gene	Nucleic Acid	Type[a]	Zone[b]	Representative Clone IDs[c]	Number of Clones Represented	Closest GenBank match (accession number)	% nt Similarity[d]
BK231	*nirS*	cDNA	Photomat	P	YNP_BP_BK231_NIR1C	1	Anaerolinea thermophila UNI-1 *nirS* gene (AP012029.1)	79
100801SLG	*amoA* (archaeal)	DNA	SBC	C	YNP_BP_SLG_AMO1D; 2D	5; 1	Uncultured archaeon clone JMYamoA01 *amoA* (FJ810654.1)	98; 98
100801SLG	*nirS*	cDNA	SBC	C	YNP_BP_SLG_NIR1C	1	Uncultured bacterium clone W7S-42 *nirS* gene (AB456948.1)	91
100801SLG-Jo	*narG*	cDNA	Sediment	C	YNP_BP_SLGJo_NAR1C	1	*Thermus thermophilus* JL-18 plasmid pTTJL1801 *narG* (CP003253.1)	92
100801SLG-Jo	*nirS*	DNA	Sediment	C	YNP_BP_SLGJo_NIR1D	4	*Thermus thermophilus* strain PRQ25 *nirS* gene (FN666415.2)	89
100801SLG-Jo	*nirS*	DNA	Sediment	C	YNP_BP_SLGJo_NIR2D;4D	1; 1	*Thermus thermophilus* SG0.5JP17-16 plasmid *nirS* gene (CP002778.1)	89; 88
100801SLG-Jo	*nirS*	DNA	Sediment	C	YNP_BP_SLGJo_NIR3D	1	*Thermus thermophilus* JL-18 plasmid pTTJL180 *nirS* gene (CP003253.1)	89
100801SLI	*amoA* (archaeal)	DNA	SBC	C	YNP_BP_SLI_AMO1D	5	Uncultured archaeon clone JMYamoA01 *amoA* (FJ810654.1)	98
100801SLI-sed	*nirS*	DNA	Sediment	C	YNP_BP_SLIsed_NIR1D	1	Uncultured bacterium clone S2T415184 *nirS* gene (JN123030.1)	80
100801SLI-sed	*nirS*	DNA	Sediment	C	YNP_BP_SLIsed_NIR2D	2	Uncultured bacterium clone 09-G-CK-S-11 *nirS* gene (HE805988.1)	80
100801SLI-sed	*nirS*	DNA	Sediment	C	YNP_BP_SLIsed_NIR3D	1	Uncultured bacterium clone NirS-707-H8_Meng1 *nirS* gene (HQ666778.1)	79
100801SLI-sed	*nirS*	DNA	Sediment	C	YNP_BP_SLIsed_NIR4D	1	Uncultured bacterium clone 3S51 *nirS* gene (DQ177110.1)	82
100801SLI-sed	*nirS*	DNA	Sediment	C	YNP_BP_SLIsed_NIR5D	2	Uncultured bacterium clone S2T415184 *nirS* gene (JN123030.1)	80
100801SLJ	*nirS*	cDNA	Photomat	P	YNP_MS_SLJ_NIR1C	3	*Thermus thermophilus* strain PRQ25 *nirS* gene (FN666415.2)	90
100801SLJ	*nirS*	cDNA	Photomat	P	YNP_MS_SLJ_NIR2C	1	*Halomonas* sp. C8 *nirS* gene (GQ384048.1)	84

[a] Photomat = photosynthetic mat.

[b] P = photosynthetic; C = chemosynthetic.

[c] "BP" denotes "Bison Pool" sample; "MS" denotes Mound Spring sample.

[d] Percent similarity is the maximum identity of the nucleotide (nt) sequence to the sequence of its closest GenBank match.

5.2.2 *amoA* gene sequence diversity and phylogenetic analysis

Representative archaeal *amoA* clones for each relevant "Bison Pool" sample are listed in Table V along with their closest GenBank match based on nucleotide sequence comparison of sample clones with the NCBI database. All "Bison Pool" clones are very closely related to one another (greater than 99% nucleotide similarity). Consequently, they are all most closely related to the same *amoA* gene sequence (maximum nucleotide identity of 99%), which was obtained from an uncultured archaeon from a subsurface groundwater sample (FJ810654) (Yagi *et al*., 2010; see Table V). Phylogenetic analysis using aligned nucleotide sequences places all "Bison Pool" *amoA* clones within a cluster containing archeael *amoA* retrieved from an uncultured subglacial clone (HQ650066) as well as an *amoA* gene obtained from a Turkish hot spring sample (unpublished data; Figure 9). This "Bison Pool" cluster (Figure 9) belongs to a larger clade containing the subsurface groundwater clone mentioned above, as well as several other clones, all belonging to organisms from mesophilic environments (HE798128, HE798076, JX010814, EU239959). Together with the "Bison Pool" clones, these mesophilic archaeal *amoA* sequences form a more deeply-rooted clade containing several *amoA* genes from other hydrothermal environments (see the bottom half of Figure 9). Although some hydrothermal *amoA* sequences cluster with the "Bison Pool" clones, the majority of the hydrothermal *amoA* genes on the phylogenetic tree in Figure 9 fall within the large clade in the top half of the arrangement, which contains the thermophilic isolate *Candidatus Nitrososphaera gargensis*. This phylogenetic reconstruction suggests that the archaeal *amoA* genes recovered from "Bison Pool" SBCs do not belong to this *Nitrosophaera* group, but instead belong to the *Nitrosopumilus*

cluster, representing a different evolutionary lineage of ammonia-oxidizing archaea than many of the hydrothermal *amoA* genes discovered, to date.

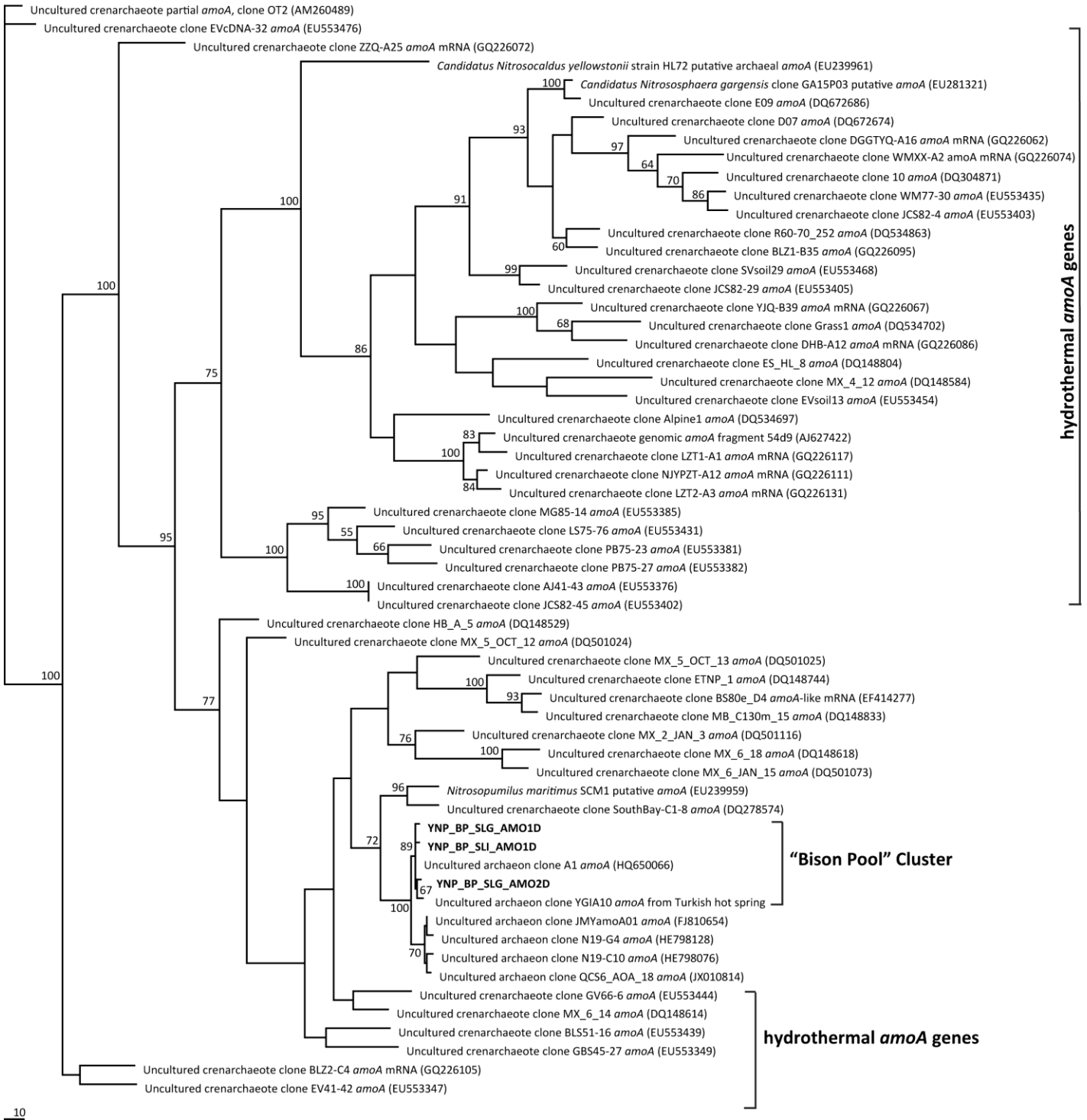


Figure 9. Maximum parsimony phylogenetic inference of archaeal *amoA* sequences obtained from environmental clones at Bison Pool. The phylogenetic reconstruction shown was generated using nucleotide sequences. Representative clones from this study (as reported in Table V) are listed in bold. This maximum parsimony arrangement was constructed using 1000 random addition replicates. Bootstrap values indicate 100 parsimony replicates; values less than 50 are not displayed. The tree is rooted with a partial archaeal *amoA* gene from an uncultured crenarchaeote extracted from a subsurface thermal spring in the Austrian Central Alps (AM260489). The scale bar indicates 10 substitutions.

5.3 Denitrification and nitrate reduction processes at Mound Spring and "Bison Pool"

5.3.1 Presence and expression of functional denitrification and nitrate reduction genes

In order to evaluate the denitrification and nitrate reduction potential at Mound Spring and "Bison Pool," a variety of functional genes (Table I) were targeted via (RT-)PCR-directed screens of DNA and cDNA from Mound Spring and "Bison Pool" sediments and biofilms. Results of this denitrification screen, along with the previous *nifH* and *amoA* results, are summarized in Table VI. *narG* appears to be found predominantly in the sediment communities at Mound Spring and "Bison Pool"; however, appropriately-sized amplicons (approximately 650 bp) were also detected in nucleic acids extracted from the lowest temperature "Bison Pool" SBC sample (DNA) as well as the Mound Spring photosynthetic mat sample (mRNA/cDNA and DNA). Most genetic evidence of *narG* in chemosynthetic communities is restricted to DNA; however, *in situ* expression of *narG* was also confirmed in 100801SLG-Jo, a "Bison Pool" chemosynthetic sediment sample collected at 62°C. These results collectively imply that, although the genetic capacity for nitrate reduction appears to be present across a range of Mound Spring and "Bison Pool" microbial communities, *in situ narG* expression may be more limited.

TABLE VI. PCR RESULTS SUMMARY OF FUNCTIONAL NITROGEN-CYCLE GENES AT MOUND SPRING AND "BISON POOL"[a]

N-Cycle Gene	Mound Spring Chemosynthetic						Mound Spring Photosynthetic	
	100801SLM		100801SLL		100801SLK		100801SLJ	
	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA
nifH		**X**	**X**	**X**	**X**	**X**	**X**	**X**
amoA								
narG				X[b]		X	X[b]	X
nirK/S							**X**	X
cnorB								
nosZ		X		X[b]	X			X

N-Cycle Gene	"Bison Pool" Chemosynthetic												"Bison Pool" Photosynthetic			
	100801SLI		100801SLI-sed		100801SLH		100801SLH-sed		100801SLG		100801SLG-Jo		BK231		100808CA	
	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA
nifH													X	n/d	X	n/d
amoA		**X**								**X**				n/d		n/d
narG				X						X	**X**	X		n/d		n/d
nirK/S	X[b]	X	X	**X**	X	X	X	X	**X**	X	X	**X**	**X**	n/d	X	n/d
cnorB														n/d		n/d
nosZ		X		X		X		X	X[b]	X[b]	X	X[b]	X[b]	n/d	X	n/d

[a] Boxes marked with "**X**" indicate confirmation of gene presence through sequenced clones; boxes marked with "X" represent possible gene presence based on amplicon size and gel banding pattern. Blank boxes indicate no detection of gene presence. "n/d" indicates that gene presence was not determined.

[b] Gene fragments were cloned and sequenced, but did not produce sequences of the anticipated functional gene.

Nitrite reduction appears to be a widespread process in both chemosynthetic and photosynthetic microbial communities at "Bison Pool," as evidenced by the abundant appearance of *nirS* genes (and *nirS*-sized amplicons) in both DNA and cDNA samples (Table VI). However, at Mound Spring, nitrite reduction seems to be limited to photosynthetic mat communities. *nirS* transcripts were recovered from both Mound Spring and "Bison Pool" photosynthetic mats indicating active nitrite reduction in both locations at the time of sampling. Furthermore, transcriptomic evidence suggests that nitrite reduction is an active, and likely important process in all of the chemosynthetic communities sampled at "Bison Pool," with *in situ nirS* expression possibly occurring in chemosynthetic sediments (100801SLI-sed) at temperatures of up to 88°C. However, though the presence of *nirS* gene markers was confirmed in the DNA extracted from this sample site, the *nirS* transcripts obtained have yet to be sequenced. Therefore, additional sequencing efforts are necessary to fully confirm *in situ nirS* expression at that site. Although a variety of *nirS* gene copies were detected, *nirK* genes were not identified in any of the Mound Spring or "Bison Pool" samples.

The presence and expression of *narG* and *nirS* in Mound Spring and "Bison Pool" microbial populations may be expected from a catabolic standpoint. Reactions coupling nitrate and nitrite reduction to both autotrophy and heterotrophy are theoretically exergonic at both Mound Spring and "Bison Pool" using a variety of electron donors (e.g. CH_4, CO, H_2O, Fe^{2+}, NH_4^+, sulfur, magnetite, $HCOO^-$) (Shock *et al*., 2010; Windman *et al*., 2007). Furthermore, metagenomic data from "Bison Pool" identified numerous organisms capable of nitrate and nitrite reduction in both chemosynthetic and photosynthetic zones (Swingley *et al*., 2012).

Although *narG* and *nirS* genes were found in several locations, *norB* and *nosZ* "gene-hunting" results offer less insight into the last two steps of the denitrification process (NO_2^- to NO and NO to N_2O). *norB* PCR results targeting the *cnorB* gene produced multi-sized amplicons for each sample, several of which were near the expected size (389 bp). Bands from several *norB* PCR products were gel excised, cleaned, cloned, and sequenced, yet none returned what appeared to be true *cnorB* sequences. Perhaps this is a result of excision of the incorrect amplicon, but this could only be confirmed through re-excision, cloning, and sequencing of c*norB* PCR products. Alternatively, it is possible that the *cnorB* gene is not present in the microbial communities of Mound Spring and "Bison Pool." This may be the most likely scenario since the *cnorB* gene has been linked to the presence of *nirK* genes in pure cultures of denitrifiers (Heylen *et al*., 2007), and no *nirK* genes were detected in any of the microbial communities sampled for this study. Although *cnorB* encodes the nitric oxide reductase utilized solely by dentrifiers, another nitric oxide reductase, encoded by *qnorB*, can be used by denitrifying organisms as well (Philippot, 2005). However, non-denitrifiers are also known to use the *qnorB* nitric oxide reductase as a protection against toxic nitrogen-containing oxides (Heylen *et al*., 2007), so further evaluation of the Mound Spring and "Bison Pool" microbial communities using *qnorB* genes could potentially identify both committed denitrifiers and non-denitrifiers, which would subsequently need to be resolved using phylogenetic analysis techniques.

No definitive results indicating organisms capable of completing the full denitrification pathway were discovered at Mound Spring or "Bison Pool." This was previously concluded at "Bison Pool" based on the results of the 2005 metagenomic study (Swingley *et al*., 2012).

However, several *nosZ*-sized amplicons (approximately 453 bp) were detected in both chemosynthetic and photosynthetic communities in both hot springs. Five *nosZ* PCR products were selected for cloning, but none of the *nosZ* clones sequenced BLASTed with known *nosZ* genes. Thus, additional sequencing efforts are necessary to verify the presence of true *nosZ* genes and transcripts at Mound Spring and "Bison Pool." Overall, the widespread genetic capacity for denitrification and nitrate reduction at both Mound Spring and "Bison Pool" provides at least a partial explanation for the downstream decrease in NO_3^- observed in both hot springs, as it would be consumed in both nitrate-reduction processes.

5.3.2 *narG* and *nirS* sequence diversity and phylogenetic analysis

Representative clones for each *narG* and *nirS* sample are listed in Table V. Only one of the three *narG* clones sequenced matched known *narG* genes in the GenBank database. This cDNA clone (YNP_BP_SLG-Jo_NARC1) is most closely related to *narG* from *Thermus thermophilus JL-18* (CP003253), with a nucleotide similarity of 98%. These results are consistent with metagenome data, which also identified most *narG* genes in a comparable chemosynthetic sample as belonging to *Thermus* species (Swingley *et al*., 2012). BLAST results indicate that several *nirS* clones are also related to sequences from *Thermus thermophilus* species. All true cDNA *nirS* clones from "Bison Pool" sediment sample 100801SLG-Jo and three cDNA clones from the Mound Spring photosynthetic mat (YNP_MS_SLJ_NIR1C) are most closely related to *nirS* from *Thermus thermophilus* species, as well (maximum nucleotide identity of 88-92%; Table V). These results highlight the importance of *Thermus* species in the microbial communities of both Mound Spring and "Bison Pool," specifically in regards to denitrification processes in the two hot springs. Additional cDNA clones from Mound Spring and "Bison Pool"

photosynthetic mat samples (YNP_MS_SLJ_NIR2C and YNP_BP_BK231_NIR1C, respectively) indicate diversity in the denitrifying populations of photosynthetic communities, with sequences being distantly related to *Halomonas* and *Anaerolinea thermophila* species (Table V). Of all clones sequenced, the clones from the 88°C "Bison Pool" sediment community (100801SLI-sed) appear to represent the most diverse population of nitrite reducing organisms. The seven true *nirS* DNA clones sequenced from this site fall into five distinct groups, each distantly related to uncultured denitrifiers from mesophilic soil and sediment environments (Table V). Similarly, the *nirS* cDNA clone obtained from SBCs at "Bison Pool" site 100801SLG (YNP_BP_SLG_NIRC1) is also most closely related to a *nirS* sequence from an uncultured soil denitrifier (nucleotide similarity of 91%) (Table V). The diversity of nitrite-reducers at "Bison Pool" was suggested in the results of the 2005 "Bison Pool" metagenomic data, which suggested that most *nirS* from chemosynthetic samples most closely grouped with a category of "unassigned" taxa (Swingley *et al*., 2012). *nirS* from *Aquificales* and *Thermus* was also discovered in the metagenome, reinforcing the prevalence of *Thermus* BLAST hits in the "Bison Pool" *nirS* sequences presented here. Although the information from GenBank BLAST searches provides insight into *narG* and *nirS* sequence diversity at Mound Spring and "Bison Pool," additional phylogenetic analyses (such as phylogenetic tree reconstructions) are needed to further resolve the evolutionary relationships of the organisms at Mound Spring and "Bison Pool" to those of other known cultured and uncultured denitrifiers.

5.4 Conclusions

Results of gene surveys targeting functional nitrification and denitrification genes indicate a marked difference between these processes at Mound Spring and "Bison Pool."

Archaeal *amoA* genes in high-temperature SBCs indicate the genetic capacity for nitrification in the chemotrophic communities at "Bison Pool." Yet, the absence of *amoA* transcripts suggests that *in situ* nitrification was not occurring at the time of sampling. Furthermore, comparison of "Bison Pool" *amoA* results from this study and a 2005 metagenomic study indicate a temporal shift in microbial community structure, highlighting the dynamic nature of hot spring microbial communties.

Genetic evidence of nitrification is absent from all Mound Spring chemotrophic and phototrophic communities sampled, and denitrification gene markers are less abundant at Mound Spring as well. Nitrate reduction and nitrous oxide reduction are the only two genetically-possible denitrification processes in chemotrophic sediment communities of Mound Spring. The phototrophic communities at Mound Spring also appear capable of these processes, in addition to nitrite reduction, which was confirmed *in situ* in photosynthetic mat communities at both Mound Spring and "Bison Pool" through recovery of *nirS* transcripts.

In contrast to Mound Spring, the microbial communities of "Bison Pool" appear to have widespread genetic capability of most denitrification processes. The extensive presence of *nirS* gene sequences and transcripts suggests that nitrite reduction is an important metabolic process in the "Bison Pool" ecosystem. *nirS* genes were detected in chemotrophic sediment communities at temperatures of up to 88°C, and *in situ* transcription of *nirS* was confirmed in SBCs at 64°C. *nirS*-sized cDNA amplicons also suggest expression of *nirS* in the 88°C sample; however, this is yet to be verified through cloning and sequencing.

Overall, denitrification gene markers are much less abundant at Mound Spring than at "Bison Pool," validating the idea that denitrification processes are less active at Mound Spring.

This, along with the lack of detectable *amoA* genes at Mound Spring, supports previous notions that the damped downstream $\delta^{15}N$ trend of Mound Spring biomass (Figure 8) is a result of not only introduction of isotopically light nitrogen by diazotrophs, but also of less active nitrification and denitrification processes at Mound Spring than at "Bison Pool" leading to less downstream enrichment of ^{15}N in biomass at Mound Spring. (See section 4.4, p. 48.)

The results of this nitrification and denitrification gene survey also highlight the importance of primer quality in functional gene hunts. Several appropriately-sized amplicons were detected for putative nitrogen cycle genes, but, when sequenced, did not match the appropriate genes from organisms in the GenBank database (see Table IX, Appendix F). Primers designed for targeting functional genes in environmental samples are typically degenerate and thus, subject to mispriming. It is possible that some of the amplicons detected in this study were the result of non-specific primer annealing capturing non-target regions of the genome. Perhaps this could be resolved through deeper sequencing in order to capture any target gene amplicons that exist. More importantly, this underscores the need for additional culturing efforts and metagenome studies of the microorganisms in hydrothermal environments. These are necessary in order to expand knowledge of the function and diversity of thermophilic organisms and to aid in design of new primer sets that are better suited for organisms in hydrothermal environments.

6. CONCLUSIONS

This study merges genomic, transcriptomic, and geochemical techniques to evaluate nitrogen cycling in two terrestrial hydrothermal systems in the LGB of YNP. The two hot springs studied, Mound Spring and "Bison Pool," are alkaline, siliceous sinter-depositing features that are fed by underground hydrothermal reservoirs. Both hot springs are boiling at the source, and fluid temperatures decrease down the outflow channel due to cooling and evaporative effects. Mound Spring is situated atop a low, wide sinter mound, whereas "Bison Pool" is topographically level with the surrounding meadow. As a result of this topographic disparity, the "Bison Pool" source and outflow channels are influenced to a greater extent by allochthonous sources of nitrogen and carbon from the surrounding meadow, which enter the hot spring ecosystem on a regular basis (as evidenced by the bison excrement, fur, insects, and exogenous grasses that are typically found scattered throughout the "Bison Pool" outflow channel). The higher elevation of Mound Spring results in greater isolation from organic and inorganic meadow inputs. During heavy precipitation events, this difference is amplified, as large amounts of meadow material wash into the "Bison Pool" source pool and outflow channels. Variations in microbial community morphology are also observed between Mound Spring and "Bison Pool." SBCs are found throughout chemosynthetic portions of the "Bison Pool" outflow channel, clinging to sinter ridges and solid organic particles in the outflow channel, such as sticks/twigs, bison excrement, and insects. These chemotrophic SBCs are visibly absent from Mound Spring.

Geochemically, Mound Spring and "Bison Pool" are very similar, with high silica and chloride content and low sulfate and sulfide concentrations as a result of water-rock

interactions with subsurface layered volcanic extrusives. Chemistry varies along the downstream profile, with increases in dissolved oxygen and nitrite, as well as decreases in nitrate with increasing distance from the source (decreasing temperature) at both Mound Spring and "Bison Pool." Nitrate is the most abundant inorganic nitrogen species in both Mound Spring and "Bison Pool" fluid, yet it is present at low concentrations, suggesting that these hot springs may be nitrogen-limited systems. Downstream changes in geochemistry can be attributed to physical processes (such as atmospheric mixing, evaporation, and cooling) as well to disequilibria-fueled redox reactions, which provide energy to microorganisms through mediation of these redox processes. Consumption of fixed nitrogen sources (NO_3^-, NO_2^-, NH_3/NH_4^+) through microbial metabolisms (e.g. nitrification, denitrification, assimilation) may partially account for the apparent decrease in overall inorganic nitrogen species with distance.

The observed geochemical trends are corroborated by genomic and transcriptomic evidence of various nitrogen cycling process at Mound Spring and "Bison Pool." Results indicate that nitrogen fixation processes are markedly different between the Mound Spring and "Bison Pool" microbial communities (see Figures 10 and 11). At "Bison Pool," diazotrophic organisms are not present in chemosynthetic communities, but are present and active in photosynthetic mats. In contrast, *nifH* genes and transcripts were identified in chemosynthetic sediment samples collected from the outflow channel of Mound Spring. The presence of *nifH* at all Mound Spring sampling sites indicates the genetic capacity for nitrogen fixation over a broad range of temperatures (57°C to 80°C). Furthermore, *in situ nifH* expression was confirmed by amplification of *nifH* transcripts from Mound Spring samples obtained from both photosynthetic (mat) and chemosynthetic (sediment) communities. Mound Spring DNA and

cDNA *nifH* amplicons form several unique phylogenetic clades, and, thus, appear to represent novel *nifH* sequences in both photosynthetic and chemosynthetic microbial communities. Although *nifH* expression was not confirmed in the highest temperature sample (80.2°C), *in situ* expression is validated in all other Mound Spring samples collected. Variability of expression downstream at Mound Spring may be affected by downstream changes in fluid chemistry and microbial community variation between sampling sites. Collectively, these results may reflect transient or selective expression of *nifH* as a result of the high energetic cost associated with nitrogen fixation, with expression occurring in response to downstream fixed nitrogen limitation.

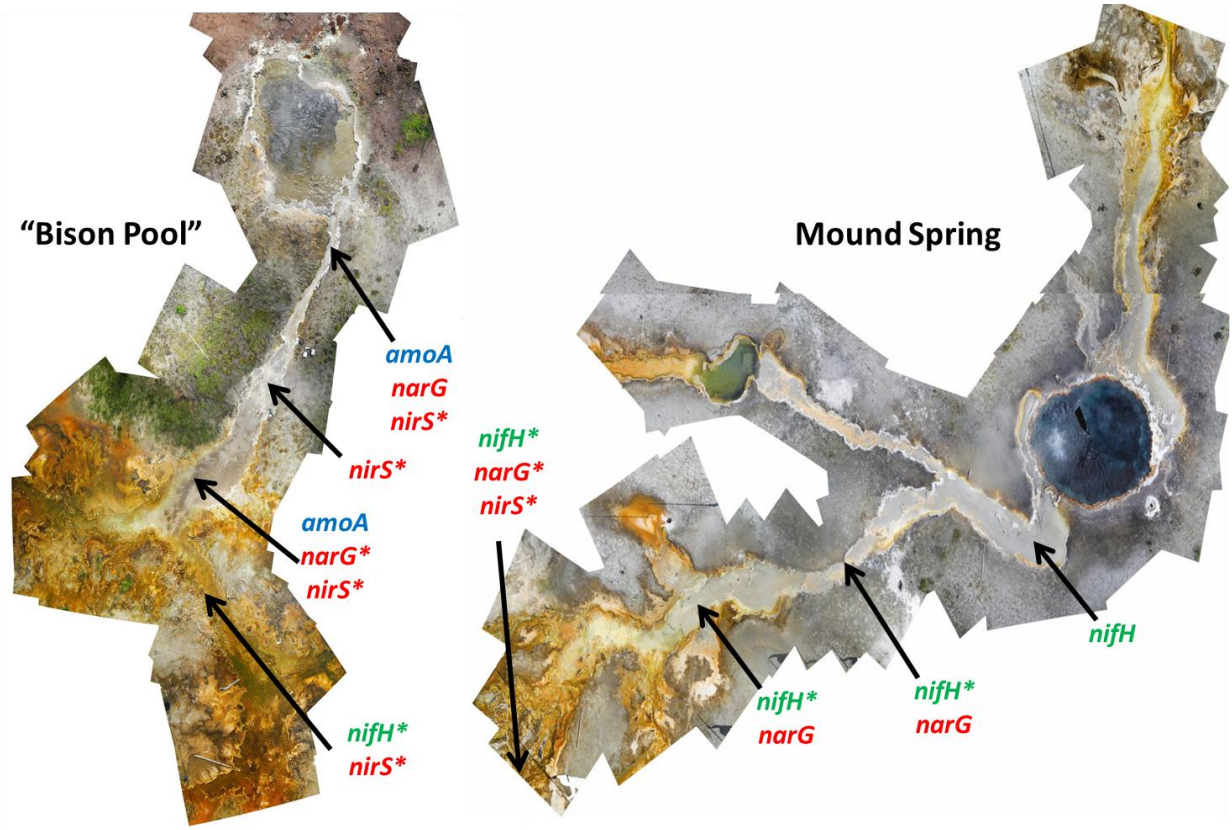


Figure 10. Summary of "Bison Pool" and Mound Spring genomic and transcriptomic results by sampling site. Gene markers and transcripts recovered from each biomass sample site are indicated on the maps of "Bison Pool" and Mound Spring. Nitrogen fixation genes are listed in green; nitrification genes are in blue; denitrification/nitrate reducation genes are in red. Asterisks (*) next to gene names indicate recovery of transcripts at that site. Note the prevalence of nitrification and denitrification gene markers and transcripts at "Bison Pool" and the abundance of nitrogen fixation gene markers and transcripts at Mound Spring.

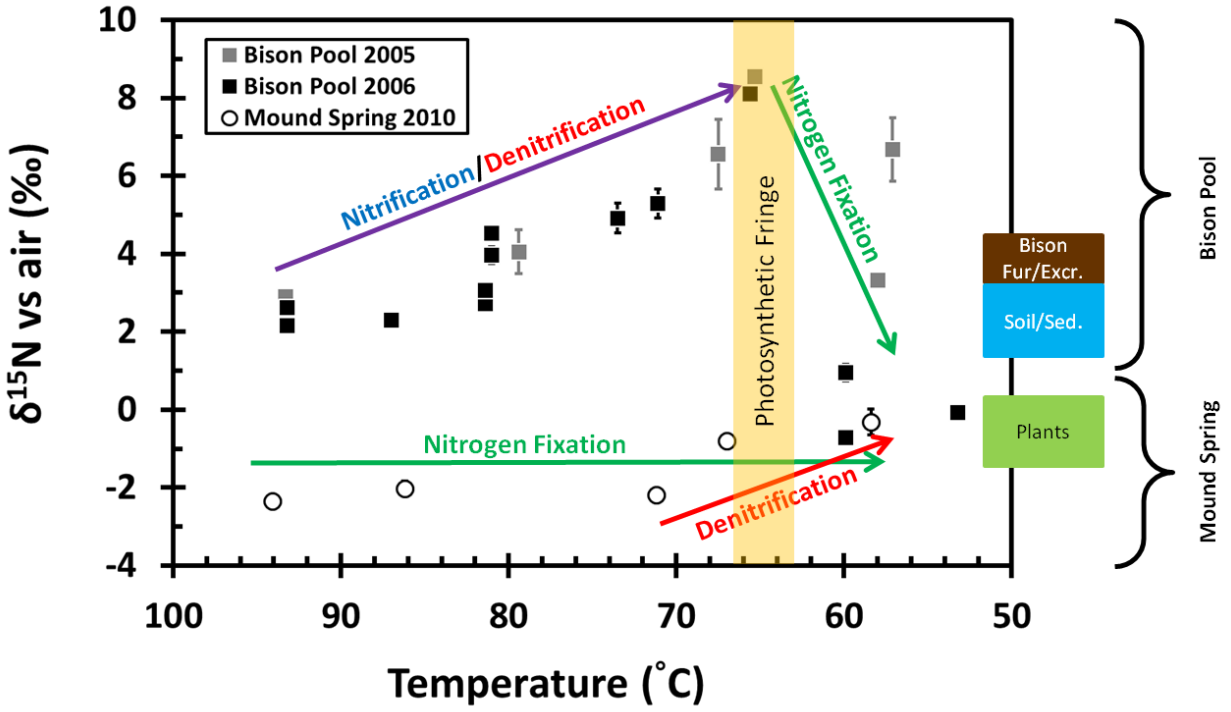


Figure 11. "Bison Pool" and Mound Spring isotope and genomic results summary. Arrows indicating the dominant nitrogen-cycling processes in the outflow channels of "Bison Pool" (top) and Mound Spring (bottom) are overlain atop isotope data, corroborating the downstream trends in biomass $\delta^{15}N$ observed in both hot springs. The approximate location of the photosynthetic "fringe" is represented by the light orange bar between 63°C and 66°C. Temperature decreases with increasing distance from the hot spring source pool. When not visible, error bars are smaller than data points. δ15N ranges for select groups of contextual samples (bison fur and excrement; soil and sediment; plants (grasses and sedges)) are represented by boxes on the right side of the plot.

Results of gene surveys also indicate a distinct difference between nitrification and denitrification processes at Mound Spring and “Bison Pool.” Genetic evidence of nitrification is absent from Mound Spring chemotrophic and phototrophic communities. However, archaeal *amoA* genes in high-temperature SBCs indicate the genetic capacity for nitrification in the chemotrophic communities at “Bison Pool.” Although genetically feasible, the absence of *amoA* transcripts suggests that *in situ* nitrification was not occurring at the time of sampling. Furthermore, comparison of “Bison Pool” *amoA* results with those of a previous study imply a temporal shift in microbial community structure, highlighting the dynamic nature of hot spring microbial communties.

The microbial communities of “Bison Pool” also appear to have widespread genetic capability of several denitrification processes. *In situ narG* transcription from a 62°C sediment sample confirms active nitrate reduction by chemotrophic communities. The extensive presence of *nirS* gene sequences and transcripts suggests that nitrite reduction is an important metabolic process in the “Bison Pool” ecosystem. *nirS* genes were detected in chemotrophic sediment communities at temperatures of up to 88°C, and *in situ* transcription of *nirS* was confirmed in SBCs at 64°C. *nirS*-sized cDNA amplicons also suggest expression of *nirS* in the 88°C sample; however, this is yet to be verified through cloning and sequencing.

Overall, denitrification gene markers are less abundant at Mound Spring than at “Bison Pool.” *In situ nirS* transcription in photosynthetic mat communities at both Mound Spring and “Bison Pool” confirm the activity of nitrite reduction in photosynthetic zones, yet evidence of other dentrification processes at Mound Spring is more limited. This, along with the lack of

detectable *amoA* genes at Mound Spring, suggests that nitrification and denitrification processes at Mound Spring are less active than at "Bison Pool" (see Figures 10 and 11).

Nitrogen stable isotope trends in "Bison Pool" and Mound Spring biomass support the differences in nitrogen fixation, nitrification, and denitrification potential and *in situ* activity observed between the two thermal features. Mound Spring biomass ^{15}N is depleted relative to atmospheric N_2 and most contextual samples, whereas "Bison Pool" biomass is enriched in ^{15}N relative to atmospheric N_2. This permits the interpretation that nitrogen fixation by Mound Spring microbial communities is repeatedly introducing isotopically light nitrogen (^{14}N) into the system. At "Bison Pool," this process is not genetically feasible, which is reflected in the positive $\delta^{15}N$ values of biomass there. Additionally, the greater potential for and activity of nitrification and denitrification at "Bison Pool" relative to Mound Spring is supported by downstream trends in $\delta^{15}N$. At "Bison Pool," incorporation of isotopically heavy nitrogen into biomass as a result of these processes is supported by a downstream enrichment in biomass ^{15}N (Figure 11). At Mound Spring, the downstream increase in $\delta^{15}N$ is damped. This can be explained not only by the repeated introduction of isotopically light nitrogen (^{14}N) through nitrogen fixation but also by less active nitrification and denitrification (Figure 11).

The overall contrast in nitrogen cycling between Mound Spring and "Bison Pool" may be a reflection of topographically-imposed nutrient variability between the two hot springs. "Bison Pool" is a more eutrophic (or periodically eutrophic) system than is Mound Spring. With greater inputs of organic and inorganic nitrogen that can be incorporated into biomass or used as a source of energy, perhaps nitrogen fixation at "Bison Pool" is unnecessary due to more frequent influx of fixed nitrogen sources, and nitrification and denitrification are important

processes for exploitation of the influx of nitrogen into the system. In contrast, Mound Spring is a more oligotrophic system, making nitrogen fixation a necessary process to provide sources of utile nitrogen to the local ecosystem.

To the author's knowledge, this study represents the first report of *in situ* transcription of several putative nitrogen cycle genes in chemosynthetic communities of terrestrial hydrothermal systems. *nifH* expression in chemosynthetic communities Mound Spring sets a new upper temperature limit (72.7°C) for nitrogen fixation in alkaline, terrestrial hydrothermal environments (Table VII, Appendix A). Furthermore, though the *narG* transcripts detected from the 62°C "Bison Pool" sediment sample in this study do not expand the upper temperature limit for nitrate reduction, it is the highest temperature reported for *in situ narG* transcription in a natural environment, to date (Table VII, Appendix A). *nirS* genes were detected in chemotrophic sediment communities at temperatures of up to 88°C, and *in situ* transcription of *nirS* was confirmed in SBCs at 64°C, also setting new upper temperature limits for *in situ* transcription of nitrite reductase in natural terrestrial hydrothermal communities (Table VII, Appendix A). *nirS*-sized cDNA amplicons also suggest expression of *nirS* in the 88°C sample; however, this is yet to be verified through cloning and sequencing.

Based on the results of this study, future work should include deeper sequencing of the 2010 Mound Spring and "Bison Pool" biomass samples in order to capture any target gene amplicons that may exist but were not detected in the sequencing efforts in this study. Additionally, culturing efforts and metagenome studies of the microorganisms in the Mound Spring and "Bison Pool" ecosystems (as well as those of other hydrothermal environments) are critical for expanding knowledge of the function and diversity of thermophilic organisms and for

aiding in design of new primer sets that are better suited for organisms in hydrothermal environments. Furthermore, since the results of this study highlighted the effects that topographically-driven nutrient influx differences can have on the Mound Spring and “Bison Pool” ecosystems, it is important for future studies to further address the effects of these differences on both short-term and long-term biogeochemical cycles. In particular, an investigation of biomass samples collected during the 2011 field season has the potential to allow comparison of short-term nitrogen cycling changes at Mound Spring and “Bison Pool” by investigating nitrogen cycle gene markers and transcripts and biomass isotopic composition of samples collected both immediately following the observed precipitation event, as well as of samples collected from “Bison Pool” over the course of several days following the event. This type of investigation would not only permit comparison of nitrogen-cycling process at Mound Spring and “Bison Pool” between 2010 and 2011, but it would also provide data for analyzing nutrient influx driven nitrogen cycling changes on a shorter (several day) time scale.

REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.

Amend, J.P. and Shock, E.L. (2000) Thermodynamics of amino acid synthesis in hydrothermal systems on the early Earth. In Perspectives in Amino Acid and Protein Geochemistry. Goodfriend, G.A., Collins, M.J., Fogel, M.L., Macko, S.A., and Wehmiller, J.F. (eds). Oxford University Press, pp. 23-40.

Amend, J.P. and Shock, E.L. (1998) Energetics of amino acid synthesis in hydrothermal ecosystems. *Science* **281**: 1659-1662.

Arvidson, R.E., Ruff, S.W., Morris, R.V., Ming, D.W., Crumpler, L.S., Yen, A.S., Squyres, S.W., Sullivan, R.J., Bell, J.F., Cabrol, N.A., Clark, B.C., Farrand, W.H., Gellert, R., Greenberger, R., Grant, J.A., Guinness, E.A., Herkenhoff, K.E., Hurowitz, J.A., Johnson, J.R., Klingelhöfer, G., Lewis, K.W., Li, R., McCoy, T.J., Moersch, J., McSween, H.Y., Murchie, S.L., Schmidt, M., Schröder, C., Wang, A., Wiseman, S., Madsen, M.B., Goetz, W., and McLennan, S.M. (2008) Spirit Mars Rover Mission to the Columbia Hills, Gusev Crater: mission overview and selected results from the Cumberland Ridge to Home Plate. *J Geophys Res-Planet* **113**: E12S33. doi: 10.1029/2008JE003183.

Barns, S.M., Delwiche, C.F., Palmer, J.D., and Pace, N.R. (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *P Natl Acad Sci USA* **93**: 9188-9193.

Barns, S.M., Fundyga, R.E., Jeffries, M.W., and Pace, N.R. (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *P Natl Acad Sci USA* **91**: 1609-1613.

Bishop, P.E., Jarlenski, D.M.L., and Hetherington, D.R. (1982) Expression of an alternative nitrogen fixation system in *Azotobacter vinelandii*. *J Bacteriol* **150**: 1244-1251.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H.W., and Stetter, K.O. (1997) *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113˚C. *Extremophiles* **1**: 14-21.

Bollmann, A., Bär-Gilissen, M.-J., and Laanbroek, H.J. (2002) Growth at low ammonium concentrations and starvation response as potential factors involved in niche differentiation among ammonia-oxidizing bacteria. *Appl Environ Microb* **68**: 4751-4757.

Boomer, S.M., Noll, K.L., Geesey, G.G., and Dutton, B.E. (2009) Formation of multilayered photosynthetic biofilms in an alkaline thermal spring in Yellowstone National Park, Wyoming. *Appl Environ Microb* **75**: 2464-2475.

Botero, L.M., D'Imperio, S., McDermott, T.R., Young, M., and Hassett, D.J. (2005) Poly(A) polymerase modification and reverse transcriptase PCR amplification of environmental RNA. *Appl Environ Microb* **71**: 1267-1275.

Boyd, E.S., Lange, R.K., Mitchell, A.C., Havig, J.R., Hamilton, T.L., Lafrenière, M.J., Shock, E.L., Peters, J.W., and Skidmore, M. (2011) Diversity, abundance, and potential activity of nitrifying and nitrate-reducing microbial assemblages in a subglacial ecosystem. *Appl Environ Microb* **77**: 4778-4787.

Braker, G. and Tiedje, J.M. (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Appl Environ Microb* **69**: 3476-3483.

Braker, G., Zhou, J.Z., Wu, L.Y., Devol., A.H. and Tiedje, J.M. (2000) Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific northwest marine sediment communities. *Appl Environ Microb* **66**: 2096-2104.

Brigle, K.E., Newton, W.E., and Dean, D.R. (1985) Complete nucleotide-sequence of the *Azotobacter vinelandii* nitrogenase structural gene cluster. *Gene* **37**: 37-44.

Brock, T.D. and Freeze, H. (1969) *Thermus aquaticus* gen. n. and sp.n., a nonsporulating extreme thermophile. *J Bacteriol* **98**: 289-297.

Bürgmann, H., Widmer, F., Sigler, W.V., and Zeyer, J. (2003) mRNA extraction and reverse transcription-PCR protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. *Appl Environ Microb* **69**: 1928-1935.

Burr, M.D., Botero, L.M., Young, M.J., Inskeep, W.P., and McDermott, T.R. (2005) Observations concerning nitrogen cycling in a Yellowstone thermal soil environment. In *Geothermal Biology and Geochemistry in Yellowstone National Park*. Inskeep, W.P. and McDermott, T.R. (eds). Bozeman: Thermal Biology Institute, Montana State University, pp. 171-182.

Cava, F., Zafra, O., da Costa, M.S., and Berenguer, J. (2008) The role of the nitrate respiration element of *Thermus thermophilus* in the control and activity of the denitrification apparatus. *Environ Microbiol* **10**: 522-533.

Cody, G.D. (2004) Transition metal sulphides and the origin of metabolism. *Ann Rev Earth Planet Sci* **32**: 569-599.

Cox, A., Shock, E.L., and Havig, J.R. (2011) The transition to microbial photosynthesis in hot spring ecosystems. *Chem Geol* **280**: 344-351.

Daesch, G. and Mortenson, L.E. (1972) Effect of ammonia on the synthesis and function of the N_2-fixing enzyme system in *Clostridium pasteurianum*. *J Bacteriol* **110**: 103-109.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M., and Stahl, D.A. (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* **10**: 810-818.

Delwiche, C.C. and Steyn, P.L. (1970) Nitrogen isotope fractionation in soils and microbial reactions. *Environ Sci Technol* **4**: 929-935.

Di, H.J., Cameron, K.C., Shen, J.-P., Winefield, C.S., O'Callaghan, M., Bowatte, S., and He, J.-Z. (2010) Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiol Ecol* **72**: 386-394.

Dixon, R. and Kahn, D. (2004) Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* **2**: 621-631.

Dodsworth, J.A., Cady, N.C., and Leigh, J.A. (2005) 2-oxoglutarate and the PII homologues $Nifl_1$ and $Nifl_2$ regulate nitrogenase activity in cell extracts of *Methanococcus maripaludis*. *Mol Microbiol* **56**: 1527-1538.

Dodsworth, J.A., Hungate, B., de la Torre, J., Jiang, H., and Hedlund, B.P. (2011a) Measuring nitrification, denitrification, and related biomarkers in continental geothermal ecosystems. *Methods Enzymol* **486**: 171-203.

Dodsworth, J.A., Hungate, B.A., and Hedlund, B.P. (2011b) Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea. *Environ Microbiol* **13**: 2371-2386.

Estep, M.L.F. (1984) Carbon and hydrogen isotopic compositions of algae and bacteria from hydrothermal environments, Yellowstone National Park. *Geochim Cosmochim Ac* **48**: 591-599.

Fouke, B.W., Bonheyo, G.T., Sanzenbacher, B., and Frias-Lopez, J. (2003) Partitioning of bacterial communities between travertine depositional facies at Mammoth Hot Springs, Yellowstone National Park, U.S.A. *Can J Earth Sci* **40**: 1531-1548.

Fournier, R.O. (2005) Geochemistry and dynamics of the Yellowstone National Park hydrothermal system. In *Geothermal Biology and Geochemistry in Yellowstone National Park*. Inskeep, W.P. and McDermott, T.R. (eds). Bozeman: Thermal Biology Institute, Montana State University, pp. 3-30.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., and Oakley, B.B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *P Natl Acad Sci USA* **102**: 14683-14688.

Gallon, J.R. (1981) The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms. *Trends Biochem Sci* **6**: 19-23.

Gotz, D., Banta, A., Beveridge, T.J., Rushdi, A.I., Simoneit, B.R., and Reysenbach, A.L. (2002) *Persephonella marina* gen. nov., sp. nov. and *Persephonella guaymasensis* sp. nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *Int J Syst Evol Micr* **52**: 1349-1359.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Roßnagel, P., Burggraf, S., Huber, H., and Stetter, K.O. (1996) *Ferroglobus placidus* gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe^{2+} at neutral pH under anoxic conditions. *Arch Microbiol* **166**: 308-314.

Halbleib, C.M. and Ludden, P.W. (2000) Regulation of biological nitrogen fixation. *J Nutr* **130**: 1081-1084.

Hall, J.R., Mitchell, K.R., Jackson-Weaver, O., Kooser, A.S., Cron, B.R., Crossey, L.J., and Takacs-Vesbach, C.D. (2008) Molecular characterization of the diversity and distribution of a thermal spring microbial community by using rRNA and metabolic genes. *Appl Environ Microb* **74**: 4910-4922.

Hallin, S. and Lindgren, P-E. (1999) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Appl Environ Microb* **65**: 1652-1657.

Hamilton, T.L., Boyd, E.S., and Peters, J.W. (2011a) Environmental constraints underpin the distribution and phylogenetic diversity of *nifH* in the Yellowstone geothermal complex. *Microbiol Ecol* **61**: 860-870.

Hamilton, T.L., Lange, R.K., Boyd, E.S., and Peters, J.W. (2011b) Biological nitrogen fixation in acidic high-temperature geothermal springs in Yellowstone National Park, Wyoming. *Environ Microbiol* **13**: 2204-2215.

Havig, J.R., Raymond, J., Meyer-Dombard, D.R., Zolotova, N., and Shock, E.L. (2011) Merging isotopes and community genomics in a siliceous sinter-depositing hot spring. *J Geophys Res* **116**: G001005.

Hennet, R.J.-C., Holm, N.G., and Engel, M.H. (1992) Abiotic synthesis of amino acids under hydrothermal conditions and the origin of life: a perpetual phenomenon? *Naturwissenschaften* **79**: 361-365.

Heylen, K., Vanparys, B., Gevers, D., Wittebolle, L., Boon, N., and De Vos, P. (2007) Nitric oxide reductase (*norB*) gene sequence analysis reveals discrepancies with nitrite reductase (*nir*) gene phylogeny in cultivated denitrifiers. *Environ Microbiol* **9**: 1072-1077.

Hirayama, H., Takai, K., Inagaki, F., Yamato, Y., Suzuki, M., Nealson, K.H., and Horikoshi, K. (2005) Bacterial community shift along a subsurface geothermal water stream in a Japanese gold mine. *Extremophiles* **9**: 169-184.

Hoehler, T.M., Alperin, M.J., Albert, D.B., and Martens, C.S. (2001) Apparent minimum free energy requirements for methanogenic Archaea and sulfate-reducing bacteria in an anoxic marine sediment. *FEMS Microbiol Ecol* **38**: 33-41.

Holl, C.M. and Montoya, J.P. (2005) Interactions between nitrate uptake and nitrogen fixation in continuous cultures of the marine diazotroph *Trichodesmium* (Cyanobacteria). *J Phycol* **41**: 1178-1183.

Hollocher, T.C. and Kristjánsson, J.K. (1992) Thermophilic denitrifying bacteria: a survey of hot springs in Southwestern Iceland. *FEMS Microbiol Lett* **101**: 113-119.

Holloway, J.M., Nordstrom, D.K., Böhlke, J.K., McCleskey, R.B., and Ball, J.W. (2011) Ammonium in thermal waters of Yellowstone National Park: processes affecting speciation and isotope fractionation. *Geochim Cosmochim Ac* **75**: 4611-4636.

Hoover, T. (2000) Control of nitrogen fixation genes in *Klebsiella pneumonia*. In *Prokaryotic Nitrogen Fixation*. Triplett, E.W. (ed). Norfolk, England: Horizon Scientific Press, pp. 131-147.

Huber, C. and Wächtershäuser, G. (2003) Primordial reductive amination revisited. *Tetrahedron Lett* **44**: 1695-1697.

Huber, H., Diller, S., Horn, C., and Rachel, R. (2002) *Thermovibrio ruber* gen. nov., sp. nov., an extremely thermophilic, chemolithoautotrophic, nitrate-reducing bacterium that forms a deep branch within the phylum Aquificae. *Int J Syst Evol Micr* **52**: 1859-1865.

Huber, T., Faulkner, G., and Hugenholtz, P. (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317-2319.

Hugenholtz, P., Pitulle, C., Hershberger, K.L., and Pace, N.R. (1998) Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* **180**: 366-376.

Inskeep, W.P. and McDermott, T.R. (2005) Geomicrobiology of acid-sulfate-chloride springs in Yellowstone National Park. In *Geothermal Biology and Geochemistry in Yellowstone National Park*. Inskeep, W.P. and McDermott, T.R. (eds). Bozeman: Thermal Biology Institute, Montana State University, pp. 143-162.

Jiang, H., Huang, Q., Dong, H., Wang, P., Wang, F., Li, W., and Zhang, C. (2010) RNA-based investigation of ammonia-oxidizing archaea in hot springs of Yunnan Province, China. *Appl Environ Microb* **73**: 4538-4541.

Junier, P., Molina, V., Dorador, C., Hadas, O., Kim, O.-S., Junier, T., Witzel, K.-P., and Imhoff, J.F. (2010) Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. *Appl Microbiol Biot* **85**: 425-440.

Konn, C., Testemale, D., Querellou, J., Holm, N.G. and Charlou, J.L. (2009) New insight into the contributions of thermogenic processes and biogenic sources to the generation of organic compounds in hydrothermal fluids. *Geobiology* **9**: 79-93.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543-546.

Kuenen, J.G. (2008) Annamox bacteria: from discovery to application. *Nat Rev Microbiol* **6**: 320-326.

Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt, E. and Goodfellow, M. (eds). Chichester, UK: John Wiley and Sons, pp. 115-175.

Lane, N., Allen, J.F., and Martin, W. (2010) How did LUCA make a living? Chemiosmosis in the origin of life. *BioEssays* **32**: 271-280.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

LaRowe, D.E. and Regnier, P. (2008) Thermodynamic potential for the abiotic synthesis of adenine, cytosine, guanine, thymine, uracil, ribose, and deoxyribose in hydrothermal systems. *Origins Life Evol B* **38**: 383-397.

Lebedeva, E.V., Off, S., Zumbrägel, S., Kruse, M., Shagzhina, A., Lücker, S., Maixner, F., Lipski, A., Daims, H., and Spieck, E. (2011) Isolation and characterization of a moderately thermophilic nitrite-oxidizing bacterium from a geothermal spring. *FEMS Microbiol Ecol* **75**: 195-204.

Loiacono, S.T., Meyer-Dombard, D.R., Havig, J.R., Poret-Peterson, A.T., Hartnett, H.E., and Shock, E.L. (2012) Evidence for high-temperature *in situ nifH* transcription in an alkaline hot spring of Lower Geyser Basin, Yellowstone National Park. *Environ Microbiol* **14**: 1272-1283.

Lotta, M., Beatriz, D., Ingvild, W., Zheng, W.W., El-Shehawy, R., and Rasmussen, U. (2009) Diazotrophic diversity, *nifH* gene expression and nitrogenase activity in a rice paddy field in Fujian, China. *Plant Soil* **325**: 207-218.

Macko, S.A., Fogel, M.L., Hare, P.E., and Hoering, T.C. (1987) Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chem Geol* **65**: 79-92.

Mariotti, A., Germon, J.C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., and Tardieux, P. (1981) Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. *Plant Soil* **62**: 413-430.

Marshall, W.L. (1994) Hydrothermal synthesis of amino acids. *Geochim Cosmochim Ac* **58**: 2099-2106.

Martin, W. and Russell, M.J. (2003) On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. *Philos T Roy Soc B* **358**: 59-83.

McCollom, T.M., Ritter, G., and Simoneit, B.R. (1999) Lipid synthesis under hydrothermal conditions by Fischer-Tropsch-type reactions. *Origins Life Evol B* **29**: 153-166.

Mehta, M.P. and Baross, J.A. (2006) Nitrogen fixation at 92°C by a hydrothermal vent archaeon. *Science* **314**: 1783-1786.

Mehta, M.P., Butterfield, D.A., and Baross, J.A. (2003) Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. *Appl Environ Microb* **69**: 960-970.

Meyer-Dombard, D.R., Shock, E.L., and Amend, J.P. (2005) Archaeal and bacterial communities in geochemically diverse hot springs of Yellowstone National Park, USA. *Geobiology* **3**: 211-227.

Meyer-Dombard, D.R., Swingley, W., Raymond, J., Havig, J., Shock, E.L., and Summons, R.E. (2011) Hydrothermal ecotones and streamer biofilm communities in the Lower Geyser Basin, Yellowstone National Park. *Environ Microbiol* **13**: 2216-2231.

Miroshnichenko, M.L., Kostrikina, N.A., Chernyh, N.A., Pimenov, N.V., Tourova, T.P., Antipov, A.N., Spring, S., Stackebrandt, E., and Bonch-Osmolovskaya, E.A. (2003) *Caldithrix abyssi* gen. nov., sp. Nov., a nitrate-reducing, thermophilic, anaerobic bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent, represents a novel bacterial lineage. *Int J Syst Evol Micr* **53**: 323-329.

Mishima, M., Iwata, K., Nara, K., Matsui, T., Shigeno, T., and Omori, T. (2009) Cultivation characteristics of denitrification by thermophilic *Geobacillus* sp. Strain TDN01. *J Gen Appl Microbiol* **55**: 81-86.

Moisander, P.H., Morrison, A.E., Ward, B.B., Jenkins, B.D., and Zehr, J.P. (2007) Spatial-temporal variability in diazotrophic assemblages in Chesapeake Bay using an oligonucleotide *nifH* microarray. *Environ Microbiol* **9**: 1823-1835.

Mulder, A., van de Graaf, A.A., Robertson, L.A., and Kuenen, J.G. (1995) Anaerobic ammonium oxidation discovered in denitrifying fluidized bed reactor. *FEMS Microbiol Ecol* **16**: 177-184.

Mulholland, M.R., Ohki, K., and Capone, D.G. (2001) Nutrient controls on nitrogen uptake and metabolism by natural populations and cultures of *Trichodesmium* (Cyanobacteria). *J Phycol* **37**: 1001-1009.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1992) Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction, 1986. *Biotechnol* **24**: 17-27.

Myrold, D.D. (1998) Transformations of nitrogen. In *Principles and Applications of Soil Microbiology*. Upper Saddle River, New Jersy: Prentice Hall, pp. 259-294.

Nakagawa, S., Nakamura, S., Inagaki, F., Takai, K., Shirai, N., and Sako, Y. (2004) *Hydrogenivirga caldilitoris* gen. nov., sp. nov., a novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium from a coastal hydrothermal field. *Int J Syst Evol Micr* **54**: 2079-2084.

National Park Service: U.S. Department of the Interior (2010) Yellowstone National Park. www.nps.gov.

Norton, J.M, Alzerreca, J.J., Suwa, Y., and Klotz, M.G. (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Arch Microbiol* **177**: 139-149.

Normand, P. and Bousquet, J. (1989) Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen-fixing microorganisms. *J Mol Evol* **29**: 436-447.

Offre, P., Prosser, J.I., Graeme, W.N. (2009) Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. *FEMS Microbiol Ecol* **70**: 99-108.

Pearson, A., Pi, Y., Zhao, W., Li, W., Li, Y., Inskeep, W., Perevalova, A., Romanek, C., Li, S., and Zhang, C.L. (2008) Factors controlling the distribution of archaeal tetraethers in terrestrial hot springs. *Appl Environ Microb* **74**: 3523-3532.

Philippot, L. (2002) Denitrifying genes in bacterial and archaeal genomes. *Biochim et Biophysica Acta* **1577**: 355-376.

Philippot, L. (2005) Denitrification in pathogenic bacteria: for better or worst? *Trends Microbiol* **13**: 191-192.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., Germon, J.C. (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. *Appl Environ Microb* **68**: 6121-6128.

Pires, A.L., Albuquerque, L., Tiago, I., Nobre, M.F., Empadinhas, N., Veríssimo, A., da Costa, M.S. (2005) *Meiothermus timidus* sp. nov., a new slightly thermophilic yellow-pigmented species. *FEMS Microbiol Lett* **245**: 39-45.

Poli, A., Romano, I., Cordella, P., Orlando, P., Nicolaus, B., and Berrini, C.C. (2009) *Anoxybacillus thermarum* sp. nov., a novel thermophilic bacterium isolated from thermal mud in Euganean hot springs, Abano Terme, Italy. *Extremophiles* **13**: 867-874.

Poly, F., Monrozier, L.J., and Bally, R. (2001) Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**: 95-103.

Priemé, A., Braker, G., and Tiedje, J.M. (2002) Diversity of nitrate reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Appl Environ Microbiol* **68**: 1893-1900.

Rabouille, S., Staal, M., Stal, L.J., and Soetaert, K. (2005) Modeling the dynamic regulation of nitrogen fixation in the cyanobacterium *Trichodesmium* sp. *Appl Environ Microb* **72**: 3217-3227.

Rasmussen, B. (2000) Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit. *Nature* **405**: 676-679.

Reigstad, L.J., Richter, A., Daims, H., Urich, T., Schwark, L., and Schleper, C. (2008) Nitrification in terrestrial hot springs of Iceland and Kamchatka. *FEMS Microbiol Ecol* **64**: 167-174.

Reysenbach, A.-L., Blanta, A., Civello, S., Daly, J., Mitchel, K., Lalonde, S., Konhauser, K. Rodman, A., Rusterholtz, K., and Takacs-Vesbach, C. (2005) Aquificales in Yellowstone National Park. In *Geothermal Biology and Geochemistry in Yellowstone National Park*. Inskeep, W.P. and McDermott, T.R. (eds). Bozeman: Thermal Biology Institute, Montana State University, pp. 129-142.

Rotthauwe, J.H., Witzel, K.P., and Liesack, W. (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microb* **63**: 4704-4712.

Rosado, A.S., Duarte, G.F., Seldin, L., and van Elsas, J.D. (1998) Genetic diversity of *nifH* gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR-amplified gene fragments. *Appl Environ Microb* **64**: 2770-2779.

Ruff, S.W., Farmer, J.D., Calvin, W.M, Herkenhoff, K.E., Johnson, J.R., Morris, R.V., Rice, M.S., Arvidson, R.E., Bell, J.F., and Christensen, P.R. (2011) Characteristics, distribution, origin, and significance of opaline silica observed by the Spirit rover in Gusev crater, Mars. *J Geophys Res-Planet* **116**: E00F23. doi:10.1029/2010JE003767.

Russell, M.J. and Arndt, N.T. (2005) Geodynamic and metabolic cycles in the Hadean. *Biogeosciences* **2**: 97-111.

Russell, M.J., Daniel, R.M., and Hall, A.J (1993) On the emergence of life via catalytic iron-sulphide membranes. *Terra Nova* **5**: 343-347.

Schink, B. (1997) Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol R* **61**: 262-280.

Schleper, C. (2010) Ammonia oxidation: different niches for bacteria and archaea? *ISME J* **4**: 1092-1094.

Setchell, W.A. (1903) The upper temperature limits of life. *Science* **17**: 934-937.

Severin, I. and Stal, L.J. (2010) *NifH* expression by five groups of phototrophs compared with nitrogenase activity in coastal microbial mats. *FEMS Microbiol Ecol* **73**: 55-67.

Shah, V.K., Davis, L.C., and Winston, J.E. (1972) Nitrogenase. I. Repression and derepression of the iron-molybdenum and iron proteins of nitrogenase in *Azotobacter vinelandii*. *BBA-Bioenergetics* **256**: 498-511.

Shock, E.L., Holland, M., Meyer-Dombard, D.R., and Amend, J.P. (2005) Geochemical sources of energy for microbial metabolism in hydrothermal ecosystems: Obsidian Pool, Yellowstone National Park. In *Geothermal Biology and Geochemistry in Yellowstone National Park*. Inskeep, W.P. and McDermott, T.R. (eds). Bozeman: Thermal Biology Institute, Montana State University, pp. 95-109.

Shock, E.L., Holland, M., Meyer-Dombard, D., Amend, J.P., Osburn, G.R., and Fischer, T.P. (2010) Quantifying inorganic sources of geochemical energy in hydrothermal ecosystems, Yellowstone National Park, USA. *Geochim Cosmochim Ac* **74**: 4005-4043.

Shock, E.L., McCollom, T.M., and Schulte, M.D. (1998) The emergence of metabolism from within hydrothermal systems. In *Thermophiles: The Keys to Molecular Evolution and the Origin of Life*. Wiegel, J. and Adams, M.W.W. (eds). London: Taylor & Francis, pp. 59-76.

Spear, J.R., Barton, H.A., Robertson, C.E., Francis, C.A., and Pace, N.R. (2007) Microbial community biofabrics in a geothermal mine adit. *Appl Environ Microb* **73**: 6172-6180.

Spear, J.R., Walker, J.J., McCollom, T.M., and Pace, N.R. (2005) Hydrogen and bioenergetics in the Yellowstone geothermal ecosystem. *P Natl Acad Sci USA* **102**: 2555-2560.

Squyres, S.W., Arvidson, R.E., Ruff, S., Gellert, R., Morris, R.V., Ming, D.W., Crumpler, L., Farmer, J.D., Des Marais, D.J., Yen, A., McLennan, S.M., Calvin, W., Bell III, J.F., Clark, B.C., Wang, A., McCoy, T.J., Schmidt, M.E., and de Souza Jr., P.A. (2008) Detection of silica-rich deposits on Mars. *Science* **320**: 1063-1067.

Steunou, A.S., Bhaya, D., Bateson, M.M., Melendrez, M.C., Ward, D.M., Brecht, E., Peters, J.W., Kühl, M., and Grossman, A.R. (2006) *In situ* analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. *P Natl Acad Sci USA* **103**: 2398-2403.

Steunou, A.S., Jensen, S.I., Brecht, E., Becraft, E.D., Bateson, M.M., Kilian, O., Bhaya, D., Ward, D.M., Peters, J.W., Grossman, A.R., and Kühl, M. (2008) Regulation of *nif* gene expression and the energetics of N_2 fixation over the diel cycle in a hot spring microbial mat. *ISME J* **2**: 364-378.

Swingley, W.D., Meyer-Dombard, D.R., Shock, E.L., Alsop, E.B., Falenski, H.D., Havig, J.R., and Raymond, J. (2012) Coordinating environmental genomics and geochemistry reveals metabolic transitions in a hot spring ecosystem. *PLoS ONE* **7(6)**: e38108. doi:10.1371/journal.pone.0038108.

Takei, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003) *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. *Int J Syst Evol Micr* **53**: 823-827.

Throbäck, I.N., Enwall, K., Jarvis, Å., and Hallin, S. (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol Ecol* **49**: 401-417.

Tourna, M., Freitag, T.E., Nicol, G.W., and Prosser, J.I. (2008) Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environ Microbiol* **10**: 1357-1364.

Vaughn, S.A. and Burgess, B.K. (1989) Nitrite, a new substance for nitrogenase. *Biochemistry-US* **28**: 419-424.

Vitousek, P.M. and Howarth, R.W. (1991) Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry* **13**: 87-115.

Völkl, P., Huber, R., Drobner, E., Rachel, R., Burggraf, S., Trincone, A., and Stetter, K.O. (1993) *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. *Appl Environ Microb* **59**: 2918-2926.

Wang, S.F., Xiao, X., Jiang, L.J., Peng, X.T., Zhou, H.Y., Meng, J., Wang, F.P. (2009) Diversity and abundance of ammonia-oxidizing archaea in hydrothermal vent chimneys of the Juan de Fuca Ridge. *Appl Environ Microb* **75**: 4216-4220.

Windman, T. Zolotova, N., Schwandner, F., and Shock, E.L. (2007) Formate as an energy source for microbial metabolism in chemosynthetic zones of hydrothermal ecosystems. *Astrobiology* **7**: 873-890.

Yagi, J.M., Suflita, J.M., Gieg, L.M., DeRito, C.M., Jeon, C.-O., and Madsen, E.L. (2010) Subsurface cycling of nitrogen and anaerobic aromatic hydrocarbon biodegradation revealed by nucleic acid and metabolic biomarkers. *Appl Environ Microb* **76**: 3124-3134.

Zani, S., Mellon, M.T., Collier, J.L., and Zehr, J.P. (2000) Expression of *nifH* genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. *Appl Environ Microb* **66**: 3119-3124.

Zehr, J.P., Jenkins, B.D., Short, S.M., and Steward, G.F. (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ Microbiol* **5**: 539-554.

Zehr, J.P. and McReynolds, L.A. (1989) Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microb* **55**: 2522-2526.

Zhang, C.L., Ye, Q., Huang, Z., Li, W., Chen, J., Song, Z., Zhao, W. Bagwell, C., Inskeep, W.P., Ross, C., Gao, L., Wiegel, J., Romanek, C.S., Shock, E.L., and Hedlund, B.P. (2008) Global occurrence of archaeal *amoA* genes in terrestrial hot springs. *Appl Environl Microb* **74**: 6417-6426.

Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol R* **61**: 533-616.

Zumft, W.G. and Kroneck, P.M. (2007) Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by Bacteria and Archaea. *Adv Microb Physiol* **52**: 107-227.

APPENDICES

APPENDIX A

TABLE VII: CURRENT UPPER TEMPERATURE LIMITS FOR SELECT NITROGEN CYCLE PROCESSES

Process	Temp.	Environment	Evidence	Organism	Reference
Nitrogen Fixation	92°C	Marine hydrothermal vent	Cultured isolate	*Methanocaldococcus* sp.	Mehta and Baross
Nitrogen Fixation	82°C; 89°C	(Acidic) terrestrial hydrothermal sediments	Acetylene reduction; PCR amplification of *nifH* from environmental DNA	Uncultured organisms	Hamilton *et al.* (2011a,b)
Nitrogen Fixation	73°C	(Alkaline) terrestrial hydrothermal sediments	*In situ nifH* transcription	Uncultured organisms	**THIS STUDY**; Loiacono *et al*., 2012
Nitrification	74°C	Marine hydrothermal vent	Cultured isolate	*Candidatus Nitrosocaldus yellowstonii*	de la Torre *et al.* (2008)
Nitrification	97°C	Terrestrial hydrothermal sediments	*amoA* gene and crenarchaeol lipid	Uncultured archaea	Reigstad *et al.* (2008)
Nitrification	94°C	Terrestrial hydrothermal sediments	*In situ amoA* transcription	Uncultured archaea	Jiang *et al.* (2010)
Nitrification	62°C	Subsurface gold mine microbial mat	*In situ amoA* transcription	Uncultured bacterium	Hirayama *et al*. (2005)
Nitrate Reduction	113°C	Marine hydrothermal vent	Cultured isolate	*Pyrolobus fumarii*	Blöchl *et al*. (1997)
Nitrate Reduction	82°C	Terrestrial hydrothermal sediments	Acetylene reduction assays; *narG* gene copies	Uncultured organisms	Dodsworth *et al.* (2011); Hedlund *et al*., (2011)
Nitrate Reduction	62˚C	Terrestrial hydrothermal sediments	*In situ narG* transcription	Uncultured organisms	**THIS STUDY**
Nitrite Reduction	64˚C (88˚C)	Terrestrial hydrothermal SBCs	*In situ nirS* transcription	Uncultured organisms	**THIS STUDY**

APPENDIX B

TABLE VIII: NITROGEN ISOTOPE ANALYTICAL RESULTS FOR CONTEXTUAL SAMPLES

Year	Contextual Sample	Sample ID	% N (by dw)	SD %N (±)	$\delta^{15}N$ (‰)	SD $\delta^{15}N$ (±)
2007	Bison Fur	JRH070710T	15.8	0.149	4.51	0.31
2007	Bison Excrement	JRH070710V	1.87	0.184	3.19	0.13
2007	Insects (in outflow)	JRH070710U	8.02	0.606	3.11	0.24
2007	Grasses (living)	JRH070710W	2.33	0.025	0.29	0.30
2007	Sedges (living)	JRH070710X	1.90	0.055	-1.35	0.35
2006	Sinter Soil 1	JRH060812N	0.17	0.002	3.19	0.14
2006	Sinter Soil 2	JRH060812R	0.16	0.005	1.48	0.10
2006	Sinter Soil 3	JRH060812S	0.77	0.042	1.42	0.05
2006	Meadow Soil 1	JRH060807U	0.09	0.002	1.90	0.11
2010	Meadow Soil 2	JRH100801X	0.48	0.004	1.22	0.13
2010	Meadow Soil 3	JRH100801Y	0.06	0.012	2.35	0.28
2010	Meadow Soil 4	JRH100801Z	0.06	0.014	2.60	0.16
2010	Meadow Soil 5	JRH100801A	0.02	0.001	3.35	0.19

APPENDIX C

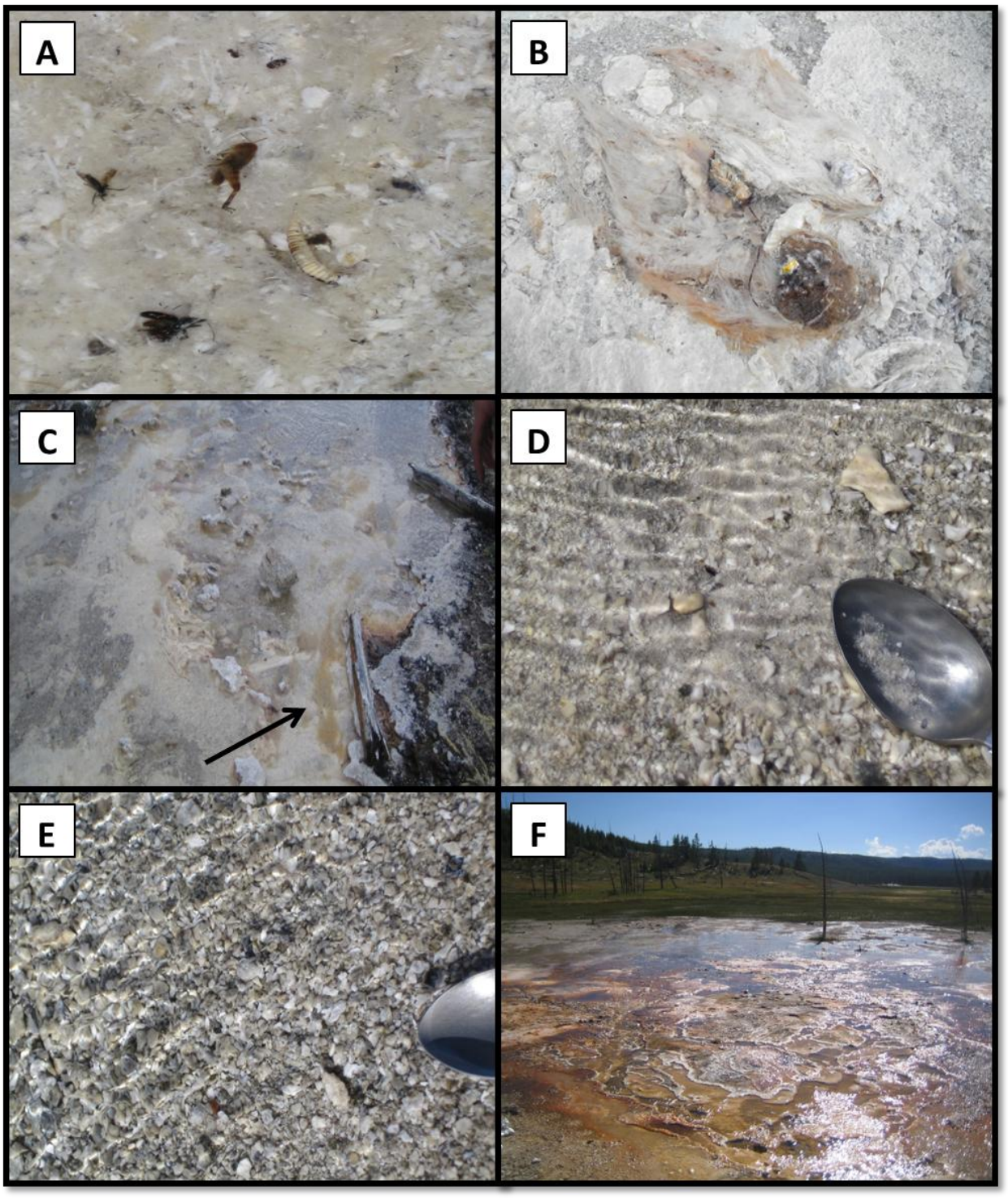


Figure 12. Additional examples of biomass at Mound Spring and "Bison Pool." A: Fluffy SBCs at "Bison Pool" (note prevalence of insects in the outflow); B: SBCs clinging to a mouse carcass in the "Bison Pool" outflow; C: SBCs (see arrow) attached to sinter rim of "Bison Pool" outflow channel; D and E: sinter sediment in Mound Spring chemosynthetic zone; F: extensive photosynthetic mat and branching outflow at Mound Spring.

APPENDIX C (continued)

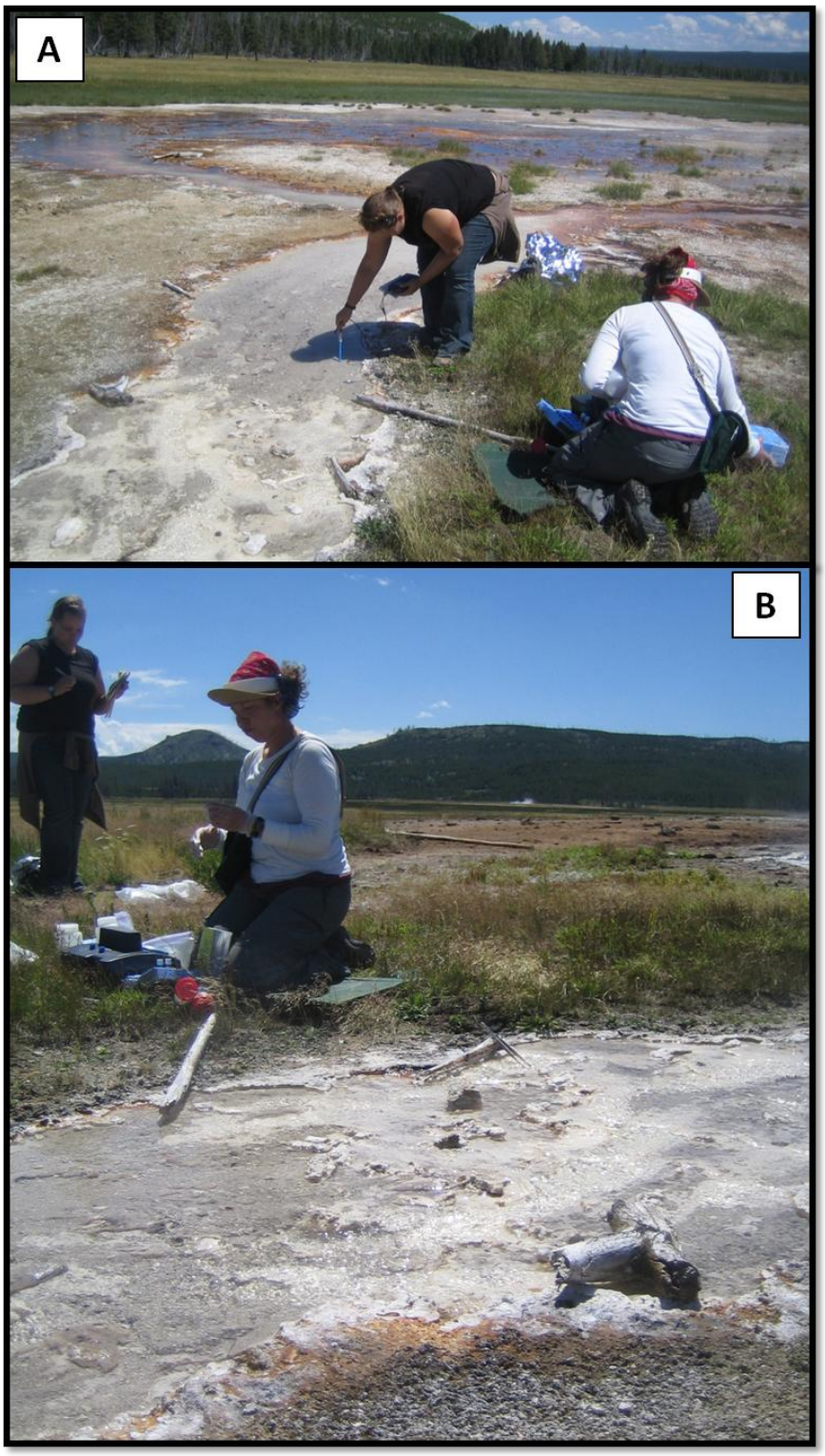
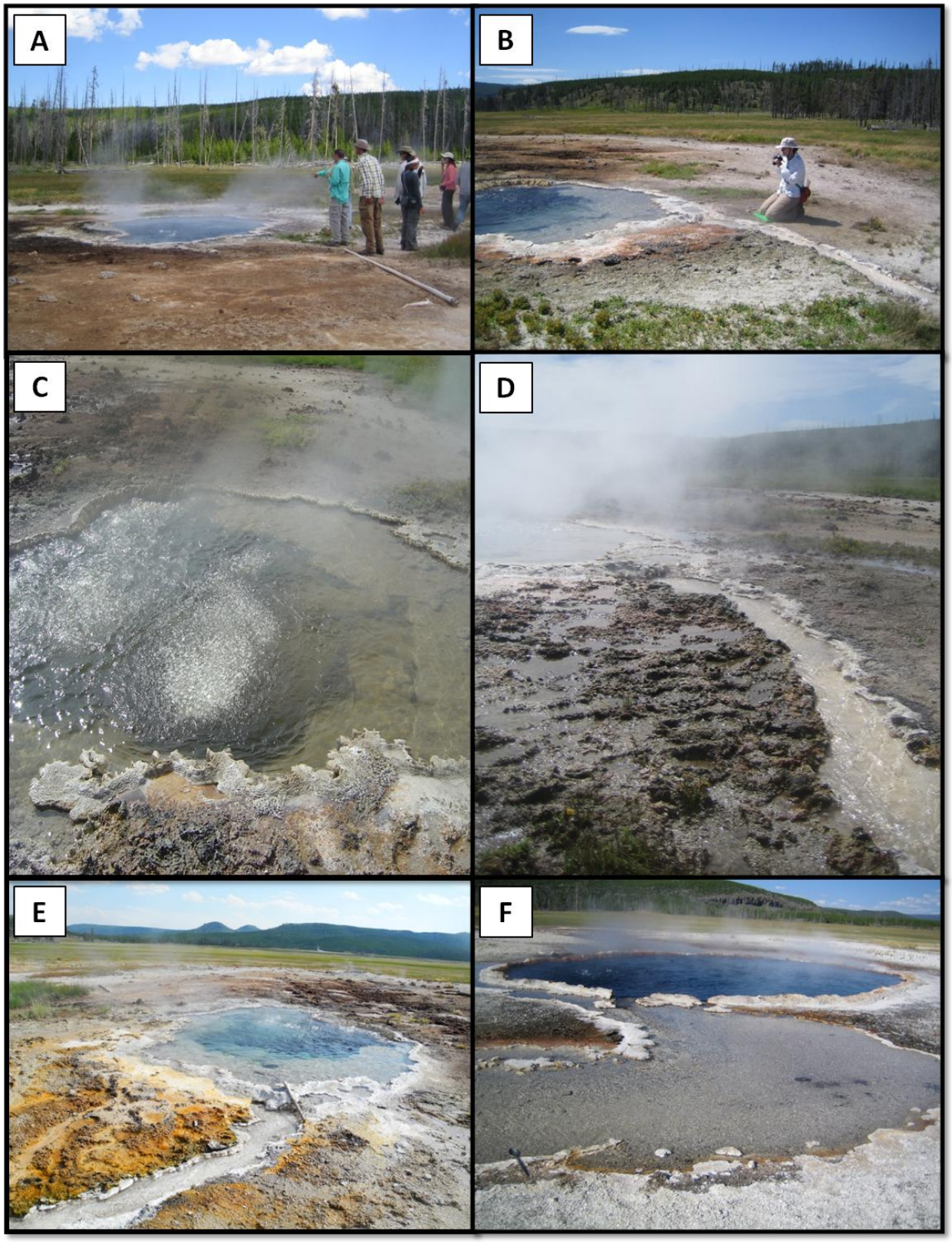


Figure 13. Geochemical sampling at "Bison Pool." A. Collecting pH and temperature measurements; B: Analyzing fluid chemistry using portable field spectrophotometers and reagents.

APPENDIX D

Figure 14. "Bison Pool" and Mound Spring source and outflow images, 2010 and 2011 sampling seasons. A and B: "Bison Pool" during August 2010 sampling season; note the brown color of the surrounding soil and bison excrement near the source in both images. C - E: "Bison Pool" during July 2011 sampling season; C and D are images taken shortly after a heavy precipitation event; note the change in source pool color relative to 2010 (from deep blue in 2010 to brown in 2011) as a result of exogenous organic input during the precipitation event; the source fluid returned to blue in color within a few days following the event (image E, taken four days later). F: Mound Spring source pool during August 2010 sampling season; note the deep blue color of the source fluid, very similar to that of "Bison Pool" in 2010 (images A and B) and 2011 after "recovery" from the precipitation event (image E); the Mound Spring source and outflow appeared this same blue color following the July 2011 precipitation event referenced above (not pictured).

APPENDIX D (continued)



APPENDIX D (continued)

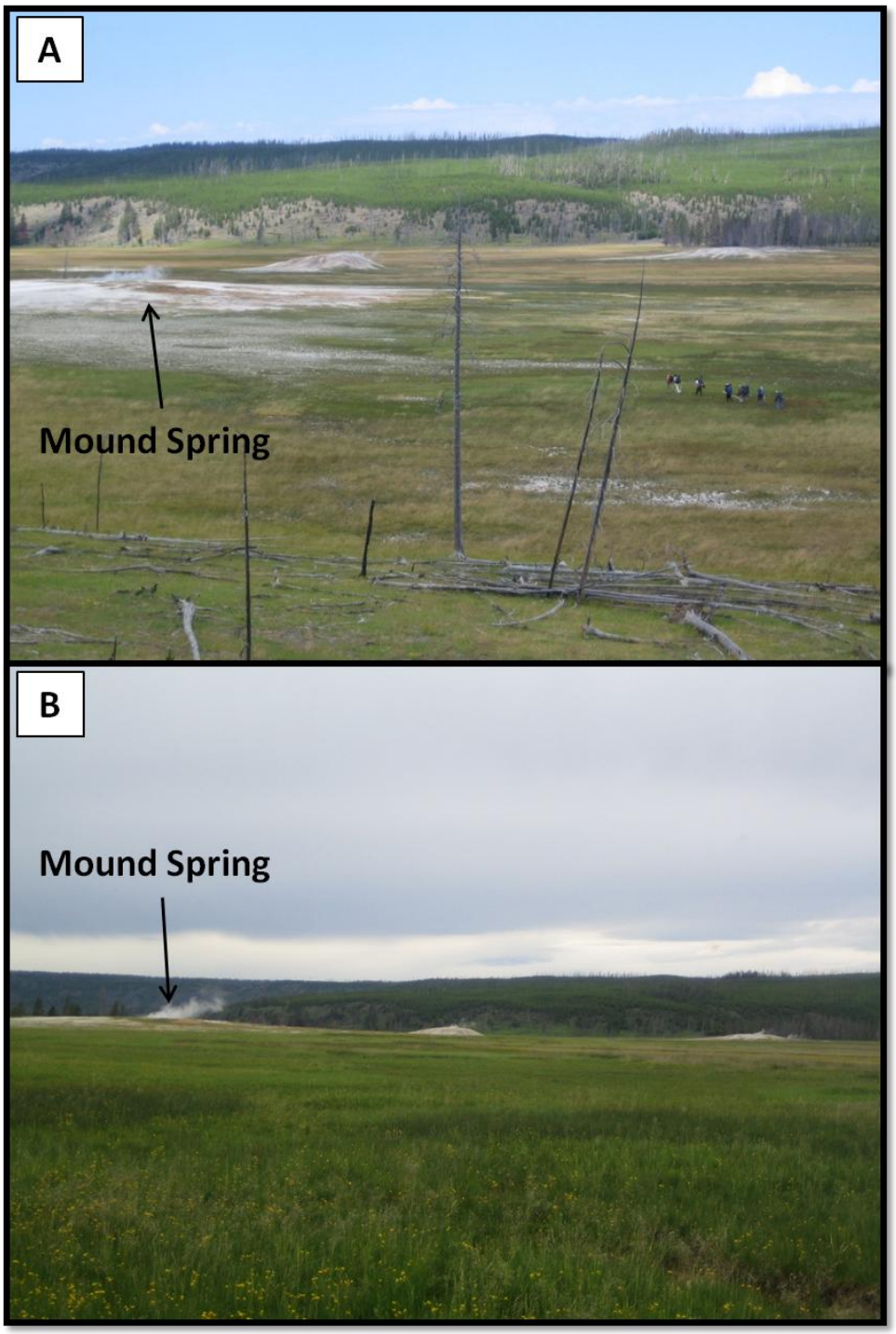


Figure 15. Additional views of the Sentinel Meadows Group thermal features. Note the topographic position of Mound Spring, which is situated atop a low, wide, sinter mound. "Bison Pool" is in the background of both images, but is not easily visible since it is topographically level with the surrounding meadow and blocked from view by other mound-forming thermal features in the foreground.

APPENDIX E

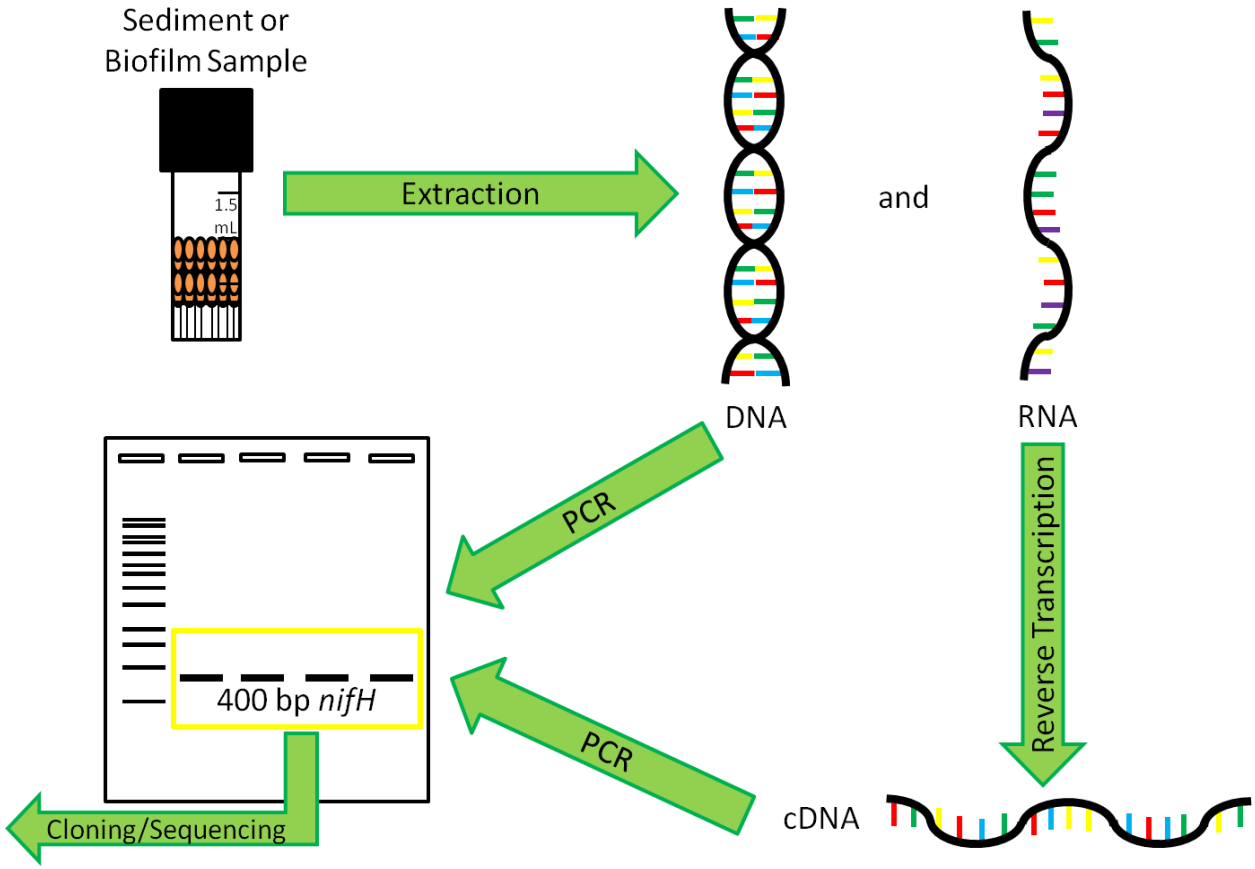


Figure 16. Molecular methods flowchart.

APPENDIX F

TABLE IX. SEQUENCING SUCCESS RATE FOR PUTATIVE NITROGEN CYCLE CLONES

	amoA **(archaeal)**	***amoA*** **(bacterial)**	***narG***	***nirK***	***nirS***	***cnorB***	***nosZ***
Total Clones Sequenced	16	282	48	8	120	88	112
Total Positive GenBank Results	11	0	1	0	20	0	0
Percentage of Clones with Positive Results	69%	0%	2%	0%	17%	0%	0%