GEOMICROBIOLOGICAL DESCRIPTION OF TWO CONTEMPORARY HYDROTHERMAL POOLS IN UZON CALDERA, KAMCHATKA, RUSSIA, AS MODELS FOR SULFUR BIOGEOCHEMISTRY

by

ELIZABETH ADRIENNE BURGESS

(Under the Direction of Juergen Wiegel)

ABSTRACT

The combination of geological activity and geographic isolation make Uzon Caldera, Kamchatka, Russia, an ideal location for geomicrobiological research. Two hydrothermal pools, Arkashin Shurf (Arkashin) and Zavarzin Spring (Zavarzin), were selected for geochemical and microbiological characterization over multiple years and scales. Arkashin has high arsenic concentrations relative to Zavarzin, which has abundant elemental sulfur. Grab samples were analyzed from a geomicrobiological perspective, to describe community structure in each pool based on sequence, lipid and stable isotope data. Based on sequence analysis and lipid distribution, Arkashin was inhabited by *Hydrogenobaculum*-related primary producers. Other community members included *Desulfurella*-, "*Sphingobacteria*"- and *Variovorax*-related microorganisms. Zavarzin was dominated by *Chloroflexus*, and heterotrophic microorganisms, including some *Crenarchaeota*. The community in Zavarzin was more diverse than in Arkashin. Additionally, nearly 20% of the sequences from Arkashin and over 50% of the sequences from Zavarzin represented uncultured and unclassified microorganisms. Core sample analyses indicated that in each pool the microbiology and geochemistry changed with depth over visible changes in color and texture. Surface sub-samples were similar to the grab samples. Autotrophic surface communities were replaced with microorganisms dependent on heterotrophic inputs or reduced hydrothermal carbon as depth increased. In Arkashin, variation in color of strata was associated with varying concentrations of As

and S. The highest As and S concentrations were associated with the lowest concentrations of phospholipid fatty acids (PLFA). In Zavarzin, the highest sulfur and PLFA concentrations were associated with fine-textured surface samples. The patterns observed indicate the biomass and composition of microbial communities can shift considerably in association with macroscopically visible changes in geochemical conditions. The described geomicrobiological data represent sulfur biogeochemistry under distinct geochemical conditions. As- and S-concentrations are linked significantly in Arkashin. The microorganisms in Arkashin play a role in As-S cycling, in part through sulfate-reduction. In Zavarzin, elemental sulfur is abundant and sulfide from sulfate-reduction is a minor component of total sulfur concentrations. Cataloging the diversity and distribution of microorganisms in contemporary thermal environments and elucidating some of the variations that occur with depth at the edges of hydrothermal pools add to knowledge about the intersection of geochemistry and microbiology.

INDEX WORDS: arsenic, thermophiles, microecology

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DEDICATION

Dedication is what it has taken to get to this point. Not just my own dedication but also the dedication of all those in my life who have shared the goal. I know my husband, Jeremy Rogers, will be glad to call me doctor at last. My parents, Rich and Carole Burgess, have worked hard to get me here. My father especially has been a constant source of encouragement and advice. I would also like to dedicate this dissertation to those who have always been dedicated to me just as I am; Rafinesque, Penelope, Minazuki, Jack, and especially Okefenokee.

To represent my aspirations with this work, here is a short poem:

Sublimation

In my quest to exalt sulfur,
as it precipitates from hot and watery depths,
may I also be purified
from vapor into a solid form.

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LITERATURE REVIEW:

THERMAL ENVIRONMENTS AND BIODIVERSITY [1]

[1]E.A. Burgess, I.D. Wagner, and J. Wiegel, J. 2007. Ch. 2, pp 13-29 in *Physiology and Biochemsitry of Extremophiles*. Gerday, C. and Glansdorff, N. eds. ASM Press, Washington, D.C. reprinted with permission;

Formatting, including Figure and Table numbers have been modified for the current document.

INTRODUCTION

Biodiversity is of interest as it relates to stability and productivity in ecosystems and sustainability of ecosystem functions and is measured generally as species or genetic diversity. When E. O. Wilson, who coined the term biodiversity, wrote *The Diversity of Life*, there were 4,800 species described in the "kingdom" *Monera* (Wilson, 1992). Presently, there are ~7,600 validly published prokaryotic (domain *Bacteria*/*Archaea*) species (http://www.bacterio.cict.fr/number.html#total, March 2006) and ~210,000 aligned 16S rRNA sequences in the Ribosomal Database Project (RDP) database (http://rdp.cme.msu.edu/misc/news.jsp#mar0206, March 2006), and every month ~30–50 more are added. Wilson (1992) estimated the number of known species of all microorganisms to be 1.4 million. Among prokaryotes alone, as many as $1x10^9$ species have been hypothesized to exist (Dykhuizen, 1998).

This vast diversity is due, in part, to prokaryotes' ability to exploit extreme environments, such as environments with elevated temperatures, as high as 121°C, referred to as thermal, thermobiotic, or hot environments. Each discovery of environments at high temperatures has opened a new reservoir of biodiversity. Prokaryotes take center stage at high temperatures, although novel eukaryotic microorganisms have also been discovered exploiting the warm edges of thermal environments (Desbruyeres and Laubier**,** 1980; Jones, 1981). Considering the wealth of data about and the amazing diversity of thermophilic prokaryotes, most of the discussion to follow will focus on *Bacteria* and *Archaea*.

Measuring the biodiversity of prokaryotes can be a daunting task in any environment. Depending on the questions to be answered and the definition of biodiversity, nearly any biological unit of interest may be used. Thus, following a brief introduction to life at high temperatures, this chapter will summarize some of the thermal environments on Earth and describe the taxonomic, genetic, metabolic, and ecological diversity of these environments. This chapter is written with the notion of illustrating that thermal environments are a fascinating source of local and global biodiversity of interest from many perspectives.

HIGH TEMPERATURES AND LIFE

Temperature, as an environmental factor, constrains all living microorganisms. In contrast to the upper temperature boundaries, the lower temperature boundaries for growth among microorganisms are not well defined (N. J. Russell, personal communication; Russell, 1990). Whether bacteria in the permafrost are "hibernating" for thousands to millions of years or just grow extremely slowly with doubling times of hundreds or even thousands of years is not yet unequivocally established. So far, no well-established growth at temperatures below –20°C (Rivkina et al., 2000) has been demonstrated for any member of the *Bacteria*, *Archaea*, or fungi (yeast, –17°C).

Broadly, microorganisms that "love" heat are known as thermophiles. A word of caution is warranted regarding the use of the term thermophilic. The term means different temperature ranges for different groups of microorganisms. For example, *Candida thermophile* is described as a thermophilic yeast with a maximum growth temperature (*T*max) of 51°C. The optimal growth temperature (*T*opt) for this microorganism is 30–35°C (Shin et al., 2001). Among *Bacteria*, this would be a thermotolerant species.

Recent observations of Pompeii worms (Wiegel, 1990, 1992) (*Alvinella pompejana*) persisting in temperatures exceeding 80°C at the tips of their tails (Cary et al., 1998) have generated new debate about the temperature tolerances of eukaryotes (Chevaldonne et al., 2000). Eukaryotes generally do not survive temperatures >60°C (Tansey and Brock, 1972). The amoeba *Echinamoeba thermarum*, found in hot springs in many places on the globe, grows optimally at 50°C and thus is one of the few truly thermophilic eukaryotes (Baumgartner et al., 2003).

Bacteria and *Archaea* can be classified according to their optimal growth temperature as follows: mesophiles (T_{opt} 20–45°C), thermophiles (T_{opt} 45–80°C), and hyperthermophiles (T_{opt} >80°C) (Stetter, 1996; Cavicchioli and Thomas, 2000). Under the classification scheme of Wiegel (1990, 1998a), using both the minimal and the maximal growth temperatures, thermophiles are further subdivided into thermotolerant, which grow optimally at mesophilic temperatures but have maximum growth temperatures >50°C, thermophiles (T_{opt} 50–70°C), and extreme thermophiles (T_{opt} >70°C). Prokaryotes

with T_{opt} >65°C and able to grow over a 35–40°C temperature span, e.g., able to grow <40°C and as high as 75°C, are considered temperature-tolerant extreme thermophiles (Wiegel, 1990, 1992). Similarly, there are hyperthermophiles, thermophiles, and mesophiles showing broad temperature spans for growth, all characterized by biphasic temperature–growth curves (Wiegel, 1990, 1998b). The record for the widest temperature span for growth, >50°C, is held by *Methanothermobacter* (basonym *Methanobacterium*) *thermautotrophicus*, able to grow from 22°C to 75°C (J. Wiegel, unpublished data). For reasons of simplification, in this chapter, the term thermophile will be used generally to include all microorganisms with T_{opt} >50°C. Hyperthermophiles, as defined by Stetter (1996), are distinguished from thermophiles only when necessary for clarity.

One might suppose that life exists in nearly any place where all the necessary requirements are met. Liquid water, essential for living microorganisms, is found at 300°C and higher under intense pressure, e.g., at deep-sea vents (Kelley et al., 2002). However, the thermostability of particular cellular constituents such as ATP, amino acids, and peptides indicates that 150°C may be the upper temperature limit for life (White, 1984; Wiegel and Ljungdahl, 1986; Wiegel and Adams, 1998). For a long time, the record for highest T_{max} was held by *Pyrolobus fumarii*, isolated from a deep-sea thermal black smoker vent chimney.

P. fumarii has a T_{max} of 113°C and a T_{opt} of 106°C and is unable to grow <90°C (Blochl et al., 1997). Strain 121, a Fe(III)-reducing *Archaea* recently isolated from a hydrothermal vent along the Juan de Fuca Ridge, is reported to have a doubling time of 24 h at 121°C and remains viable after exposure to temperatures as high as 130°C (Kashefi and Lovley, 2003); however, this record has yet to be verified with more detailed analyses. In addition, some thermophiles, such as those belonging to the phylogenetic branch of gram-type positive (Wiegel, 1981) bacteria (i.e., *Firmicutes*) (Wiegel et al., 1981), form highly heat-resistant spores (Onyenwoke et al., 2004). The current record for the most heat-resistant spore is held by *Moorella* (basonym *Clostridium*) *thermoacetica* strain JW/DB-4. When grown autotrophically and sporulating at 60°C, this bacterium forms spores with a decimal reduction time (time of exposure to

reduce viable spore counts by 90%) of nearly 2 h at 121°C. A subpopulation of spores apparently requires ~1 h at 100°C to become fully activated before germinating (Byrer et al., 2000).

Thermal environments have been the source of a wide variety of biotechnological advances. *Thermus aquaticus*, an aerobic, thermophilic bacterium, was isolated from Yellowstone National Park, Wyoming, in the late 1960s (Brock and Freeze, 1969), and the microorganism's DNA polymerase, coined *Taq*, has become an essential component of molecular biology. Additional polymerases and many other thermostable enzymes from thermophiles are now on the market, and their use in applications will increase in the future. Many chemical industrial processes employing high temperatures have benefited by using different groups of thermophiles for various applications (Wiegel and Ljungdahl, 1986; Lowe et al., 1993), such as anaerobic fermentative microorganisms for waste treatment and fuel production or sulfur-metabolizing organisms to remove sulfur compounds from crude oil.

The anaerobic, hyperthermophilic microorganisms branched early in the universal phylogenetic tree (Stetter, 1996). Considering that these microorganisms persist today in habitats that may have been present, and at times predominant, throughout Earth's geologic history, some assume that they represent linea closely descendent from the first living microorganisms on the planet (Baross, 1998; Schwartzman, 1998; Wachtershauser, 1998). The argument has been made that, considering an absence of geologic evidence from very early Earth and the effects of temperature on organic molecules, life did not originate in hyperthermal environments (Forterre, 1998; Miller and Lazcano, 1998). Still, in the evolution of eukaryotic life, there is evidence for thermophilic ancestors (Lake, 1988; Lake et al., 1998). The biphasic temperature–growth curves of many thermophiles growing at elevated temperatures and the existence of cryptic thermophiles are considered as additional arguments for the start of life in the range of 60–90°C and that hyperthermophiles as well as mesophiles and psychrophiles are adaptations to changed environments (Wiegel, 1998).

Looking for evidence of early life on Earth is similar to looking for the evidence that life, and the necessary water, that once existed on Mars (Westall, 2005). As our perception of habitable environments for life has expanded to include hyperthermobiotic environments and combinations of acidic, alkaline,

saline, or piezoelectric conditions in environments at elevated temperatures, our technological ability to investigate these habitats and identify evidence of life has also advanced, both on Earth and on other planets (Des Marais and Walter, 1999). For example, thermal intraterrestrial habitats have been recently identified on Earth, which may also persist on planets with assumed uninhabitable surface environments (Gold, 1992).

The island-like nature of thermal environments has made them popular models to test biogeographical hypotheses. Using similar strains of the thermophilic archaeon *Sulfolobus* originating from hot springs in Yellowstone National Park and Italy, Zillig et al. (1980) formulated the hypothesis that "geographical barriers between habitats of the same type do not exist for microorganisms." This hypothesis also corresponds to the oft-quoted hypothesis that "everything is everywhere and the environment selects" (Baas-Becking, 1934 citing Beijerinck, 1913). However, Whitaker et al. (2003) attribute genetic divergence detected by multilocus sequence analysis of strains of *Sulfolobus solfataricus* from five sites to geographic isolation. Papke and Ward (2004) reasoned that biogeography requires physical separation and gave several examples of mechanisms that could mediate such separation among prokaryotes. Experiments with thermophilic cyanobacterial mat samples from several locations supported these arguments (Papke et al., 2003). Multilocus enzyme electrophoretic analysis of *Rhodothermus marinus* isolates from hot springs in Iceland attributed variances to both genetic drift and differences among the four locations, or local selection (Petursdottir et al., 2000). Different species of thermophiles appear to have different modes of population distribution. Examples of cosmopolitan microorganisms from thermal environments include *Methanothermobacter thermautotrophicus*, *Thermanaerobacter* (basonym *Clostridium*) *thermohydrosulfuricus*, *Thermoanaerobacterium thermosaccharolyticum*, and *Geobacillus stearothermophilus* (Wiegel and Ljungdahl, 1986). Although spore formation is certain to be an advantage for high population dispersal, *M. thermautotrophicus* is nonsporulating. Alternatively, examples of species regarded as endemic include members of the aerobic, hyperthermophilic, archaeal genus *Aeropyrum*, which have yet to be isolated from anywhere other than the coastal geothermal fields of southwest Japan (Nakagawa et al., 2004a), and the genus *Methanothermus*, thus far found only in

Iceland (Lauerer et al., 1986). Among macroscopic organisms, island biogeography is being reexamined (Lomolino, 2000), and the universality of new paradigms should be enhanced by recent research with thermophiles.

The interaction correlation between biogeography and biogeochemistry in thermal environments is also worthy of note. As an example, when examining the thus-far described anaerobic alkalithermophiles, three combinations can be defined: (i) relaxed biogeography and biogeochemistry, (ii) relaxed biogeography and restricted biogeochemistry, and (iii) restricted biogeography and relaxed biogeochemistry. For example, *Thermoanaerobacterium thermosaccharolyticum* and *Thermobrachium celere* have a relaxed biogeography and biogeochemistry. They have been isolated from a variety of environments from several locations including thermobiotic, mesobiotic, slightly alkaline and acidic environments. *Clostridium paradoxum* and *Clostridium thermoalcaliphilum*, isolated from sewage sludge on four different continents, but only from sewage sludge, thus have a relaxed biogeography but apparently restricted biogeochemical requirements. *Anaerobranca horikoshii* ($pH^{60°C}_{min}$ 6.9; $pH^{60°C}_{opt}$ 8.5; $pH^{60°C}_{max}$ 10.3) is an example of restricted biogeography and relaxed biogeochemistry. *A. horikoshii* has only been isolated from a specific area behind the Old Faithful ranger station in Yellowstone National Park, but from several pools in that area, representing a spectrum of pH values from acidic (pH ~5) to alkaline (pH ~8.5). Although relatively easy to isolate, strains of *A. horikoshii* have not been obtained from other areas of Yellowstone National Park or other countries, nor has its sequence been found in environmental 16S rRNA gene libraries (Engle et al., 1995).

Finally, biogeology/biogeochemistry is especially of interest in thermal environments, where mineralization is active and the role of prokaryotes in mineralization is being examined. An increasing number of studies in thermal environments are linking geochemical conditions and phenomena with specific microbial species and metabolisms (Nubel et al., 2002; Donahoe-Christiansen et al., 2004). Electron microscopy and denaturing gradient gel electrophoresis (DGGE) were well coupled with geochemical analyses in the investigation of arsenite-oxidizing communities at Yellowstone National Park (Jackson et al., 2001; Langner et al., 2001). Prokaryotes can couple mineral formation with energy

generation, e.g., the formation of pyrite by *Thiomonas thermosulfatus*, strain 51 (Popa and Kinkle, 2004). Thermophilic metal ion reducers contribute to the deposition of precious metals by dissimilatory reductive precipitation (Kashefi et al., 2001 and references therein). Fe(III) reducers (e.g., Wiegel et al., 2003) frequently can reduce a variety of heavy metal ions and are assumed to have been involved in the deposition of low-temperature banded iron formations. The presence of different types of microorganisms may influence the morphology of siliceous sinters laid down along the edges of hot springs (Frankel and Bazylinski, 2003 and references therein; Konhauser et al., 2004). The formation of such sinters may serve to protect the sinter-forming microorganisms from ultraviolet radiation (Phoenix et al., 2001). Additionally, understanding the role of microorganisms in mineralization in contemporary thermal environments will enhance our understanding of ore deposition, biogeochemistry, and paleogeology (e.g., Karpov and Naboko, 1990; Russel et al., 1998).

TYPES OF THERMAL ENVIRONMENTS

Considering the constraint of high temperatures as an environmental requirement, thermophiles are still able to exploit a diverse array of habitats on Earth. The environment at the surface of a solfatara may have a pH of <1 (Stetter, 1989), and microorganisms at the carbonate-rich Lost City hydrothermal field (Kelley et al., 2005) or sun-heated salt lakes (Mesbah and Wiegel, 2005) may experience pH as high as 11. Water is readily available in circumneutral, freshwater hot springs, but there are thermal environments having low water potentials; e.g., in intraterrestrial environments because of high surface-area-to-water ratios (Pedersen, 2000) or in solarheated soils and sediments because of evaporation and high salinity (Mesbah and Wiegel, 2005). Thermal environments may be grouped into several types, some of which are described below. Within each type, a variety of factors generate additional diversity.

Terrestrial, Non-anthropogenic Environments

Hot springs, geysers, thermal pools, and solfatares (mud or paint pots) are found concentrated in volcanically active regions throughout the world, including Iceland, Western North America, New

Zealand, Japan, Eastern Russia, and the rest of the so-called Pacific Ring of Fire (Waring, 1965). The Yellowstone National Park area of North America contains the greatest concentration of geothermal features on Earth and was the site of some of the earliest studies of life at high temperatures, dating back to 1897 (as referenced within Reysenbach and Shock, 2002). Depending on local water chemistry and hydrology, such environments can represent a spectrum of pH and geochemical conditions that can vary greatly across small distances (<20 m in the Uzon Caldera, Kamchatka, Russian Far East) (personal observation). Generally, solfatares are sulfur rich and acidic, and isolates from such environments are acidophilic and frequently sulfur metabolizers (Stetter, 1989). Alternatively, waters are more neutral to alkaline if richer in chloride salts or carbonate (Hedenquist, 1991; Zhao et al., 2005). Temperatures in these environments may range from freezing to boiling, even within a single pool or spring (Combie and Runnion, 1996). Such environments are also frequently enriched in elements such as arsenic (As), antimony (Sb), and mercury (Hg), which are toxic to humans, and gold (Au), silver (Ag), and copper (Cu), which are valuable commercially (Karpov and Naboko, 1990).

Solar-heated environments may occur anywhere on Earth receiving solar energy inputs. Such environments are likely inhabited by mesophilic, thermotolerant, and thermophilic microorganisms because solar energy can heat some soils to 60°C and shallow waters to 50°C at certain times of the day or year, as already pointed out by Brock (1970). Thermal environments on Earth's surface also experience evaporation, and thus many environments have elevated salinity and, therefore, halophilic inhabitants. For example, *Thermohalobacter berrensis*, a thermophilic and halophilic bacterium, was isolated from a solar saltern in France (Cayol et al., 2000). In the author's J. Wiegel's laboratory, halophilic (up to 25% NaCl 4.5 M Sodium ion as $NaCl/Na_2CO_3$), thermophilic (up to 75°C), and alkaliphilic (up to $pH^{25°C}$ 10.5) triple extremophiles, coined haloalkalithermophiles, have been isolated from dry salts from salt flats in Nevada and from sediments of athalassohaline lakes in Wadi An Natrun, Egypt (Mesbah and Wiegel, in press). Note that especially for acidic and alkaline conditions the pH at 25°C can differ from the pH at 60°C by >1 pH unit, and a superscript indicating the temperature at which pH was determined (e.g., $pH^{25°C}$) is recommended (Wiegel, 1998a).

Marine Environments

Marine thermal environments may occur at beaches, e.g., Hot Water Beach (Whitianga, New Zealand), Pozzuol: (Italy), or Savusavu (Fiji Island) (Waring, 1965); under <8 m of water, e.g., vents off the coast of Mílos Island, Greece (Sievert et al., 2000a); or under an abysmal 2,500 m of water, e.g., at deep-sea hydrothermal vents first discovered in 1977 near the Galápagos Islands (Corliss et al., 1979). Venting water can exceed 300°C, but in deep-sea vents it cools quickly upon mixing with cold, deep-sea water, and habitat types range from those preferred by hyperthermophiles to temperatures habitable by psychrophiles (Cary et al., 1998; Kelley et al., 2002). Black smoker chimneys, associated with volcanic spychrophiles activity, plate spreading zones generally are fueled by high concentrations of sulfides (Kelley et al., 2002). Serpentinite-hosted systems, like the Lost City hydrothermal field, are enriched in hydrogen and methane as energy sources (Kelley et al., 2005).

Organisms living at deep-sea hydrothermal vent areas generally cope with additional environmental extremes. For example, they must be at least piezotolerant and may be piezophilic (barophilic). *Thermococcus barophilus*, obtained from the Snake Pit region of the Mid-Atlantic Ridge, requires elevated pressure for growth at or above 95°C (Marteinsson et al., 1999), and *Pyrococcus* strain ES4 shows an extension of T_{max} under increased pressure (Pledger et al., 1994; Summit et al., 1998). At hydrothermal vents, the level of natural radioactivity can be 100 times greater than that at Earth's surface because of increased occurrence of elements such as ^{210}Pb, ^{210}Po, and ^{222}Rn (Cherry et al., 1992, cited in Jolivet et al., 2003). The archaea *Thermococcus gammatolerans*, isolated from a hydrothermal site in Guaymas Basin (Jolivet et al., 2003), *Thermococcus marinus* from the Snake Pit hydrothermal site on the Mid-Atlantic Ridge, and *Thermococcus radiotolerans* from a hydrothermal site in the Guaymas Basin (Jolivet et al., 2004) were isolated from enrichment culture subjected to γ-irradiation on the basis of the rationale that microorganisms from deep-sea hydrothermal vent areas would be adapted to elevated levels of radiation. Additionally, all organisms existing in marine environments also have some tolerance for moderate (around 3%) salinity.

Subsurface Environments

Subsurface thermal environments include petroleum reservoirs and geothermally heated lakes and aquifers. Activity in subsurface environments varies with the availability of nutrients, water, and energy based on depth, surrounding matrix, and source materials. Owing to heterogeneity in the composition of Earth's crust, a variety of sources for energy are readily available (Gold, 1992). Lethal temperatures may not occur until as much as 10,000 m below the surface (Pedersen, 2000), though in some areas, e.g., Uzon Caldera, temperatures well above 100°C can occur at depths of only a few meters (personal observation). A depth record for culturable life has been established at 5,278 m (Szewzyk et al., 1994).

Elevated temperatures found within petroleum reservoirs can be up to 130°C (Grassia et al., 1996). The geochemical conditions in reservoirs are variable because of age, source material, and surrounding geology, and prokaryote communities therein vary as well (Orphan et al., 2003) and include several thermophilic Fe(III) reducers (Slobodkin et al., 1999). Takahata et al. (2000) have proposed that microorganisms in these environments may face oligotrophic conditions. Microorganisms in petroleum reservoirs may be endemic or introduced during drilling or water injection (L'Haridon et al., 1995; Pedersen, 2000). The same is true of ultradeep gold mines in South Africa, where service water containing mesophilic bacteria and fungi can contaminate communities of thermophilic sulfate-reducing bacteria indigenous to the hot, subsurface rocks (Onstott et al., 2003).

Subsurface geothermal aquifers such as the well-known and expansive Great Artesian Basin of Australia are nonvolcanically heated but experience temperatures up to nearly 103°C (Kimura et al., 2005). Nonvolcanic geothermal environments are temporally stable as compared with volcanically heated environments and have low flow rates and long recharge times (e.g., 1,000 years). Thus, the Great Artesian Basin contains microbial communities of ancient composition (Kimura et al., 2005). Microorganisms isolated from these habitats include *Desulfotomaculum australicum* (Love et al., 1993), *Fervidobacterium gondwanense* (Andrews and Patel, 1996), and *Caloramator indicus*, isolated from an artesian aquifer in Surat District, Gujarat, India (Chrisostomos et al., 1996).

Anthropogenic Environments

Anthropogenic habitats include household compost piles and water heaters and industrial process environments and thermal effluent from power plants (Brock, 1970; Stetter, 1989). One of the earliest well known anaerobic thermophiles, *Thermoanaerobacter* (basonym *Clostridium*) *thermohydrosulfuricus*, was isolated from an Austrian sugar factory (Klaushofer and Parkkinen, 1965; Lee et al., 1993). Other thermophiles have been isolated from thermally polluted effluent from a carpet factory (Carreto et al., 1996), the smoldering slag heap of a uranium mine (Fuchs et al., 1996), and mushroom compost (Korn-Wendisch et al., 1995). Strains of *T. aquaticus* have been isolated from various anthropogenic thermal environments including hot tap water and greenhouse soil (Brock and Freeze, 1969).

Temporary Environments and Mesobiotic Environments with Thermal Microniches

Thermophiles can be isolated from various environments, such as animal droppings, manure piles, or compost, temporarily heated by biodegradation of organic material. One example is the acetogenic bacterium *Moorella thermoacetica* (Fontaine et al., 1942). Interesting temporary thermal environments also include sun-heated soils and sediments at the edges of lakes and puddles at the ocean, which can have temperatures up to 50°C but are frequently around 35–45°C (J. Wiegel unpublished data). Whereas most of the thermophiles isolated from these environments are *Firmicutes*, i.e., endospore-forming species, there are exceptions. One example is the archaeon *Methanothermobacter* (basonym *Methanobacterium*) *thermautotrophicus*, for which no resting cell forms are known. This species (or alikes) can be easily isolated from sun-heated black sediments of lakes and mesobiotic sewage plants, but it also has been isolated from sun-heated wood stumps in Georgia, United States (J. Wiegel and L. G. Ljungdahl, unpublished data), and mesobiotic environments such as cold stream sediments in Germany (Wiegel et al., 1981) or sediments of Lake Mendota, Wisconsin, for which temperatures have never been measured <16°C.

The chapter authors believe that many environments that are classified as mesobiotic from their grab temperature measurements contain temporary thermal microniches, created by localized biodegradation of organic material. An example for this phenomenon could be *Methanothermobacter thermautotrophicus* strain ΔH (isolated from sewage sludge) as well as strain JW500 (isolated from river sediment in Georgia, United States), which have been shown to grow in complex media (simulating a more natural environment) at temperatures as low as 22°C (Wiegel, unpublished). In mineral media, T_{min} is given as 35°C (Zeikus and Wolfe, 1972). However, this methanogen still metabolizes and produces methane at temperatures as low as 16°C (but not measurable within 3 months at 12°C). Metabolic activity and growth at even lower temperatures could be possible (but probably with doubling times of years and thus difficult to measure in laboratory settings). *M. thermautotrophicus* can grow over a temperature span of 55°C, the record of the widest (measured) temperature span over which a microorganism can grow, and thus, in classification of Wiegel, is a temperature-tolerant thermophile (Wiegel, 1990). *C. thermoalcaliphilum*, for which no spore formation could be demonstrated, and although having a lower temperature optimum and smaller temperature span for growth than the methanogen, has been isolated from similar lowtemperature sewer sludge (Li et al., 1994).

A second exemplary case is the recent isolation of the alkalithermophilic *T. celere* from several meadows and floodplains (Engle et al., 1996). Interestingly, from the many obtained strains for this species, those isolated from mesobiotic environments have doubling times below 20 min and as low as 10 min, whereas isolates from hot springs have doubling times >30 min (Wiegel, unpublished). A possible explanation for this difference in doubling times is that thermophilic microorganisms in mesobiotic environments must be able to maximize the use of temporarily occurring high-temperature growth conditions and must be able to start growing quickly and grow at high growth rates. Such properties have been found, for example, for the thermoalkaliphile *Clostridium paradoxum*, isolated from mesobiotic anaerobic digestor and aerobic oxidation basin of the Athens and Atlanta, GA, Municipal Sewage Plant; it has a doubling time of 16 min; and is able to start growing exponentially from a spore solution within 30 min from the time of inoculating spores into prewarmed, prereduced media (Y. Li and J. Wiegel,

unpublished data). In contrast, thermophiles living in stable thermal environments such as hot spring pools and sediments, living all the time at their growth temperature and more or less at constant,—although possibly low-substrate concentrations, do not have that selection pressure for very rapid growth as long as their residence time in the pool is longer than their doubling time.

CULTURAL DIVERSITY

Most of the microorganisms from nearly all environments they inhabit are presently uncultured (Hugenholtz, 2002). Considering the extreme conditions in which most thermophiles thrive, some require special handling or novel approaches for their enrichment, culturing, and isolation (Mesbah and Wiegel, in press; Wiegel, 1986). An overview of the validly published and well-characterized (i. e, those with reported optimal growth conditions) thermophilic and hyperthermophilic microorganisms in isolation can be given in graphs using the cardinal data pH_{opt} versus T_{opt} (Figs 1.1 and 1.2, http:// www.bacterio.net, March 2006). Figures 1.1 and 1.2 further indicate the extreme optima beyond which no microorganisms have been isolated, because either they do not exist or applied isolation conditions were insufficient for their growth. As already pointed out by Stetter in 1989, thermophiles that grow optimally at low pH tend to be aerobic, especially among *Bacteria* (Fig. 1.2). At the highest known growth temperatures, most of the microorganisms appear to be neutronphilic and *Archaea* (Fig. 1.1). Such trends among microorganisms in culture are likely the result of multiple interacting factors of the environments and the microorganisms as well as the objectives and limitations of the enrichments and isolations. However, enrichments for specific types of microorganisms can yield commercially useful resources (Wiegel and Ljungdahl, 1986) or reveal information about how various environmental factors alter the distribution of different microorganisms (Grassia et al., 1996).

In 1996, Stetter identified 54 species of hyperthermophilic prokaryotes in 25 genera and 11 orders, and as of March 2006, among the well-characterized and validly published microorganisms, ~64 species of hyperthermophilic prokaryotes and >350 species of thermophiles have been isolated. Well-represented among the ~30 genera of *Archaea* are *Thermococcus, Sulfolobus*, and *Methanococcus*. Other

genera such as *Thermococcus*, *Sulfurisphaera*, *Methanotorris*, and *Acidilobus* at present contain few isolated species. Most of the hyperthermophiles are *Archaea*, though the bacterial genus *Aquifex* is represented by a hyperthermophilic strain and members of the bacterial genus *Thermotoga* have T_{opt} ≥80°C. Of the *Bacteria* depicted in Fig. 1.2, there are >110 different genera represented. Except for the genera *Geobacillus*, *Thermoanaerobacter*, *Desulfotomaculum*, and *Clostridium*, each genus is represented by <10 isolates. This does not take into consideration recent debates on the taxonomy of these genera. For example, there are many species that do not belong to the genus *Clostridium* sensu stricto (Rainey et al., 2006).

PHYLOGENETIC AND GENETIC DIVERSITY

Upon an extensive analysis of bacterial evolution, Woese (1987) presented the small subunit rRNA gene as an espalier on which to train our universal phylogenetic tree. Among prokaryotes, within the 16S rRNA phylogeny, thermophily is polyphyletic. As mentioned above, hyperthermophiles, in general, branch early in the universal phylogenetic tree (Stetter, 1996). However, among thermophilic bacteria, there are mesophilic and thermophilic members in some of the same phylogenetic groups and genera (e.g., *Firmicutes*), perhaps as a result of adaptation to mesobiotic temperatures from thermophilic ancestors or via lateral gene transfer from thermophilic taxa (Wiegel, 1998b). Amplification of 16S rRNA genes directly from environmental DNA has revealed an astonishing amount of diversity among prokaryotes; e.g., Barns et al. (1994) and Hugenholtz et al. (1998) identified novel lineages of thermophilic *Archaea* and *Bacteria* in Obsidian Pool (OP) in Yellowstone National Park, via amplification of 16S rRNA genes in environmental DNA extracted from sediment samples. Sequences similar to the OP sequences have since been identified in many locations, including the Great Artesian Basin, Australia (Kimura et al., 2005), and thermal pools in Kamchatka, Russian Far East (E. A. Burgess, unpublished data). Environmental 16S rRNA sequence similarities to characterized species do not always directly correspond to the same *in situ* niche or physiology but have led to targeted culturing of novel microorganisms first identified in clone libraries (Huber et al.,1995; Ghosh et al., 2003).

In general, PCR biases, also present in the real-time PCR approach, limit the ability to determine quantitatively the relative abundance among all prokaryote populations and, consequently, the diversity in thermal environments via environmental 16S rRNA gene analyses (Wintzingerode et al., 1997; Baker and Cowan, 2004). Variations in experimental design and implementation among studies also inhibit direct comparisons. However, a brief, qualitative summary of richness as number of environmental 16S rRNA restriction enzyme phylotypes in a selection of environments is presented (Table 1.1). In general, these studies were coupled with some sequencing. Differences among dominant phylogenetic groups were observed. For example, from an artesian well in the Great Artesian Basin, Australia, nine *Archaeal* groups were dominated by methanogen-like sequences from *Euryarchaeota* (35 of 59 clones) (Kimura et al., 2005). From a hot spring in Thailand, 17 of 25 clones grouped with noncultivated *Crenarchaeota* sequences (Kanokratana et al., 2004). Sequences from deep-sea hydrothermal vents obtained by enrichment of *in situ* microorganisms within an innovative growth chamber (first deployed at the Mid-Atlantic Ridge and since then at other deep-sea hydrothermal vent locations) led to the identification of novel lineages among *Archaea* and *Bacteria* (Reysenbach et al., 2000).

Although many novel and diverse 16S rRNA sequences have been described from various thermal environments, some thermal environment communities may contain only a few phylogenetic types. Reysenbach and Shock (1994) identified only three major phylogenetic groups out of 35 clones analyzed from pink filaments collected at Octopus Spring, Yellowstone National Park. Environmental DNA from siliceous sinters in Yellowstone National Park contained predominantly 16S rRNA sequences related to *Thermocrinis ruber*, and the dominating abundance of *T. ruber* in the sinter community was verified by fluorescent *in situ* hybridization (FISH) (Blank et al., 2002). Interestingly, the populations were represented by diverse morphotypes when examined with electron microscopy (Blank et al., 2002). Thus, the resolution of 16S rRNA to represent genetic diversity in prokaryote communities appears to be limited. The diversity of other ribosomal operon sequences, such as internal transcribed spacer (ITS) regions and 23S rRNA genes, has been useful to differentiate among closely related strains of thermophilic prokaryotes (Moreno et al., 2002; Papke et al., 2003; I. D. Wagner, unpublished data).

Besides using ribosomal RNA genes for describing biodiversity, the sequences of "housekeeping" genes have proven to be very useful, especially when sequences from many strains are available (Santos and Ochman, 2004). However, not all genes are informative, as revealed by the presence/absence of sporulation-specific genes in the genomes of non- and asporulating *Firmicutes* (Onyenwoke et al., 2004). The selection of useful genes also depends on the physiological groups of interest. For example, novel lineages of dissimilatory sulfite reductase (*dsr*) genes have been identified in thermal environments and associated with high rates of sulfate reduction (Fishbain et al., 2003). Analysis of *dsr* has been used to compare prokaryote communities and sulfate-reducing bacterial lineages in thermal environments from underground mines in Japan to multiple hydrothermal vents (Nakagawa et al., 2002, 2004b) and ultradeep gold mines in Africa to basalt aquifers in Washington state (Baker et al., 2003), revealing that closely related *dsr* sequences are often found in distant locations.

Genomic analyses are the new avenue of investigation of genetic diversity. The information from genomic sequences (http://www.genomesonline.org) and the resulting proteomic information need to be linked to the geochemical processes occurring in the environment (Reysenbach and Shock, 2002) or the geographical distribution of prokaryote diversity (Whitaker et al., 2003). Furthermore, metagenomics has become a popular tool and, for example, has been used to identify a thermostable polymerase related to that of *Thermoplasma acidophilum* from a deep-sea vent sample (Moussard et al., 2006). The metagenomic approach is predicted to become much more frequently applied in a large variety of environments as sequencing becomes more affordable. Eventually, there will be a wealth of information available on the physiological and biogeochemical potential of prokaryote communities in the studied environments. Mining this metagenomic data will lead to in silico models of how the interwoven interactions between microorganisms, their metabolic activities, and the geochemistry of their environments occur *in situ*, and such models should be, at least in part, experimentally verifiable.

METABOLIC DIVERSITY

Although nearly all types of microbial metabolism are observed in thermal environments,

chemolithotrophy (obtaining energy from inorganic electron-donating and -accepting reactions) is a cornerstone of hyperthermophilic communities. In general, chemolithotrophy has been observed both in autotrophic (obtaining carbon from inorganic sources such as CO_2) and in heterotrophic organisms (obtaining carbon from organic sources such as sugars or volatile fatty acids). Chemolithoautotrophy is considered the source of primary production in sunless environments and environments too hot for photoautotrophic production. For example, Blank et al. (2002) suggest that because chemolithoautotrophs, in particular bacteria of the order *Aquificales*, inhabit hot springs in Japan, Iceland, Kamchatka, and Yellowstone National Park, these types of microorganisms may be the primary producers in these ecosystems.

Many thermal environments are anaerobic or of reducing nature, either because of the remoteness of the environment from the atmosphere, the low solubility of oxygen in water at elevated temperature, as well as hypersalinity in some cases, or because of the inputs of gasses such as H_2 and reducing H_2S (Brock, 1970; Stetter, 1996). Thus, there is abundant chemical energy for chemolithotrophs, and anaerobic respiration predominates among respiring microorganisms. Amend and Shock (2001) calculated the energetics of many metabolic reactions in environments with elevated temperatures. Notably, anaerobic thermophilic *Bacteria* are generally unable to grow at acidic pH, and most acidophilic, thermophilic *Archaea* are obligate aerobes (Wiegel, 1998). There are neutrophilic, facultatively aerobic *Archaea*, such as *Pyrobaculum oguniense* (Sako et al., 2001), as well as examples of anaerobic acidophilic *Archaea*, such as *Stygiolobus azoricus* (Segerer et al., 1991). The most acidophilic *Archaea* are *Picrophilus oshimae* and *Picrophilus torridus*, growing optimally at pH ~0.7 (Schleper et al., 1995). The most acidophilic anaerobic bacteria presently are *Thermoanaerobacterium aotearoensis*, $pH^{60°C}_{opt}$ 5.2 and $pH^{60°C}$ range 3.8–6.8 (Liu et al., 1996), and *Lebetimonas acidiphila*, pH_{opt} 5.2 and pH range 4.2–7.0 (Takai et al., 2005), though for *L. acidiphila* the temperature of pH measurement has not been reported.

Most of the hyperthermophilic microorganisms are *Archaea*, and most of these perform chemolithotrophic metabolism for energy, including methanogenesis, sulfate reduction, sulfur oxidation,

sulfur reduction, nitrate reduction, and hydrogen oxidation (Stetter, 1989; Slobodkin et al., 1999). Kletzin et al. (2004) provide a review of elemental sulfur metabolism in *Archaea*. Examples of physiological types among the *Archaea* are *Archaeoglobus, Thermodiscus*, *Thermoproteus*, *Acidianus*, and *Desulfurococcus* reducing sulfur or sulfate, *Sulfolobus* species generally oxidizing H2S or elemental sulfur, *Methanothermus*, *Methanococcus*, and *Methanopyrus* being methanogenic, and *Pyrobaculum* and *Pyrolobus* reducing nitrate (Stetter, 1996).

Among *Bacteria* are anaerobic *Firmicutes* (Gram-type positive, although among the thermophilic Firnmicutes, many stain Gram reaction negative at all growth phases; Wiegel, 1981), such as the facultative chemolithoautotrophs *Moorella thermacetica*, producing acetate from CO_2/H_2 or from CO (100% gas phase) but also carrying out homoacetogenic fermentations
from carbohydrates, and the anaerobe *Ammonifex degensii*, capable of forming ammonium from nitrate via chemolithoautotrophic growth (Huber et al., 1996). Other examples of chemolithoautotrophic thermophilic *Bacteria* are Fe(III) reducers such as *Thermolithobacter ferrireducens*, isolated from various hot springs, and *Thermolithobacter* (basonym *Carboxydothermus*) *carboxydivorans*, a hydrogenic CO utilizer (Sokolova et al., in press;
Svetlichny et al., 1991; Wiegel et al., 2003). Recently, several novel, some obligately hydrogenic, CO utilizers have been described (Sokolova et al., 2005 and references therein). Photoheterotrophs such as *Chloroflexus aggregans*, *Chloroflexus aurantiacus*, *Heliobacterium modesticaldum*, and *Roseiflexus castenholzii* use light for generating energy but use organic carbon as their carbon source; i.e., they are unable to utilize CO2 as would a photoautotroph (Pierson and Castenholz, 1974; Hanada et al., 1995, 2002; Kimble et al., 1995).

In some examples, *in situ* geochemistry of thermal environments may be shaping the dominant metabolisms or perhaps is shaped by the dominant metabolisms (Orphan et al., 2003). Microorganisms have been isolated which are obligate users of gasses escaping in volcanic regions, such as CO, as mentioned above. They are using a novel chemotrophic metabolism, i.e., using 100% CO as sole carbon and energy source via the reaction $CO + H_2O \rightarrow H_2 + CO_2$ (Svetlichny et al., 1991; Sokolova et al.,

2005). Many thermal environments are enriched in elements that are toxic to humans, such as As and selenium (Se), and some microorganisms in these habitats use toxic, redox-active elements to gain energy, via either oxidation or reduction (Huber et al., 2000; Donahoe-Christiansen et al., 2004). Respirationally diverse Fe(III) reducers are frequently able to reduce many redox-active compounds in thermal environments (Kieft et al., 1999; Kashefi et al., 2001).

In situ radiolabel rate measurements and stable isotope analyses are yielding increasing amounts of information about the metabolism and ecology of microorganisms in thermal environments. Even with little knowledge of specific microorganisms responsible for activities *in situ*, metabolically active microorganisms can be quantified, the metabolic potential of the communities identified, and the distributions of different metabolic types determined (Burgess, unpublished; Harmsen et al., 1997; Slobodkin et al., 2001). Temperature limits for sulfate reduction have been expanded to nearly 100°C by radiotracer experiments in marine sediments (Jorgensen et al., 1992).

ECOLOGICAL DIVERSITY

There are diverse ecological interactions associated with the biology in geothermally and anthropogenically heated environments, and thermophilic prokaryotes have opened our eyes to many novel modes of life. The discovery of deep-sea hydrothermal vent communities demonstrated that life can exist at temperatures >100°C as well as at 2°C in the surrounding ocean, without inputs of solar energy, on the basis of associated microbial vent community (Corliss et al., 1979). Novel symbioses between eukaryotes and prokaryotes have been identified at deep-sea vents, such as the association between the tube worm *Riftia pachyptila* and chemosynthetic, sulfur-oxidizing bacteria (Cavanaugh et al., 1981) or the Pompeii worm, which may derive some of its thermo-tolerance from the eurythermal enzymes of a community of prokaryotes living on its back (Cottrell and Cary, 1999; Chevaldonne et al., 2000).

Microecology is a relatively young and rapidly expanding field, and thermal environments may be model systems for examining microecological concepts. Generally, the unit of ecological diversity is the species (Magurran, 1988). The species unit has become increasingly difficult to define among

prokaryotes (Kampfer and Rossello-Mora, 2004; Gevers et al., 2005). Small subunit rRNA sequence diversity can be used as a proxy for species diversity (Stackebrandt et al., 2002) (Table 1.1). Moreover, an increasing number of statistically powerful tools have become available for comparison of 16S rRNA sequences (Singleton et al., 2001; Cole et al., 2005; Schloss and Handelsman, 2005). For example, the diversity of microorganisms within mats of cyanobacteria inhabiting hot springs has been examined with the intent to demonstrate the importance of a prokaryote species concept based on the role of the microorganisms in their environments (Ward, 1998; Ward et al., 1998). Classical ecological concepts such as energy partitioning and trophic transfer have been examined using models developed at Yellowstone National Park (Wiegert and Fraleigh, 1972). Viruses have been hypothesized as a top-down control on prokaryote communities (Torsvik et al., 2002). Perhaps the importance of this control may be measured in model thermal environment systems where viruses are present, novel, and quite diverse (Prangishvili and Garrett, 2004 and references therein).

Molecular tools have been largely responsible for many advances in our perception of the ecology in thermal environments, and the application of culture-independent methods has greatly enhanced our knowledge of the diversity and physiological potential of difficult-to-culture microorganisms that inhabit these environments. The effect of different geochemical parameters, including temperature, on the distribution and community structure of prokaryotes has been examined through genetics and the distribution of different metabolic types (Sievert et al., 1999, 2000b; Ramsing et al., 2000; Skirnisdottir et al., 2000; Norris et al., 2002; Orphan et al., 2003), and some of these data are demonstrating trends of prokaryote communities changing in predictable ways with changing environmental conditions. Methods such as FISH enable the examination of the structural distribution of microorganisms of known phylogenetic affiliations (Nubel et al., 2002). Lipids present within the membranes of prokaryotes can be diagnostic for various types of microorganisms and have provided insight into the distribution of microorganisms among different environments. For example, analysis of glycerol dialkyl glycerol tetraethers (GDGTs) from selected hot springs in Nevada revealed the presence of the archaeal lipid crenarchaeol, previously found only in low-temperature, marine environments. Additionally, DGGE band

sequences of 16S rRNA genes from these springs were related to thermophilic *Crenarchaeota*, demonstrating that the presence of crenarchaeol is not exclusive to the cold-adapted, marine branch of the *Crenarchaeota* (Pearson et al., 2004). This conclusion has substantial implications for the theory of the origins of *Crenarchaeota* (Zhang et al., 2006).

CONCLUSIONS

As described above, thermal environments are an amazing reservoir of biodiversity. Especially among prokaryotes, some *Archaea* have optimum temperatures for growth in excess of 100°C. This vast biodiversity in turn has been a source of advancement in the fields of biotechnology, evolutionary biology, astrobiology, biogeography, biogeochemistry, and biogeology. Among the different types of environments with elevated temperatures, ranging from volcanic and geothermally heated to anthropogenic sources, to temporary thermal microniches in otherwise mesobiotic environments, there is a diverse array of unique habitats where often many extreme conditions converge, e.g., sun-heated haline and alkaline salt lakes. An important point that becomes evident when examining these environments and their biodiversity of archaea, bacteria, viruses, and even a few eukaryotic species is the vast diversity of approaches and methods that have been used in an attempt to measure it. A count of cultured thermophiles, >350 wellcharacterized and validly published, does not represent the much larger number of prokaryotes presently known only through environmental 16S rRNA sequences. Measuring the genetic diversity of 16S rRNA and functional genes, which has been an avenue for discovery of many enzymes for biotechnological applications and the isolation of novel microorganisms, provides only limited information about their *in situ* abundance and activity. Among the many modes of life, including metabolic pathways, found to be present in thermal environments are some that are unique to thermophiles and some that are employed by their mesophilic relatives. These diverse species, sequences, and their metabolisms in turn enhance the ecological diversity of the planet and play an important role in the evolution and stability of the planet's biota. In general, analysis of multiple approaches applied in single environments combined with that of similar approaches in different environments has enhanced the

robustness of our understanding of the various high-temperature environments and the biodiversity they harbor. Considering how the initial discovery of life in shallow and deep-sea vents expanded our notion of global biodiversity, future approaches and discoveries, perhaps also in extraterrestrial thermal environments, will likely reveal additional information relevant to many fields of basic and applied science.

Acknowledgments. Owing to the wealth of research related to biodiversity of thermal environments and the pages allowed for this chapter, it was inevitable that we have been unable to include all the pertinent publications, and thus we apologize to all colleagues whose relevant work has not been cited. Details about the diverse adaptations of thermophiles to elevated temperatures are well covered in other chapters of this book. J. W. is especially indebted to Lars G. Ljungdahl (University of Georgia), who is celebrating his 80th birthday and—30 years ago—introduced him to the wonderful world of anaerobic thermophiles. The writing of this review and much of the cited work from the laboratory of J. Wiegel was supported through the NSF—Microbial Observatory grant NSF-MCB 0238407 and NSF-MIP 0348180.

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Table 1.1 Comparison of richness of restriction enzyme phylotypes (types) from clone libraries of environmental 16S rRNA genes from a small selection of thermal environments

Environment sampled	Specificity of primers used	No. of clones analyzed	No. of types identified	Reference
Hot spring, Yellowstone National Park	2 universal sets and bacterial set	>300	95 total from all three libraries	Hugenholtz et al., 1998
Hydrothermal vent, Mid-Atlantic	Archaeal set and bacterial set	85	38 (11 archaea, 27 bacteria)	Reysenbach et al., 2000
Two hydrothermal vents, Juan de Fuca	Archaeal set and bacterial set	141	64 and 59 total from each site	Alain et al., 2002
Petroleum reservoirs, California	Universal set and archaeal set	68	9 archaea	Orphan et al., 2003
Hot spring, Thailand	Archaeal set and bacterial set	200	24 archaea, 32 bacteria	Kanokratana et al., 2004
Artesian well, Australia	Archaeal set and Bacterial set	59 in each library	9 archaea, 7 bacteria	Kimura et al., 2005

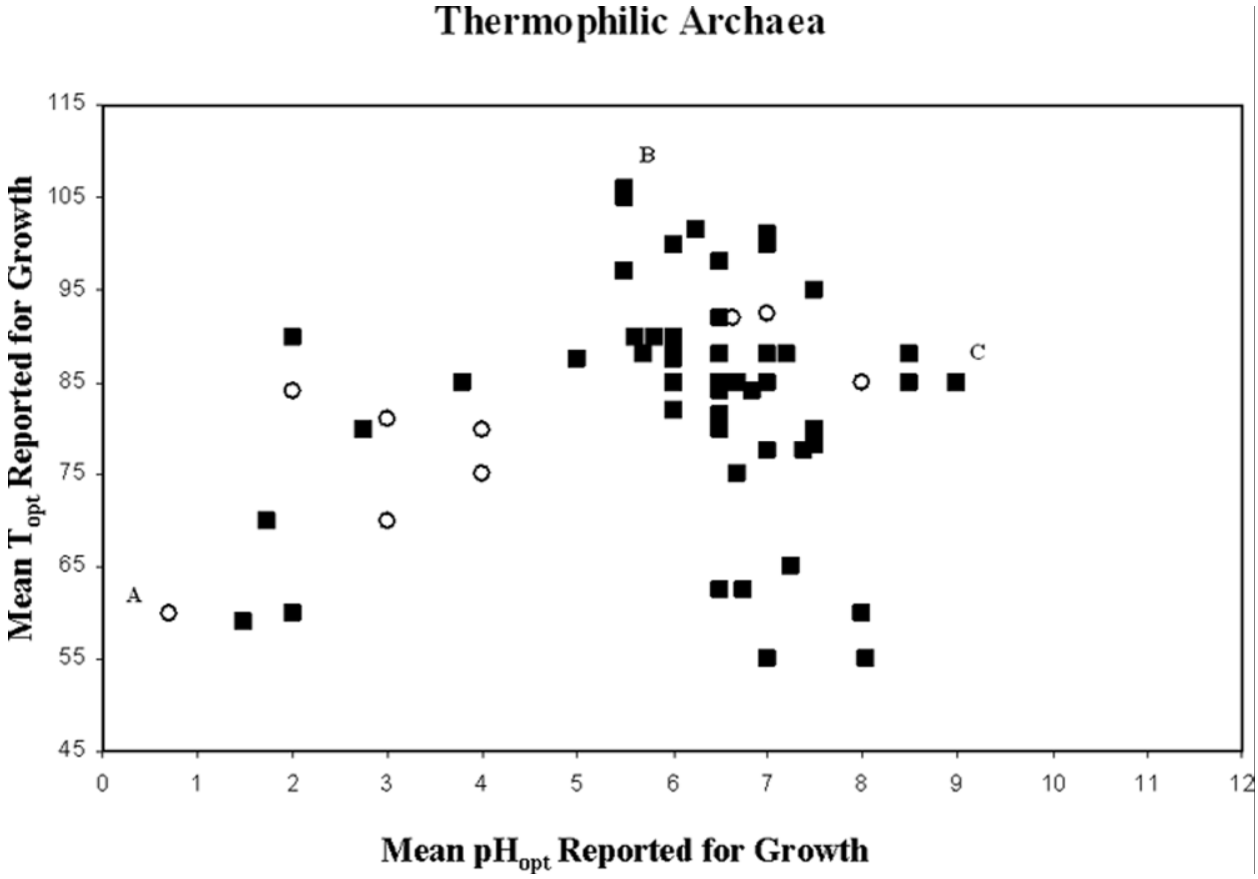

Figure 1.1 Thermophilic archaea: aerobic/microaerophilic/facultative aerobic archaea (o) and anaerobic/facultative aerobic archaea (■). A, *P. oshimae* and *P. torridus*: optimal growth at pH 0.7, 60°C (Schleper et al., 1995). B, *P. fumarii*: optimal growth at 106°C, pH 5.5 (Blochl et al., 1997). C, *Thermococcus acidaminovorans*: optimal growth at pH 9, 85°C (Dirmeier et al., 1998).

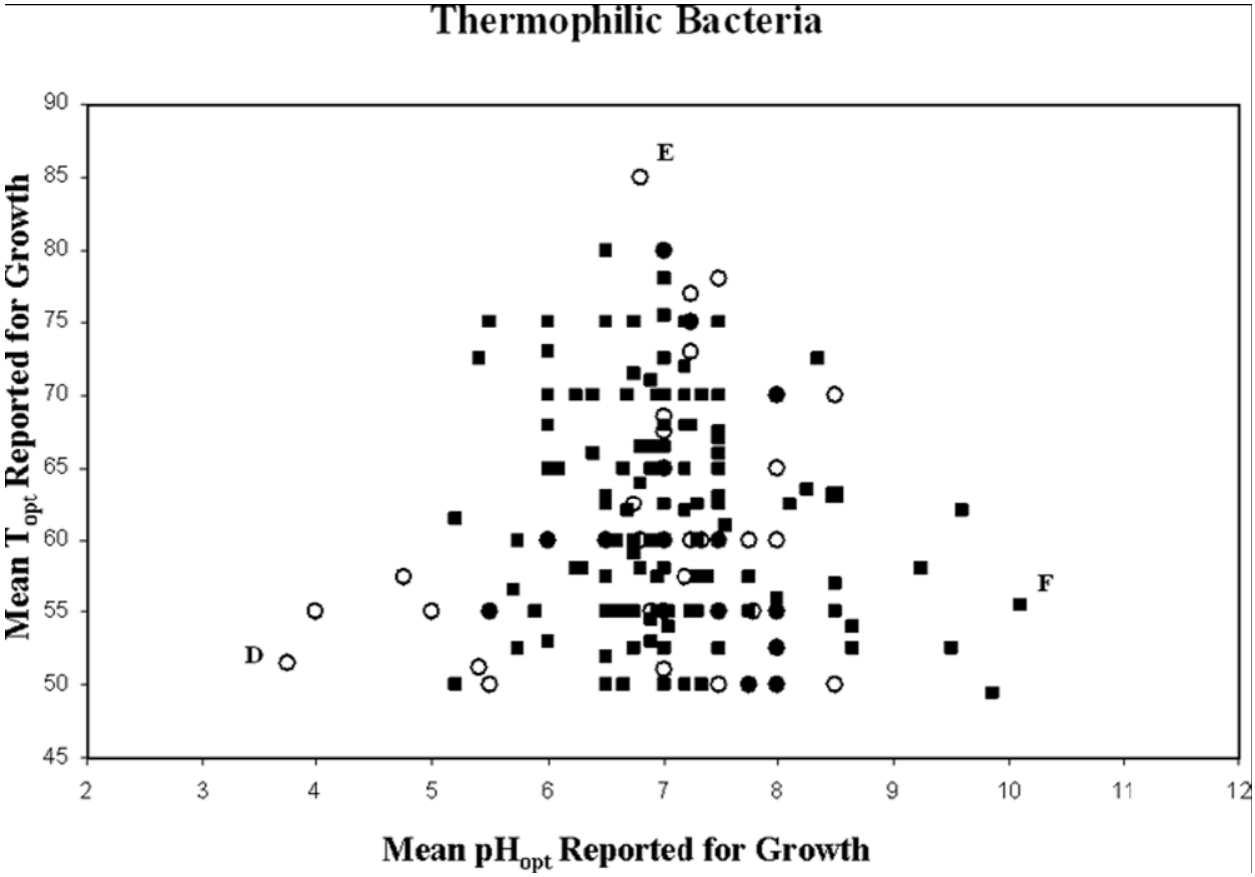

Figure 1.2 Thermophilic bacteria: aerobic/microaerophilic/facultative aerobic bacteria (o) and anaerobic/facultative aerobic bacteria (■). D, *Alicyclobacillus hesperidum*: optimal growth at pH 3.5–4.0, 50–53°C (Albuquerque et al., 2000). E, *Aquifex pyrophilus*: optimal growth at 85°C, pH 6.8 (Huber et al., 1992). F, *C. paradoxum*: optimal growth at pH25°C 10.1, 55–56°C (Li et al., 1993).

CHAPTER 2

INTRODUCTION: UZON CALDERA,

AN OVERVIEW WITH ADDITIONAL DESCRIPTION OF TWO POOLS

INTRODUCTION

Part of the Pacific Ring of Fire, the Uzon Caldera (Uzon) is on the Kamchatka peninsula, Russia, bordered by the Sea of Okhotsk on the west and the Pacific Ocean on the east. Along the Pacific coast of the peninsula, subduction of the oceanic Pacific tectonic plate under the continental Eurasian plate generates active volcanism (Karpov and Naboko 1990; Waltham 2001; Nechayev 2003). There are two volcano ranges running the length of Kamchatka. Uzon is located in the more active, eastern Vostochny range in the Semyachik geothermal region (Belousov et al. 1984; Karpov and Naboko 1990; Waltham 2001; Nechayev 2003).

Most of Kamchatka remains as it was when first described by Russian explorers in the 17th century, and the value of the pristine habitats has led to the designation of preserves, such as the Kronotsky Zapovednik, of which Uzon is a part (Waltham 2001; Nechayev 2003). Unlike the frequently-visited Yellowstone Caldera in Yellowstone National Park (YNP), United States, Uzon is considerably more isolated. Additionally, Uzon is at a lower elevation than YNP, allowing for liquid water at higher temperatures. Thus the combination of geological activity and geographic isolation make Uzon an ideal location for geomicrobiolgical research.

The research described in this dissertation was conducted as part of an international, interdisciplinary endeavor funded by the National Science Foundation Microbial Observatory Program, the Kamchatka MO. The aim of this chapter is to briefly describe the merits of research in Uzon, the geological context of the caldera and the relevant features therein, summarize the current knowledge of microbiology in Uzon, and set forth the objectives of the remaining dissertation chapters.

THE MERITS OF RESEARCH IN UZON

Research in Uzon is relevant to examining the physicochemical limits of life. A recent amoeboflagellate isolated from Uzon, with a temperature range of 28-54°C and a pH range of 1.2-5 for growth, is a new strain of thermopilic (T_{opt} 45°C) eukaryote (Baumgartner et al. in press). Another representative at the limits of the biological definition of life, the virus AFV9, was isolated from an Uzon enrichment culture at pH 3.5 and 75°C (Bize et al. 2008). There also have been many novel bacteria isolated from hydrothermal features in Uzon, as described below.

Additionally, research in Uzon has resulted in biotechnological advances. Proteases isolated from an Uzon strain of *Thermoanaerobacter* have been demonstrated as potentially useful for the neutralization of prions (Tsiroulnikov et al. 2004). The quest for thermostable agarases led to the isolation of two agarolytic strains from Uzon (Bannikova et al. 2008). Although the agarase characterized in that research was of a *Thermoanaerobacter wiegelii* isolate from the Lake Baikal region, one of the Uzon isolates has been published as a new species of *Caldanaerobacter* (Kozina et al. 2009). Additional isolates have been recovered upon enrichment for thermophiles with hydrolytic activity on various polymeric substrates (Kublanov et al. 2009).

Finally, Uzon has been cited multiple times as an analog for extraterrestrial thermal environments. In 1968, Uzon was mentioned in a comparative analysis of crater morphology on the Earth and the Moon (Steinberg 1968). The unique thermal lake Bannoe in Uzon was the source of one sulfurous sample among many characterized for interpretation of the sulfur-rich geology of Jupiter's moon Io (Kargel et al. 1999). More recently, a survey of five thermal features in Uzon provided a context for biosignature preservation in siliceous sinters that should be useful for interpretation of any such rocks observed on another planet (Goin and Cady 2009). The length of time such biosignatures might be preserved in the rock record is also relevant. Coupling of electron microscopy and microprobe analysis for two Uzon pools pointed to aluminum laminations as a preservation mechanism for the siliceous tubes known to form around actively growing cells (Kyle et al. 2007).

GEOLOGICAL CONTEXT

The contemporary appearance of the Uzon Caldera is the result of a series of events starting in the late part of the Middle Pleistocene (175-225 thousand years ago) and continuing into the Holocene (<10 thousand years ago; Belousov et al. 1984; Karpov and Naboko 1990). In the Middle Pleistocene, northeastern trending faults occurred in the area as part of a larger volcanic wide-separation fault on the peninsula, forming the southeast boundary of the depression (Belousov et al. 1984). Accompanying volcanic activity included explosive eruptions of andesitic and basaltic magma (Belousov et al. 1984; Karpov and Naboko 1990). Arcurate, low-magnitude faults, invaded by dike-forming magma, contribute to the southeast depression boundary (Belousov et al. 1984).

During the early Late Pleistocene, a series of sublatitudinal faults formed on the long axis of the Uzon-Geyser Valley depression. Two cycles of volcanic eruptions are associated with this period (Belousov et al. 1984). Collapse of the volcano formed the caldera's western boundary, the characteristic terrace of peak Barany (also known as Mt. Uzon), about 40 thousand years ago (Waltham 2001; Karpov and Naboko 1990; Figure 2.1). Eventually up to 200 m of subsidence along the sublatitudinal faults formed the north and northwest boundaries of the depression. Additional arcurate faults and dikes formed during this period as well (Belousov et al. 1984).

More recent activity in Uzon includes formation of a resurgent dome, Mt. Belaya, about 10-12 thousand years ago and the dramatic maar Lake Daln'eye sometime in the early Holocene (Belousov et al. 1984; Karpov and Naboko 1990). Lake Bannoe, the thermal pool where scientists, tourists and rangers can enjoy a warm soak, experienced a phreatic explosion as recently as the late 1980s (Karpov 1998; Waltham 2001). These intervening periods of activity and quietude have resulted in a spectacular caldera approximately 10 km in diameter, layered with inputs from basaltic and andesitic magmas and their alterations, lucastrine sediments, and ashfall from surrounding volcanoes (Bazhenova et al. 1998; Simoneit et al. 2009; Karpov and Naboko 1990; Belousov et al. 1984).

The combination of the network of faults, the heterogeneous texture of deposits, and the volcanic heat source has resulted in diverse hydrothermal features in Uzon (Belousov et al. 1984; Hollingsworth 2006). These include springs, pools, mud cauldrons and volcanoes, fumaroles, spouting prospector wells, and large thermal lakes (Karpov et al. 2000; Hollingsworth 2006; Goin and Cady 2009). A recent summary of hydrothermal features in Uzon defined a pH range of 3.1 to 9.8 and a temperature range of 20 to 90°C (Zhao et al. 2005). Unpublished measurements include temperatures of 5-7°C in a freshwater stream, at times even colder, to 105°C detected approximately 1 m in the soil near hydrothermal features. Additionally, two prospector wells, K4 and K1, spew super-heater steam.

The hyddrothermal features occur in several distinct thermal fields collectively spanning approximately 5 km of the caldera floor (Figure 2.1; Karpov and Naboko 1990; Bazhenova et al. 1998). The alternating layers of sediments and magmas with varying degrees of acid alteration described above are equivalent to alternating layers of porous and non-porous materials (Belousov et al. 1984; Waltham 2001). Deep, magmatic water occurs in the porous layers (coarse-clastic tuffs), and meteoric water percolates downward through the faults described above and collects on overlying fine-grained tuffs or in low-lying, porous textured areas (Belousov et al. 1984; Hollingsworth 2006; Karpov 1991; Karpov 1992). The heterogeneous distribution of these features provides a setting for myriad local hydrothermal fluids defined by their $\delta^{18}O_{H2O}$ and δD_{H2O} values and chemical constituents (Belousov et al. 1984; Hollingsworth 2006; Karpov et al. 2000). In the higher-elevation thermal fields, magmatic volatiles pass through perched metoric water and acid-sulfate type hydrothermal fluids are generated (Kusakabe et al. 2000; Hollingsworth 2006). Alkali-chloride fluids occur in thermal fields on the caldera floor; the alkali-chloride component is magmatic, but the hydrothermal features represent diverse mixtures of meteoric and magmatic inputs (Hollingsworth 2006; Migdisov and Bychkov 1998; Karpov et al. 2000). In 2000, upon analysis of $\delta^{18}O_{H2O}$ and δD_{H2O} values in several sites in Kamchatka, the Uzon lakes Bannoe and Fumarol'noye were described as dominated by meteoric and magmatic waters respectively (Karpov et al. 2000).

More recently, $\delta^{18}O_{H2O}$ and δD_{H2O} values were determined for additional hydrothermal features (Figure 2.2), and these data were combined with characterization of fluid chemistry and $\delta^{34}S$ values for sulfur species to develop a model of sulfur isotope fractionation in Uzon (Hollingsworth 2006). According to this model, sulfur inputs into acid-sulfate waters are dominated by H_2S gas in a narrow range of $\delta^{34}S$ values -1.5 to +1.6‰ due to the biotic and abiotic oxidation of the volatile inputs. Low-lying thermal fields receive magmatic S inputs of multiple species, including $H_2S_{(g)}$ and aqueous sulfate, with $\delta^{34}S$ value ranges of -1.5 to +1.6‰ and +25.3 to +27.1‰ respectively (Hollingsworth 2006). The variation among $\delta^{34}S$ values in the low-lying thermal fields is further influenced by *in situ* cycling by microorganisms, mineral formation and dissolution reactions, and the allocthonous inputs of light S oxidized in the acid-sulfate pools (Hollingsworth 2006; Karpov 1991). Stable isotope $\delta^{34}S$ values may provide some insight to how these sources of variation influence a unique feature in Uzon; namely that it is "the largest sulphide ore-forming hydrothermal system now active in the Kurile-Kamchatka region" (Karpov and Naboko 1990).

Magmatic alkali-chloride waters, in Uzon and elsewhere, are known for the presence of ore-forming elements (Karpov and Naboko 1990). The active mineralization in Uzon results from the combination of high concentrations of ore-forming elements, especially As and S, with the oxidation and cooling of magmatic waters as they reach the surface in the central part of the caldera (Karpov 1991). Two As-S mineralization zones have been identified: one near the surface in the central thermal field and the other in the sediments of Fumarol'noye Lake (Karpov and Naboko 1990). Mineralization in the central thermal field is better characterized (Karpov and Naboko 1990; Migdisov and Bychkov 1998; Cleverley et al. 2003). The As-S minerals in the central thermal field are forming in an "overall mushroom shape" along isotherms defined by orientation along the faults and temporal fluctuations (Karpov and Naboko 1990; Migdisov and Bychkov 1998; Karpov 1991). Along these isotherms, temperature, redox and pH conditions control the availability of various As and S species for mineral formation and thus, which minerals occur (Karpov and Naboko 1990; Eary 1992; Migdisov and Bychkov

1998; Cleverley et al. 2003). Quantification of even a subset of the relevant species, especially under field conditions, is a sophisticated undertaking (Migdisov and Bychkov 1998).

Uzon is also home to considerable elemental sulfur (S^o) deposition (Karpov 1991). There are at least two mechanisms for S^o formation: disproportionation reactions below the surface (i.e., at temperatures between 250-400°C; Hollingsworth 2006) and precipitation from polysulfanes ($H_2S \cdot S_n$) at the surface (Migdisov and Bychkov 1998; Slobodkin et al. 1999). The latter mechanism generates especially fine-grained sulfur, considered ideal for microbial reduction (Bonch-Osmolovskaya 1994; Slobodkin et al. 1999).

MICROBIOLOGICAL CONTEXT

A considerable contribution to the knowledge of thermophilic microorganisms has been made by research associated with the Winogradsky Institute of Microbiology, Russian Academy of Sciences, especially the lab of E.A. Bonch-Osmolovskaya, in Uzon. This research has encompassed targeted enrichment and isolation and characterization of metabolic pathways in hot spring samples. There have been four archaeal and 14 bacterial type strains isolated from Uzon, seven of which have been published since 2008 (Table 2.1). These isolates represent one class and two families of *Archaea*, as well as seven classes and eight families of *Bacteria*. Three of the type strains represented novel genera when published (Miroshnichenko et al. 2009; Bonch-Osmolovskaya et al. 1990; Golovacheva et al. 1985). A recent isolate representing another novel genus not yet validly published is "*Fervidococcus fontis*" Kam940 (Perevalova et al. 2008). Other isolates not yet validly published may represent novel species of previously described genera (Kublanov et al. 2009; Sokolova et al. 2009). Strains of *Sulfolobus* species (Whitaker et al. 2003) and *Thermodesulfovibrio yellowstonii* (Zavarzina et al. 2000) isolated from Uzon represent species with type-strains from other locations. These isolates represent diverse metabolisms, including autotrophy, heterotrophy, fermentation, and aerobic and anaerobic respiration (Table 2.1). Radioisotope experiments to characterize metabolic pathways in Uzon have demonstrated sucrose oxidation in acidic samples from Orange Thermal Field and the central sector of East Thermal Field (Prokofeva et al. 2006); CO oxidation

in a silt and biofilm sample collected from an Uzon hot spring (Slepova et al. 2007); and bicarbonate conversion to methane, acetate and/or biomass in samples from several locations representing multiple pH and temperature combinations (Bonch-Osmolovskaya et al. 1999). The last of these studies also examined the potential for multiple anaerobic metabolisms in Uzon hot springs, including sulfate, S^o, and Fe(III) reduction, through enrichment culturing and quantification of target end-products (Bonch-Osmolovskaya et al. 1999). Studies from Uzon were also included in two reviews of thermophilic anaerobic respiration and metal reduction (Slobodkin et al. 1999; Slobodkin 2005). Although not directly characterized, sulfidogenesis and arsenate reduction may influence the As-S mineralization in Uzon (Karpov 1991; Huber et al. 2000). Microbial activity is expected to influence biogeochemical cycling of C and N as well. Nitrification in Uzon hydrothermal features has been suggested indirectly by the presence of *amoA* genes (Reigstad et al. 2008) and by 16S rRNA gene sequences that are related to sequences associated with *amoA* genes and nitrification activity elsewhere (Perevalova et al. 2008).

Other molecular studies in Uzon have included DNA hybridization with group specific probes, 16S rRNA gene sequencing, and membrane lipid profiling. Probes for the genus *Thermoanaerobacter* were used to characterize two isolates from Uzon, one as a member of the genus and the other not (Subbotina et al. 2003). *Crenarchaeota*-specific primers and *Desulfurococcus* probes identified the presence of *Desulfurococcus* in an environmental sample from Uzon (Perevalova et al. 2003). The same primer set was used to sequence 16S rRNA genes from additional environmental samples from Uzon. The data collected from that study placed the crenarchaeoic sequences in the context of similar data from other locations and suggested pH and temperature ranges for some of these groups (Perevalova et al. 2008). Sequencing of 16S rRNA genes has also been used to characterize anaerobic enrichment cultures with organic substrates under acidic conditions (Prokofeva et al. 2005) and to identify a previously undescribed phylum of Archaea, the "Nanoarchaeota" (Hohn et al. 2002), in samples from Uzon and elsewhere. However, sequence surveys in Uzon have not included characterization of 16S rRNA genes with commonly-used "universal" primers for *Archaea* and *Bacteria*. Membrane lipid profiling has included both archaeal and bacterial lipids (Pearson et al. 2008; Zhao 2008). An extensive survey of

glycerol dialkyl glycerol tetraethers (GDGT) in environmental samples from hydrothermal features in Uzon, YNP, Nevada, and China, as well as in pure cultures, indicated the relative importance of pH, temperature and geochemistry in controlling GDGT distributions (Pearson et al. 2008). Although comparisons among different hydrothermal features are possible with this data, they do not indicate variation within individual sites.

STUDY SITES AND OBJECTIVES

Two hydrothermal features were selected for the research described in this dissertation, Arkashin Schurf (Arkashin) and Zavarzin Spring (Zavarzin). Both of the hydrothermal features sampled in this study are pool-like rather than spring-like (Karpov 1998). The two hydrothermal pools are located in different sectors of the low-lying East Thermal Field, on opposite sides of a fault (Belousov et al. 1984; Karpov 1992; Karpov and Naboko 1990). Arkashin is located in the central sector of the East Thermal Field (CTF; Figure 2.1). The CTF can be described as a gravellite-textured depression in the surrounding terrain of forested hummocks. Arkashin was named after Arkadiy Loginov who dug the schurf during a prospecting expedition to Uzon. This pool has been stable in size and shape since its first description over 20 years ago (Karpov et al. 1988 in Russian; Sokolova pers. comm.). Arkashin, rich in As, is in the region of As-S mineralization described above. Zavarzin is located in the eastern sector of the East Thermal Field (ETF; Figure 2.1) near the mouth of a stream that carries inputs of freshwater into the area, especially during annual snowmelts (Karpov 1998). The ETF is larger and more heterogeneous than the CTF, and the area where Zavarzin is located is level with the surrounding terrain. Zavarzin, a natural hydrothermal feature, is named after G.A. Zavarzin, who was a pioneer of microbiology in Uzon (as cited in Bonch-Osmolovskaya 2004). The size and shape of Zavarzin have varied somewhat as vents have emerged and collapsed and the pools' margins have expanded and contracted (Karpov 1998). Zavarzin is the site of considerable elemental sulfur deposition.

The objectives of this research were to:

1. Catalog the communities in each pool in order to provide clues about the pool's ecology and enable comparisons to other locations. This objective was met in the third chapter of this dissertation with a description of 16S rRNA gene sequences, phospholipid fatty acids (PLFA) and GDGTs for each pool. The information from these descriptions is coupled with analysis of C, N and S stable isotopes and knowledge from cultured microorganisms to provide an ecological description of each pool.

2. Determine if the geochemistry and microbiology co-vary across a visible scale with depth. This objective was met in the fourth chapter where changes in geochemistry, C and N stable isotope δ-values, and PLFAs are characterized in core samples from each pool, demonstrating the variation observable across centimeters from the surface.

3. Examine the relationship between biological sulfate-reduction and As-S mineralization. This objective was met in the fifth chapter with measurement of sulfate-reduction rates and S stable isotope δ-values in core samples from each pool.

4. Isolate arsenate and sulfate reducing prokaryotes. Although isolation attempts were not successful for the target metabolisms, two appendices are provided detailing the methods used.

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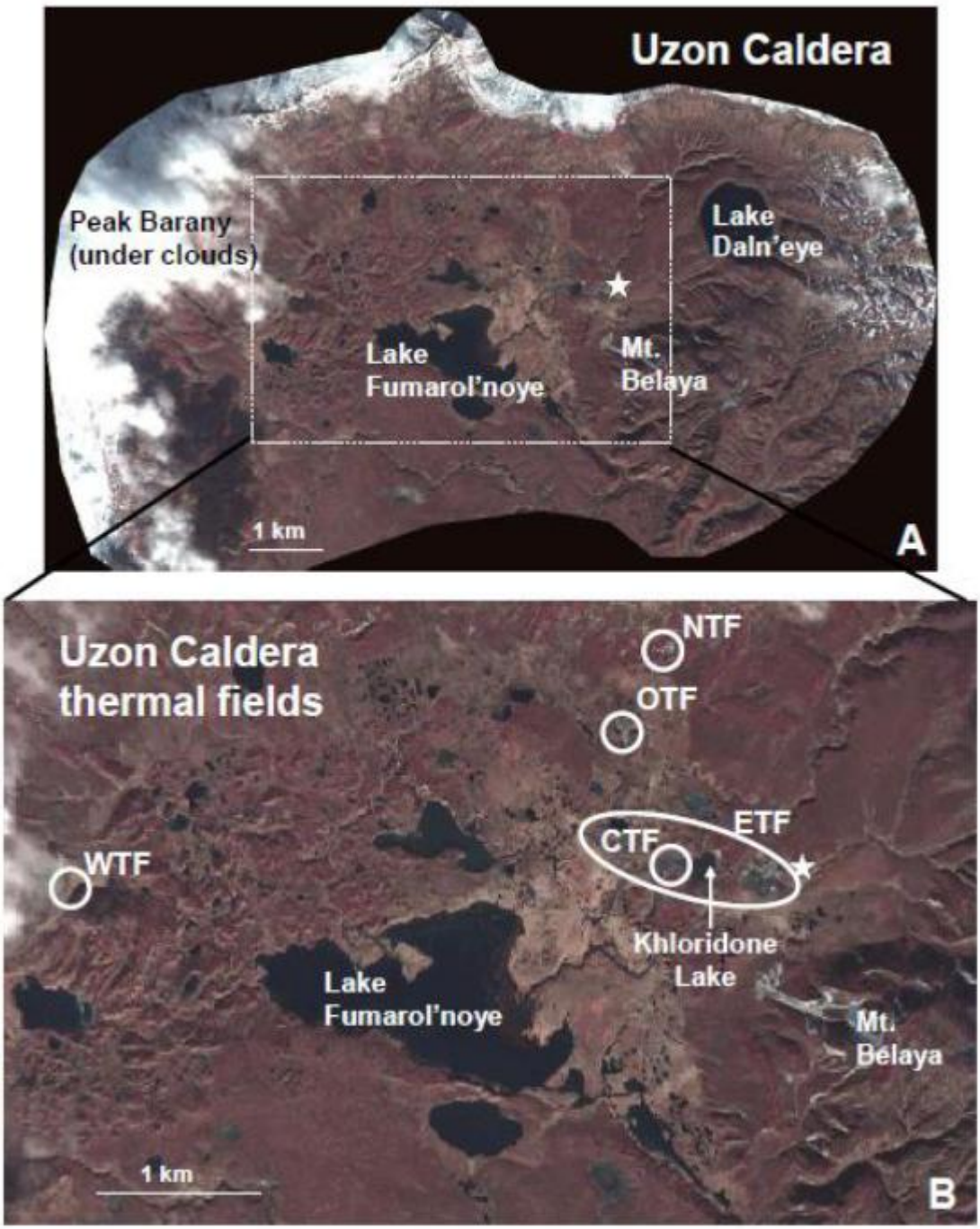
Table 2.1 Type-strain isolates from Uzon Caldera

Name	Phylum*	Class	Family	Location	Temperature range, optimum (°C)	pH range, optimum	H, A or both + additional e-sources**	F, R or both**	e- acceptors**	Year 1st Author
Archaea										
Desulfurococcus fermentans Z-1312^T	*"Crenarchaeota"*	*Thermoprotei*	*Desulfurococcaceae*	freshwater hot spring	63-89, 80-82	4.8-6.8, 6.0	H	F	S^o stimulates	2005 Prevalova
Desulfurococcus kamchatkensis 1221n^T	*"Crenarchaeota"*	*Thermoprotei*	*Desulfurococcaceae*	Izvilistyi Spring, CTF	65-87, 95	5.5-7.5, 6.5	H	F	S^o stimulates	2009 Kublanov
Sulfurococcus mirabilis INMI AT-59^T	*"Crenarchaeota"*	*Thermoprotei*	*Sulfolobaceae*	couldn't get location informaiton	50-86, 70-75	1.0-5.8, 2.0-2.6	H + S^o	R	O_2	1987 Golovacheva via Chan
Thermoproteus uzoniensis Z-605^T	*"Crenarchaeota"*	*Thermoprotei*	*Thermoproteaceae*	hot springs, soil and geothermal waters	74-102, 90	6.6-6.8, 5.6	H	both	S^o	1990 Bonch-Osmolovskaya
Bacteria										
*Dictyoglomus turgidum (*basonym *D. turgidus)* Z-1310^T	*"Dictyoglomi"*	*"Dictyoglomia"*	*"Dictyoglomaceae"*	Oranzhevoe Pole Spring	48-86, 72	5.2-9.0, 7.0-7.1	H	F	n/a	1988 Svetlichnii & Svetlichnaya
Ammonifex thiophilus SR^T	*"Firmicutes"*	*"Clostridia"*	*"Thermo-anaerobacteraceae"*	Treshchinnyi Spring. ETF	60-82, 75	6.0-7.5, 6.8	A + H_2, formate	R	S_2O3^{-2}, SO_4^{-2}, S^o	2008 Miroshnichenko
Caldanaerobacter uzonensis K67^T	*"Firmicutes"*	*"Clostridia"*	*"Thermo-anaerobacteraceae"*	Thermophilny Spring, ETF	50-70, 68-70	4.8-8.0, 6.8	H	both	$S^2O_3^{-2}$	2009 Kozina
Thermoanaerobacter sulfurophilus L64^T	*"Firmicutes"*	*"Clostridia"*	*"Thermo-anaerobacteraceae"*	hot pond, sulfur-containing cyanobacterial mat	44-75, 55-60	4.5-8.0, 6.9-7.2	H	both	S^o, $S_2O_3^{-2}$	1997 Bonch-Osmolovskaya
Thermoanaerobacter uzonensis JW/IW010^T	*"Firmicutes"*	*"Clostridia"*	*"Thermo-anaerobacteraceae"*	small pool feeding Winding Spring, CTF	32.5-69, 61	4.2-8.9, 7.1	H	both	$S_2O_3^{-2}$	2008 Wagner
Thermo-anaerobacterium aciditolerans 761-119^T	*"Firmicutes"*	*"Clostridia"*	*"Thermo-anaerobacteraceae"*	hydrothermal vent, OTF	37-68, 55	3.2-7.1, 5.7	H	both	$S_2O_3^{-2}$, SO_3^{-2}	2007 Kublanov
Thermovenabulum ferriorganovorum Z-9801^T	*"Firmicutes"*	*"Clostridia"*	*"Thermo-anaerobacteraceae"*	Zhelezisti (Ferrous) freshwater hydrothermal source	45-76, 63-65	4.8-8.2, 6.7-6.9	H + H_2	both	Fe(III), Mn(IV), NO_3^{-2}, SO_3^{-2}, $S_2O_3^{-2}$, S^o fumarate	2002 Zavarzina
Caldalkalibacillus uzonensis JW/WZ-YB58^T	*"Firmicutes"*	*"Bacilli"*	*Bacillaceae*	Zavarzin Spring, ETF	42-64, 50-52	6.4-9.7, 8.2-8.4	H	R	O_2	2008 Zhao
Hydrogenobacter hydrogenophilus Z-829^T *(*basonym *Calderobacterium hydrogenphilum)*	*"Aquificae"*	*Aquificae*	*Aquificaceae*	couldn't get location information	50-82, 72-76	nr, 6.0-7.0	A + H_2	R	O_2	1983 Kryukov
Sulfurihydrogenibium rodmanii UZ3-5^T	*"Aquificae"*	*Aquificae*	*Hydrogen-othermaceae*	Jenn's pool, CTF	55-80, 75	5.0-7.2, 6.0	A + S^o, $S_2O_3^{-2}$	R	O_2	2008 O'Neill
Desulfurella acetivorans A63^T	*"Proteobacteria"*	*d-Proteobacteria*	*Desulfurellaceae*	hot water pool	44-70, 52-57	4.3-7.5, 6.8-7.0	H + H_2	R	S^o	1990 Bonch-Osmolovskaya
Desulfurella propionica U-8^T	*"Proteobacteria"*	*d-Proteobacteria*	*Desulfurellaceae*	high sulfide hot pond, cyanobacterial mat	33-63, 55	nr, 6.9-7.2	H + H_2	R	S^o, $S_2O_3^{-2}$	1998 Miroshnichenko
Thermanaerovibrio velox Z-9701^T	*"Synergistetes"*	*Synergistia*	*Synergistaceae*	hot spring, cyanobacterial mat	45-70, 60-65	4.5-8.0, 7.3	H + H_2	R	S^o	2000 Zavarzina
Caldimicrobium rimae DS^T	*"Thermo-desulfobacteria"*	*Thermo-desulfobacteria*	*Thermo-desulfobacteriaceae*	Treshchinnyi Spring. ETF	52-82, 75	6.8-7.4, 7.0-7.2	both	R	$S_2O_3^{-2}$, S^o	2009 Miroshnichenko

*taxonomy as per the List of Prokaryotic Names with Standing in Nomenclature (J.P. Euzéby, www.bacterio.net), " " indicate a name that has not been validly published

** H=heterotrophic, A= autotrophic, e-=electron, F=fermentation, R=respiration, n/a=not applicable

Figure 2.1 Quickbird satellite images of the caldera indicating major features (A) and the thermal fields (B) described in the text. The white star in both panels indicates the location of the Kamchatka Microbial Observatory research camp. WTF=West Thermal Field; NTF=North Thermal Field; OTF=Orange Thermal Field; ETF=East Thermal Field; CTF=Central Thermal Field, sometimes referred to as the central sector of ETF.


Uzon Caldera
Peak Barany
(under clouds)
Lake
Daln'eye
Lake
Fumarol'noye
Mt.
Belaya
1 km
A
Uzon Caldera
thermal fields
NTF
OTF
ETF
CTF
WTF
Khloridone
Lake
Lake
Fumarol'noye
Mt.
Belaya
1 km
B

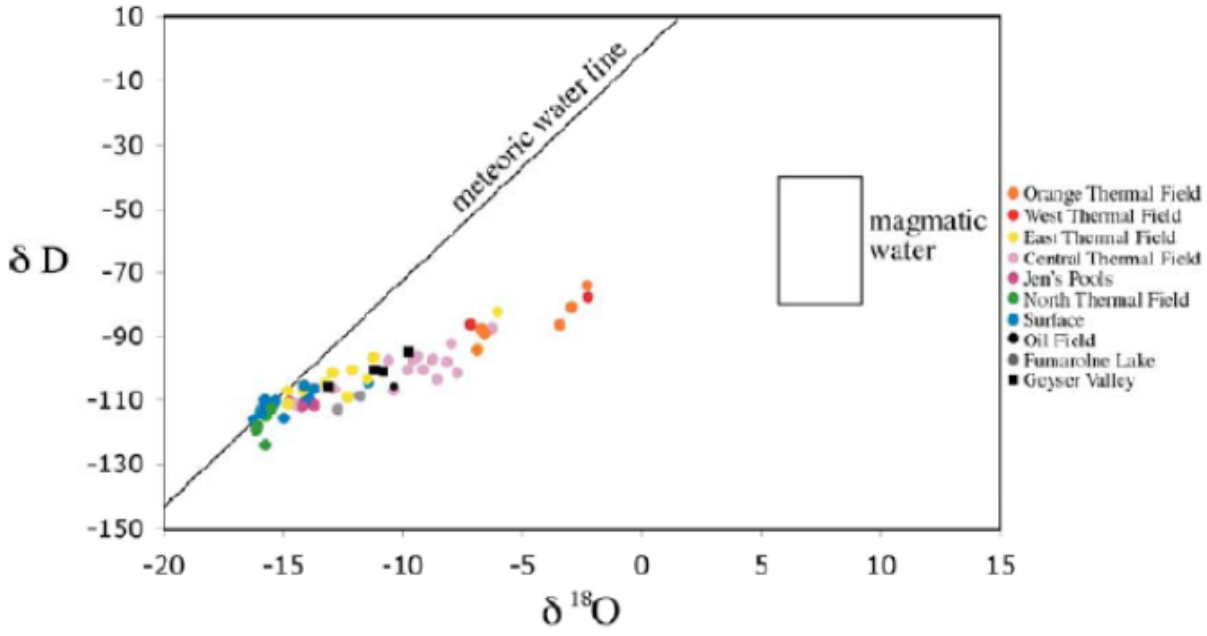


Figure 2.2 Plot of δD and $\delta^{18}O$ values for waters of the Uzon Caldera, grouped by thermal field or relevant feature (NSF Kamchatka MO Annual Report 2004-2005, based on data from C.S. Romanek and D. Crowe).

CHAPTER 3

COMPARATIVE GEOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF TWO THERMAL POOLS IN THE UZON CALDERA, KAMCHATKA, RUSSIA[2]

[2]Burgess, E. A., Unrine, J.M., Mills, G. L., Romanek, C.S., Wiegel, J. In preparation.

ABSTRACT

Contemporary hydrothermal systems on Earth are of interest as ecological systems and for cataloging the occurrence of microorganisms that inhabit them. Two active thermal pools in the Uzon Caldera, Kamchatka, Russia, (Arkashin Schurf (Arkashin) and Zavarzin Spring (Zavarzin)), were sampled to characterize geochemical conditions and bacterial and archaeal communities and to determine if either pool contained novel microoganisms. Each pool was geochemically and microbiologically distinct. Sediments in Arkashin relative to Zavarzin demonstrated high concentrations of total As, 4252 mg kg^{-1}. The glycerol dialkyl glycerol tetraether (GDGT) profiles from Arkashin were dominated by GDGT-4. From Arkashin, 183 to 350 μg g^{-1} of phospholipid fatty acids (PLFA) were recovered and included biomarkers for *Aquificales*. In Arkashin, 206 Bacterial 16S sequences were assigned to 19 OTUs. The most abundant OTUs in Arkashin contained sequences related to the "*Sphingobacteria*", *Hydrogenobaculum* of the *Aquificales* and *Variovorax* of the β-*Proteobacteria*. Twenty-percent of Arkashin clone sequences had <90% sequence identity to isolated, type-strain microorganisms. Relative abundance among archaeal GDGTs was more evenly distributed in Zavarzin than in Arkashin. Ninety-one archaeal 16S rRNA sequences were recovered from Zavarzin and assigned to 31 operational taxonomic units (OTUs) representing the *Crenarcheota* and "*Korarchaeota".* Approximately one quarter of the Zavarzin archaeal sequences appeared to represent unique clades of *Archaea.* Biomarkers in the 436 to 455 μg g^{-1} of PLFA recovered from Zavarzin included those for Chloroflexus as well as for heterotrophic bacteria. The 300 Bacterial 16S sequences analyzed from Zavarzin were assigned to 59 OTUs. The most abundant of these contained sequences was related to *Rosiflexus* and other abundant groups were related to the δ-*Proteobacteria* and the "*Clostridia*." In Zavarzin, nearly 50% of clone sequences had <90% identity to isolated type-strain microorganisms. These data, coupled with stable isotope data indicate that *Hydrogenobaculum* is a primary producer in Arkashin and *Chloroflexus* may be a primary producer in Zavarzin. The sequences related to uncultured microorganisms in each pool indicate these pools are suitable ecosystems for further characterization.

INTRODUCTION

A large portion of current knowledge about microorganisms in hydrothermal features is from culture independent methods such as sequence, lipid, and stable isotope analyses. For example, early experiments with environmental DNA from Obsidian Pool in Yellowstone National Park (YNP) revealed previously unrealized diversity (Barns et al. 1994; Hugenholtz et al. 1998). Lipid biomarker profiles and stable isotope data have provided additional insights (Jahnke et al. 2001; House et al. 2003; Zhang et al. 2004). The combination of these data can be interpreted such that sequences identify community members; bacterial phospholipid fatty acid profiles semi-quantitatively represent the active and abundant community members; and C, S and N stable isotope δ-values can provide clues to biogeochemical cycling.

Through examination of multiple pools in multiple locations, factors influencing the diversity of a community in a thermal pool, including temperature, pH, in-situ chemistry and biogeography, have been identified (e.g. Skirnisdottir et al. 2000; Mathur et al. 2007; Pearson et al. 2008)). The cataloging and characterization of such communities continues and can be expected to further inform hypotheses about the structure, dynamics and distributions of microbial populations.

Uzon Caldera is an active hydrothermal system in the Eastern volcanic zone of Kamchatka, Far East Russia. Although well-represented in culture-collections (Bonch-Osmolovskaya 2004), Uzon has remained relatively under-sampled by culture-independent methods until recently (Perevalova et al. 2008). The thermal fields in Uzon are heterogeneous and include multiple distinct hydrothermal features such as the two pools selected for this study (Figure 3.1). Arkashin Schurf and Zavarzin Spring are characterized here geochemically and their community composition, structure, and function by 16S rRNA sequence, phospholipid fatty acid (PLFA), glycerol dialkyl glycerol tetraether lipid (GDGT), and stable isotope analyses are described. The objectives of this study were to describe the microbiological communities and to determine if any novel microorganisms occur in these pools.

METHODS

Sampling sites description

Uzon is comprised of multiple thermal fields delineated by the underlying fault system that generates diverse hydrothermal features (Belousov et al. 1984; Karpov 1998). Both of the hydrothermal features sampled in this study are pool-like rather than spring-like as compared, for example, to features such as Thermophile Spring (Karpov 1998) or Amphitheater Springs (Mathur et al. 2007). Both pools are in the low-lying East Thermal Field, which is comprised of multiple, hydrologically distinct sectors (Belousov et al. 1984; Karpov 1992). Arkashin Schurf (Arkashin) is located in the central sector of the East Thermal Field. The sector can be described as a gravellite-textured depression in the surrounding terrain of forested hummocks. This pool has been stable in size and shape since its first description over 20 years ago (Karpov et al. 1988; T. Sokolova, pers. comm.).

Zavarzin Spring (Zavarzin) is in the eastern sector of East Thermal Field, located in the southeastern edge of the sector near the mouth of a stream that carries inputs of freshwater into the area, especially during annual snowmelts (Karpov 1998). The size and shape of Zavarzin have varied somewhat as vents have collapsed or emerged and the pools' margins have expanded and contracted (Karpov 1998).

Sample collection and storage

Water and sediment for sample A04 were collected in August 2004 into sterile polypropylene 50-mL tubes from several spots in Arkashin using a sampling dipper. Samples A05 and Z05 were collected in September 2005 from several spots in Arkashin and along the inside wall of the largest vent in Zavarzin into sterile 100-mL glass bottles fitted with rubber stoppers and screw-caps. Additionally, temperature and pH were measured in several locations in each pool by multiple methods (digital probes, liquid-in-glass thermometers, pH paper; etc.) over several days. Upon returning to the lab, samples were homogenized and aliquoted for storage at -80°C until further processing.

DOC measurements

Surface water samples were collected from each pool and filtered into premuffled vials with 10 μL saturated $HgCl_2$ solution. After returning from the field, 5 mL of each sample was combined with 20 μL of 2 N HCl in VOC vials and analyzed on a TOC-5000A (Shimadzu Corp.; Kyoto, Japan).

Particle Size Characterization

A spatula was used with aseptic techniques to collect approximately 2-5 g of sediment for particle size characterization by a modification of the micro-pipette method (Miller and Miller 1987). Sediment weights were recorded and sediment combined with 40 mL of dispersant solution (0.05% sodium metaphosphate, 0.01 M NaOH) in 50 mL centrifuge tubes and shaken horizontally for approximately 96 hours. Samples were removed from shaker, shaken thoroughly by hand and left to settle for 110 minutes. From each tube, 2.5 mL was collected from a depth of 2.5 cm, dispensed into a pre-weighed aluminum tin, dried overnight and weighed to determine percent clay content. These values were corrected by the weight of salts in the dispersing solution. The remaining sediment was poured through a 2 mm sieve and the >2mm fraction weighed to determine percent gravel content. Considered as a single fraction, the sand and silt content was determined as the remaining percent of total weight.

Trace Element Chemistry

For elemental analysis, approximately 2 g sub-samples were weighed, dried at 60°C, re-weighed, and ground using an acid-washed mortar and pestle. Two approximately 0.25 g replicates from each dried and ground sediment sub-sample were suspended in 10 mL trace metal grade nitric acid and digested at 180°C for 10 min in sealed fluoropolymer bombs using a MARSXpress microwave digestion system (CEM, Mathews, NC, USA; EPA Method 3052H (EPA 1996). The digestates were then diluted to 50 mL with 18 MΩ deionized H_2O. Analytical method blanks and standard reference materials (SRM) were included in each digestion batch. Following digestion, samples, blanks and standards were analyzed for

24 elements (Li, Be, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Cd, Cs, Ba, Tl, Pb, U, Hg) via inductively coupled plasma mass spectrometry (ICPMS) on an Elan DRC Plus (PerkinElmer SCIEX; Waltham, MA, USA). Detection limits for the 2005 samples ranged from 0.014 $\mu g\ kg^{-1}$ Cs to 12.4 $\mu g\ kg^{-1}$ Al; relative percent difference (RPD) of replicate dilutions from 0.53% for Al to 7.41% for Pb; average RPD of replicate digests from 4.16% for Cs to 18.2% Mg; spike recovery from 95% for Zn to 131% for Ca; recovery from standard reference materials (SRM) was 32% for Sr to 97% for Cu. Results are presented in $mg\ kg^{-1}$ of original sample weight.

S, C and N Content and Stable Isotope Ratios

The sulfur content and $\delta^{34}S$ values were determined by oxidation of total sulfur in sediment sub-samples to SO_2 (Ueda and Krouse 1986). In short, oven-dried and ground samples were further ground together with V_2O_5, SiO_2, and Cu powders, packed with quartz wool into quartz glass tubes, and reacted under vacuum at 1050°C. SO_2 was extracted using a conventional vacuum line equipped with a variable temperature trap. Purified SO_2 was analyzed on a Finnigan MAT 252 mass spectrometer. Standard reference materials IAEA-S1 ($\delta^{34}S$ = -0.3 ‰) and NBS-123 ($\delta^{34}S$ = +17.1 ‰) were prepared and analyzed with samples. Sample isotopic results were normalized to these standards using a two-point scale, thus $\delta^{34}S$ values are reported relative to Vienna Cañon Diablo Troilite (VCDT). Replicate analyses of standard reference materials indicated a resolution of the method of +/- 0.4 ‰ .

To determine total C (C_{tot}) and N percent composition and $\delta^{13}C_{tot}$ and $\delta^{15}N$ values, oven-dried, ground and homogenized sub-samples were weighed into combustion tins and analyzed at the Ecology Soil Analysis Laboratory (Univ. of GA). Replicate analyses of standards indicated a resolution of < +/- 0.3 ‰. Among repeated measures of the same samples, the highest variance was +/- 1.3 ‰. For organic C (C_{org}), oven-dried and ground sediments were weighed into ceramic crucibles. Sediments were then acidified with 1 N HCl until effervescence subsided and re-oven-dried. Acidification and oven-drying were repeated until no effervescence was observed upon addition of HCl (Ryba, et al. 2002). Due to

changes in sample consistency during this process, samples were re-homogenized with a spatula before being packed into tins and analyzed as described above for C_{tot}. Variation among repeated measures was greater for C_{org} than for $C_{tot,}$ up to +/- 3.4 ‰. The C_{tot}, C_{org} and N δ-values are presented as $\delta^{13}C_{tot}$ vs. Vienna Pee Dee Belemnite (VPDB), $\delta^{13}C_{org}$ vs. VPDB and $\delta^{15}N$ vs. Air respectively. Content results are presented in percent composition of original sample weight.

Lipid extraction, fractionation and analysis

Sediments for lipid analysis were stored at -80°C until extraction by common methods (White, et al., 1998). Before lipid extraction, sediments were freeze-dried and approximately 10 g weighed out into glass centrifuge tubes. A sample blank was processed along with the samples and, for PLFA extractions, a fatty-acid methyl ester (FAME) internal standard, 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (PL, 19:0), was added to each tube. Total lipids were extracted overnight in 1:2:0.8 chloroform:methanol: 50 mM phosphate buffer. A total of four independent total lipid extractions were performed for each sample A05 and Z05, two each for PLFA and GDGTs.

From the first set of extractions, polar lipids were recovered via silicic acid column chromatography. PLFA were transesterfied with methanol to fatty acid methyl esters (FAME) (White and Ringelberg 1998). FAME were separated by gas chromatography (GC) on a Agilent HP 6890 Series GC system with a DB-5 column (30 m; 0.25 mm id) and flame ionization detector using He as the carrier gas and 250°C as the inlet temperature. The temperature program for FAME analysis was as follows: 80°C for 1 min, 10°C/min to 150°C, 2°C/min to 190°C, 3°C/min to 235°C, and finally 10°C/min to 280°C held for 20 min.

Individual chromatographic peaks were identified by retention time as compared to those of standard reference components. In the instance where an individual PLFA could not be identified due to the tendency of multiple fatty acids to exhibit the same retention time, possible identifications are

indicated as a co-eluting group. A number of co-eluting groups were further resolved using an Agilent 5890 series GC with a DB-5MS column (30 m; 0.25 mm id) and an Agilent 5973 mass selective detector.

Sample profiles were compared to the profile of the sample blank. Unidentifiable peaks occurring in the sample blank were removed from analysis in the samples. The calculated μg of identifiable peaks occurring in the sample blank was subtracted from the μg of the same peaks in the samples.

PLFA were named as the number of carbons in the chain, followed by a colon and the number of double bonds. The ω notation indicates the position from the terminal carbon where the double bond occurs, followed by "c" or "t" to indicate *cis* or *trans* orientation in the case of single double bonds. A methyl-branched carbon chain length is preceded by an "I" for iso- or an "a" for anteiso- to indicate branch location.

The second set of samples were subjected to methanolysis then fractionated on a 500 mg Octadecyl Solid Phase Extraction column (Allied Signal, Inc., Burdick & Jackson, B&J Inert SPE System, B & J EnviroPrep, cat # 7004GW). The GDGT fraction was recovered in 1:3 ethyl acetate:hexane, transferred to 2 mL chromatography vials, then dried under N_2 and shipped to Dr. Ann Pearson for LC-MS analysis and chromatogram interpretation as per Pearson (2008). The GDGTs are named as GDGT-0 through -6 indicating the number of cyclopentane rings present. The well-known GDGT unique in having a cyclohexane ring and four cyclopentane rings is named as crenarchaeol. GDGT regioisomers were not observed.

DNA extraction

Environmental DNA was extracted from ~15 g of A04 (Arkashin 2004) sediment using 1 g in each of 15 Ultra-Clean Soil DNA Kit preps (MoBio) following the manufacturer's instructions. The extraction products were pooled and ethanol precipitated. Environmental DNA was also extracted from ~10 g each of sediment from samples A05 (Arkashin 2005) and Z05 (Zavarzin 2005) using the PowerMax Soil DNA Isolation Kit (MoBio) following the manufacturer's instructions.

PCR, clone library construction, sequencing

DNA extraction products were used as template in PCR for 16S rRNA genes with universal primer sets 27f & 1492r (Bacteria-specific) 21f & 1492r (Archaea-specific). The final PCR mix per 25 uL reaction had 0.4x each primer, 0.2 mM each dNTP, 0.5x bovine serum albumin, 1x PCR buffer, 1 mM $MgCl_2$, and 1.25 units JumpStart Taq (Sigma). Tremplate was added in multiple concentrations and the reaction yielding the cleanest product was chosen for ligation. Optimized reaction conditions for bacterial primers were 95°C for 5 min; 95°C for 45 s, 60°C for 30 s, and 72°C for 2 min 30 times; and 72°C for 4 min. Conditions for archaeal primers were the same except the annealing temperature was 50°C rather than 60°C.

PCR products were ligated into Topo-TA kit vector pCR 2.1 and the ligation products were used to transform Top10 *E. coli*, following manufacturer's instructions (Invitrogen). The transformation culture was spread-plated onto S-Gal™/LB Agar (Sigma-Aldrich) with 50 mg/L ampicillin, incubated overnight at 37°C, then stored at 4°C for several hours. White colored colonies were hand-picked and inoculated into 96-deep well plates of Luria-Broth with 50 mg/L ampicillin. Deep well plates were incubated at 37°C with shaking at 200 rpm for 24 to 48 hours. Additional Luria-Broth with 50 mg/mL apicillin and glycerol were added to the plates to a final concentration of 15% glycerol. An aliquot from each well was transferred to a small-volume 96-well plate. Plates were sealed and stored at -80°C. Small-volume plates, after freezing, were shipped on ice for sequencing to SeqWright or the Sequencing and Synthesis Facility (Univ. of GA). Cultures were plasmid-prepped and bacterial clones were sequenced from the 27f primer. Archaeal clones were sequenced from vector primers M13f and M13r, but were analyzed from the 21f direction only. Sequences from each library were designated by sample site and primer set such that ZA indicates sequences from the Zavarzin archaeal library, and ZB and AB indicate sequences from the Zavarzin bacterial and Arkashin bacterial libraries respectively.

Database Information

All sequences analyzed in this publication were deposited to GenBank and were assigned accession numbers GQ328121 through GQ328717.

Analyses

Relative abundance of various PLFA in each profile was determined based on recovery of an internal standard. These data are presented in mol% estimated from retention time. The mol% of unknown peaks was summed prior to calculation of relative abundance. Diversity of PLFA profiles was determined using Estimate S (Colwell, R.K., http://purl.oclc.org/estimates).

Chromatograms from sequence libraries were uploaded to Sequencher v. 4.5 (GeneCodes, Ann Arbor, MI). Vector sequences were removed. Sequences were trimmed for quality using criteria modified such that <10 ambiguities remained in most sequences after trimming. Sequences <450 bp were removed from analysis. Remaining ambiguities were corrected manually. Chimeras were identified using Bellerophon (Huber et al. 2004) and removed prior to further analysis.

Initial identification of sequences from each library was done using RDP Classifier (Wang et al. 2007). Sequences were assigned to various taxonomic Classes based on >80% confidence in the classification.

Sequence alignments were performed using the on-line NAST alignment tool and gaps common to all sequences in the alignments were removed (DeSantis et al. 2006). Alignments were trimmed to the length of the shortest sequence(s) in the alignment using GeneDoc (Nicholas, K.B., http://www.psc.edu/biomed/genedoc).

Operational taxonomic units (OTUs) were identified using DOTUR (Schloss and Handelsman 2005) and a Jukes-Cantor distance matrix generated using Mega 3.1 (Kumar et al. 2004). Representative sequences from each OTU defined by 0.03 average distance among neighbors were chosen for further analysis. The 0.03 average distance among neighbors was chosen following comparative assessment of all three sequence libraries as a balanced representation of diversity in each.

Using myRDP and SeqMatch (Cole et al. 2009), sequences closely related to OTU representatives were identified and downloaded for 16S rRNA gene trees. The current List of Prokaryotic names with Standing in Nomenclature (Euzéby, J.P., http://www.bacterio.net) was taken into consideration in taxonomic interpretation and additional sequences were downloaded as type representatives of the various taxonomic Classes identified by RDPClassifier. Taxonomically and/or phylogenetically unresolved classes are presented in quotes (e.g. "Clostridia") or indicated by the distinction *incertae sedis* (meaning "of uncertain placement").

Sequences for trees were aligned with the NAST tool as described above. Gene trees were made from by neighbor-joining from a Jukes-Cantor distance matrix based on pairwise deletion of missing data using Mega 3.1. Bootstrap values represent 100 replicates, only values >50% are shown. Clades with strong bootstrap support were generally also strongly supported in trees generated using different distance algorithms (*e.g.*, Tajima-Nei and Kimura 2-parameter models) and minimum evolution and maximum parsimony tree-building methods (data not shown). Delineations of described Classes in trees are based on taxonomic as well as phylogenetic groupings, therefore many branches were flipped to bring together sequences from the same taxonomic groups although they may not belong in the same clade in the trees presented.

Comparison of AB and ZB libraries was performed using [Web]Libshuff (Singleton et al. 2001) and a DNAML distance matrix generated using Phylip (ver 3.6; Felsenstein, J., http://www.phylip.com) from the NAST alignment described above. Coverage for all three libraries was calculated for OTUs at 0.03 distance among members as [1 - (n / N)] x 100; n is the number of singleton OTUs and N is the total number of sequences ((Park et al. 2008) citing Good 1953).

RESULTS

Field Observations and physico- and geochemical setting

Arkashin was characterized in the field as a small, approximately 1 m^2, pool with a single bubbling vent at its center, formed over 30 years ago as a prospector-dug shurf (Figure 3.1). Although no

mats were present in Arkashin, flocs of unknown composition were abundant and varied in color from pale yellow-orange to bright orange-red. Temperatures measured in Arkashin ranged from 99°C at ~10 cm in the venting sediments to 32°C near the edge of the pool, and pH varied from 3.7 to 7.0 when measured at multiple locations on multiple days. Texture in Arkashin was mostly of the sand & silt size fraction. Dissolved organic carbon (DOC) values were 1.01 mM in Arkashin water (Table 3.1).

Zavarzin was approximately 10 m^2 and sourced from as many as 20 vents of various sizes from a few centimeters to nearly half a meter in diameter. Greenish mats were found along the edges of Zavarzin. Thicker brown and green mats were found within the pool, under water and a surface dusting of fine yellow-white sediment. Temperature in Zavarzin was 26-74°C, and the pH was from 5.5-7.5. The temperature deeper in the sediments closer to the vents may have been higher, but was out of reach for measurement. Zavarzin had higher clay content and larger pieces of gravel than did Arkashin, which was more coarse-textured overall. DOC in Zavarzin water was 1.52 mM (Table 3.1).

Arkashin and Zavarzin sediments were geochemically distinct (Table 3.1). Arkashin was higher in total As, Rb, Ca, and Cs than Zavarzin. Zavarzin was higher in total V, Mn, Co, Zn, Sr, Ba, Fe and S. The two pools were essentially equivalent in concentrations of Mg, Al, Cu, Pb, N, and C.

The Archaeal Community

Sequence Analysis

No archaeal 16S rRNA genes were successfully amplified and cloned. Archaeal 16S rRNA genes were sequenced from Zavarzin, although with some difficulty, such as the requirement for sequencing with primers for the vector rather than the insert sequence. Ninety-one sequences were included in analysis of the Zavarzin archaeal (ZA) library (Table 3.1). Thirty-one representative sequences were chosen for each OTU defined by 0.03 average distance among OTU members. The Chao estimated 0.03 distance OTU richness (95% CI) was 45 (34-84). Good's coverage indicated that the ZA library represents 85% of the community at 0.03 distance among OTU members in the Zavarzin archaeal community. Representative sequences for ZA OTUs 1 through 31 had only 77.4 to 91.5 % similarity to

sequences from type-strains in the RDP database, but 95.6 to 99.5 % similarity to sequences in the database when all quality sequences were included in the comparison. The relationship among ZA OTU sequences, sequences from archaeal type-strains and sequences from environmental DNA clone libraries revealed that the archaeal community of Zavarzin is not well represented with respect to isolated microorganisms (Figure 3.2). A number of the ZA OTUs were more closely related to one another than to any sequences in the RDP database. Additionally, a clade of unclassified sequences indicated the presence of Archaea outside the currently described phylogenetic groups. The majority (56%) of the ZA sequences were classified in the Class *Thermoprotei* (Figure 3.3A). Twenty-one percent of the sequences were classified in the phylum Korarchaeota with high confidence (100%). The remaining ZA sequences could not be classified to any archaeal group with at least 80% confidence in the classification.

Lipid Analysis

Due to challenges with 16S rRNA gene sequence recovery, Archaea were also characterized by glycerol dialkyl glycerol tetraether (GDGT) analysis. Although not quantitative, GDGTs were recovered from both sites. GDGTs 0 through 6 as well as crenarchaeol were identified. Crenarchaeol was not abundant at either site. The relative abundances of the remaining GDGTs were slightly different (Figure 3.3B). GDGT-4 was the most abundant in Arkashin, GDGTs 2 and 3 occurred at moderate abundances and GDGTs 0, 1, 5 and 6 were less abundant. The relative abundance of GDGT-0 was higher in Zavarzin than in Arkashin. In Zavarzin, the abundances of GDGTs 0, 2, 3 and 4 were nearly equivalent and GDGT-1 was slightly less abundant. The relative abundance of GDGT-5 in Zavarzin was equivalent to that in Arkashin, though GDGT-6 was less abundant. Although GDGTs are not well-characterized as taxonomic biomarkers, these data demonstrated the presence of an archaeal component of the microbiological community that was distinct in each pool.

The Bacterial Community

Sequence Analysis

Two-hundred-six and 300 sequences were analyzed from the Arkashin bacterial (AB) and the Zavarzin bacterial (ZB) libraries respectively (Table 3.1). The ZB and AB libraries were significantly different according to Libshuff analysis (ΔC_{ABZB} was 18.53 and ΔC_{ZBAB} was 16.06, both at p=0.001). Coverage as determined by Libshuff and Good's coverage were generally in agreement. Both libraries had at least 90% coverage at the 0.03 distance level; the lower coverage in Zavarzin suggests a more diverse community. The Chao estimated OTU richness (95% CI) at 0.03 distance was 91 (69-148) and 21 (18-35) for the ZB and AB libraries, respectively. AB OTUs at 0.03 distance were represented by 19 sequences. These OTU representatives had high similarities, 95 to 100 %, to sequences in the RDP database, however only 81.1 to 100 % similarity to sequences from type-strain isolates. Sequences from the ZB library were chosen to represent the 59 OTUs defined at 0.03 distance. These ZB OTU representative sequences had 81.3 to 99.9 % similarity to sequences in the RDP database. When compared to type-strain sequences only, the similarity scores were 71.6 to 99.9 %.

The relationships among OTU representative sequences from the AB library and sequences in various classes were examined with a 16S rRNA gene tree (Figure 3.4). A number of sequences from the AB library were closely related to members of the genus *Variovorax*; these and clones related to the genus *Thiomonas* formed a distinct clade of *β-Proteobacteria*. Additional distinct clades were formed with clones related to members of the *"Sphingobacteria"*, "*Dictyoglomia"* and *Aquificae*, as well a clade of clones related to so-called division OP8 environmental sequences. Two clusters of *"Clostridia"* were observed; one OTU, AB OTU 9, was related to the genus *Thermodesulfobium* of the family *Thermodesulfobiaceae*, and another OTU, AB OTU 12, to *Thermoanaerobacter brockii* of an unresolved family, Incertae Sedis III. *Acidobacteria*, *α-Proteobacteria* and δ-*Proteobacteria* were also represented in the phylogeny of AB sequences, as were additional clades of unclassified sequences. Approximately half of the sequences from the ZB library shared at least 95% identity to sequences from type-strains (Figure

3.5). Close relationships of sequences to the type-strains *Desulfurella multipotens*, *Rosiflexus castenholzii*, *Chlroflexus aurantiacus*, *Dictyoglomus thermophilum* and *Sulfurihydrogenibium azorense* and to isolated members of the *Thermodesulfovibrio* and *Calditerrivibrio* genera were supported in all analyses. The sequences from type-strains were retained for comparison to a number of ZB OTU representatives most closely related to uncultured, unclassified microorganisms (Figure 3.6). Many of these ZB OTUs were classified with *δ-Proteobacteria* and *"Clostridia"* when compared to sequences from type-strains only, however, additional analysis with environmental clones showed that these sequences were more closely related to one another or to other environmental sequences than to any isolated microorganisms. Those sequences from the ZB library closely related to environmental sequences from the database included members of the classes "*Nitrospira*," *"Clostridia,"* and *Acidobacteria*, as well as candidate groups BRC1 and OP10 and some unclassified clades (Figure 3.6).

The sequence libraries were compared by the proportion of clones in various taxonomic or phylogenetic classes (Figure 3.7A). The AB library was dominated by sequences in the *"Sphingobacteria"*, though *Aquificae*, *β-* and *δ-Proteobacteria*, and *"Clostridia"* were also well represented. Approximately 20% of sequences in the AB library and nearly 35% of sequences in ZB library were grouped with unclassified environmental sequences. *Aquificae* were less abundant in ZB than in AB, and *β-Proteobacteria* were absent. Members of the *δ-Proteobacteria* were of nearly equal abundance in the ZB and AB libraries. However, the *Deferribacteres, "Nitrospria"* and *incertae sedis* groups OP10, OP11 and BRC1 were present in the ZB library and absent in the AB library. Additionally, the ZB library was dominated by "*Chloroflexi,"* also absent in the AB library.

Lipid Analysis

To confirm 16S rRNA gene sequence data interpretation, phospholipid fatty acids (PLFA) were also analyzed. As determined from the added standard, 19:0, calculated recovery of PLFA ranged from 83-124% (Table 3.1). Diversity of PLFA profiles from Arkashin and Zavarzin were nearly equivalent,

although the diversity of the Zavarzin replicates was slightly higher. The concentration of PLFA recovered was higher in Zavarzin than in Arkashin, and the PLFA profiles of each site were distinct (Figure 3.7B). The PLFA 20:1ω9c/20:3ω3 and 18:0 and unidentified PLFA each were a >10 mol% proportion of both replicates from Arkashin. PLFA recovery from the second replicate was higher than from the first, had a higher proportion of unknown PLFA, and additionally contained 20:4ω6. Zavarzin also had >10 mol% of unknowns and of 16:0. Of the PLFA occurring in co-eluting groups, 20:1ω9c/20:3ω3 peaks were identified as 20:1ω9c in both pools and 18:1ω9t/18:1ω7c represented 18:1ω9t in Zavarzin. As detailed in the discussion, PLFA biomarkers were in agreement with sequence data.

Stable Isotope Analysis

In Arkashin, sediment $\delta^{34}S$ was +1.22‰, $\delta^{15}N$ was -7.50‰. Values for $\delta^{13}C_{tot}$ and $\delta^{13}C_{org}$ in the sediments were nearly equivalent at -18.68 and -19.35 ‰ respectively (Table 3.1, Figure 3.8). Although lower, at -25.70 and -24.81‰ respectively, $\delta^{13}C_{tot}$ and $\delta^{13}C_{org}$ were also equivalent to one another in Zavarzin sediments. The $\delta^{15}N$ and $\delta^{34}S$ values, -3.87‰ and +1.54‰ respectively, were higher in Zavarzin than in Arkashin.

DISCUSSION

This study examined two geochemically distinct hydrothermal pools using multiple complementary methods with the objectives of describing the microbiological communities and determining if any novel components occur therein. The results demonstrate that the microbiological communities in the pools are distinct and different. Some of the ecological structure of the microbiological communities can be described by the combination of sequence, membrane lipid and stable isotope δ-values from both sites as presented below. In addition, novel sequences, sequences related to uncultured microorganisms, and unknown lipids were found.

Physicochemical and geochemical setting

The different textures observed in the different pools were consistent with the geological history of Uzon (Belousov et al. 1984; Migdisov and Bychkov 1998). This history also contributes to heterogeneous hydrology and the unique geochemical conditions in each pool. For example, Arkashin was enriched in As relative to Zavarzin. This coincides with higher concentrations of chloride waters and active arsenic-sulfur mineralization near Arkashin (Karpov and Naboko 1990; Cleverley et al. 2003; Reed and Palandri 2006).

The DOC concentrations in Akrashin and Zavarzin represented a moderate abundance of carbon available for local consumption. Although high compared to many hydrothermal features at Yellowstone National Park (McCleskey et al. 2005), water samples in that study were filtered at 0.1 μm rather than at 0.45 μm as in this study.

The Archaeal Community

The GDGT profiles described in this study closely matched those described previously for the same pools (Pearson et al. 2008). The relatively low amount of crenarchaeol was in accordance with expectations from similar pH and temperature conditions measured in other studies (Schouten et al. 2003; Schouten et al. 2007; Pearson et al. 2008). Although GDGTs are not as useful as PLFA for taxonomically relevant biomarkers, they do confirm the presence of Archaea in both pools and indicate that communities in each are distinct. The differences in GDGT profiles from each pool may represent sampling at a slightly higher temperature in Arkashin (as correlated with a lower relative abundance of GDGT-0), or the effect of different geochemical conditions on the Archaeal community.

The difficulties encountered recovering archaeal 16S rRNA genes from these hydrothermal features were consistent with other studies (Meyer-Dombard et al. 2005) and do not indicate a lack of Archaea. Rather, the archaeal 16S rRNA gene diversity was likely under-sampled by the described

methods. Similar problems with DNA recovery in Arkashin specifically have been encountered by others (T. Sokolova, pers. comm.), as well as in pools of similar chemistry (Meyer-Dombard et al. 2005; Hetzer et al. 2007). One interference factor may have been the As-rich nature of Arkashin. In Champagne Pool, Waopitu, NZ, a much larger but chemically similar pool, As toxicity has been indicated as a possible limitation on biomass (Hetzer et al. 2007). However, the recovery of abundant GDGTs and PLFA in the present work did not support this interpretation. Despite a low annealing temperature required in optimizing the archaeal PCR, sequencing of 16S rRNA gene clones from Zavarzin required use of vector-specific primers. Other archeal primer sets, Parch519f-Arch815r (Coolen et al. 2004) and Arc85f-Arc313r (Lima and Sleep 2007), were not well-represented in the ZA library. These primers may also have been poor matches to sequences in Arkashin, confounding Archaeal sequence recovery. Recent advances in sampling environmental sequences related to Archaea have been the result of enrichment and primer development (Hohn et al. 2002; Perevalova et al. 2008; Kublanov et al. 2009). Further research in the field is likely to reveal additional interesting Archaea.

The ZA library contained more archaeal groups than indicated by the classification of sequences available in RDP. The closest relationship to any type-strain sequences was approximately 91% similarity of two OTUs to *Staphylothermus hellenicus* of Class *Thermoprotei*. Multiple studies in thermal pools have documented the presence of uncultured *Korarchaeota* and *Crenarchaeota* in such environments (Skirnisdottir et al. 2000; Meyer-Dombard et al. 2005; Kvist et al. 2007). The abundance of *Crenarchaeota* in the ZA library was reflected in the recent recovery of novel sequences and isolates from Uzon thermal pools (Perevalova et al. 2008; Kublanov et al. 2009). The ZA library also contained sequences related to *Korarchaeota* known only from sequence libraries (Auchtung et al. 2006).

The Bacterial Community

The bacterial communities in each pool represented a combination of cultured and uncultured microorganisms, demonstrating the continuing need for isolation efforts. Many of the sequences recovered from both pools are related to microorganisms previously isolated from Uzon hydrothermal

features, including multiple *Desulfurella* species (Miroshnichenko et al. 1998) and a recent *Sulfurihydrogenibium* species (O'Neill et al. 2008). Strains related to *Thermoanaerobacter uzonensis* (Wagner et al. 2008) have been isolated from both Arkashin and Zavarzin (Burgess, unpublished; I. Wagner, pers. comm.).

Approximately 20% of clones from Arkashin and more than 35% of clones from Zavarzin remained unclassified. These microorganisms may also be represented to some degree in the high proportions of unidentified PLFA from both pools. Additional comparisons will be possible as more novel microorganisms are brought into isolation. For example, only recently has an microorganism from the phylogenetic clade known colloquially as OP10, previously known only from sequence libraries (Hugenholtz et al. 1998), been isolated (Stott et al. 2008). In the ZB library, 19 clones clustered with an environmental sequence belonging to OP10 *genera incertae sedis.*

Although many of the sequences in the AB and ZB libraries had closest relatives among sequences known only from environmental DNA samples, there were also close matches to sequences of cultured microorganisms. Of these, AB sequences related to *Variovorax paradoxus*, *Hydrogenobaculum acidophilum*, and *Pedobacter caeni* of class "Sphingobacterium" made up 12, 10 and 31% of the clones in the library. Fatty acid characterization of these strains provides data for comparison to PLFA profiles. The major fatty acids of *V. paradoxus* are 16:1, 16:0 and 18:1w7c (Willems et al. 1991); of *H. acidophilum* they are 18:0, 18:1w9c, and 20:1w9c (Stohr et al. 2001); and of *Pedobacter caeni*, i17:0(3-OH) and i15:0 (Vanparys et al. 2005). The fatty acids of *V. paradoxus* collectively made up 6.03% of the PLFA profile from Arkashin and the fatty acids of *H. acidophilum* made up 35.8%. Athough i17:0(3-OH) could not be identified with the standards of this study, i15:0 was not detected. Thirty-one percent of the sequenced clones in Zavarzin were related to type-strain of *Roseiflexus castenholzii*, characterized by fatty acid composition of 16:0, 14:0 and 15:0 (Hanada et al. 2002). Approximately 29% of the PLFA profile from Zavarzin was composed of these fatty acids. PLFA representative of heterotrophic bacteria, e.g. i15:0 and 15:0 in *Thermoanaerobacter* (Wagner et al. 2008), were 9% of the Zavarzin, but only up to 2% of Arkashin profiles.

Stable Isotope Analysis

The C, S and N stable isotope δ-values measured in this study indicated the presence of metabolic pathways linked to the microorganisms identified in sequence libraries and PLFA profiles. Source values for $\delta^{13}C$ in CH_4 ($\delta^{13}C_{CH4}$) were measured as approximately -28‰ in both Arkashin and Zavarzin and $\delta^{13}C_{CO2}$ as -5.3‰ and -4.2‰ in Arkashin and Zavarzin respectively (Zhao 2008). These are within described ranges for hydrothermal reduced carbon and hydrothermal CO_2 (Des Marais 1996). The range of atmospheric CO_2 is within that of hydrothermal CO_2, and the range of hydrothermal reduced carbon is within that of organic matter. Values for $\delta^{13}C_{org}$ reported above most likely represent autotrophic CO2 fixation in both pools (Hayes 2001; House et al. 2003). However, the $\delta^{13}C$ values in Zavarzin are lighter than would be expected by CO_2 fixation by *Chloroflexus* alone, suggesting the Calvin cycle as a C source in Zavarzin relative to Arkashin. The $\delta^{15}N$ values were within the range values measured for ammonia in Uzon (-8 to 8‰) but were depleted relative to values for nitrate (2 to 9‰; Kamchatka MO NSF Annual Report (2006). The $\delta^{15}N$ values of Arkashin and Zavarzin suggest ammonia as a nitrogen source for these pools (Estep and Macko 1984). Possible pathways of nitrogen cycling in Zavarzin include denitrification by *Calditerrivibrio* (Iino et al. 2008) and nitrification by uncharacterized Crenarchaea (Reigstad et al. 2008). In a recent characterization of sulfate $\delta^{34}S$ values in Uzon, sulfate was 7.6‰ in Arkashin and 8.0‰ in Zavarzin, and a value between -1.5 to 1.6‰ was predicted for hydrothermal sulfide (Hollingsworth 2006). The $\delta^{34}S$ values measured in this study were intermediate between those. Source mixing and microbial metabolism are two possible explanations for this observation (Bonch-Osmolovskaya 1994; Habicht and Canfield 2001; Hollingsworth 2006). Sequences related to a variety of microorganisms likely to play a part in sulfur cycling were recovered from both pools.

Ecological structure in Arkashin and Zavarzin

The data described above lend themselves to an ecological description of the two thermal pools investigated in this study. In Arkashin, primary production can be attributed to CO_2 fixation by *Hydrogenobaculum acidophilum* via the reverse tri-carboxylic acid (rTCA) cycle. Additionally, considering the high concentration of arsenic in Arkashin, *Hydrogenobaculum* may be participating in redox cycling of this element (Jackson, et al. 2001). The presence of *H. acidophilum*, along with *Variovorax paradoxus* and members of the *Desulfurella* genus may indicate the importance of H_2 as an energy source. Members of the *Chloroflexi* and the genus *Sulfurihydrogenibium* represented possible primary producers in Zavarzin; however, both groups also contain heterotrophic or facultatively heterotrophic representatives. Additional heterotrophic community members were represented in both pools and included the genera *Desulfurella*, *Thermoanaerobacter*, *Thermodesulfovibrio* and *Calditerrivibrio*, known for using short-chain organic acids for C and energy sources and a variety of electron acceptors. The stable isotope δ-values for both S and N indicated some *in situ* cycling. In both pools, an additional source of C by allocthonous inputs was suggested. In Zavarzin, microorganisms capable of degrading the recalcitrant carbohydrates characteristic of allocthonous inputs were suggested by sequences related to members of the candidate phylum OP10 and to members of the *Crenarchaeota*. Sequences related to *Pedobacter canei* were recovered from Arkashin may also represent consumers of allocthonous inputs. Both *Pedobacter*- and *Variovorax*-like sequences in Arkashin may represent mesophilic microorganisms at the low temperature edges of this pool or novel previously thermophilic members of these groups.

Additional ecological comparison of Arkashin and Zavarzin can be made in terms of diversity. Although nearly equivalent in terms of PLFA diversity, the sequence diversity in Arkashin was much lower, suggesting the limitation was not biomass per se. The actual richness and the estimated richness of sequence OTUs were also lower, and the coverage was higher, despite the lesser number of sequences analyzed. Arkashin was characterized as a smaller pool with a larger, and thus steeper, temperature

gradient. Hypotheses for the lower diversity detected in Arkashin may include limitations imposed by of high concentrations of the potentially toxic element, As.

Comparison with Other Culture-Independent Studies

All of these sequences from the AB and 92% of the sequences from the ZB libraries were at least 95% similar to sequences already in the RDP database. Eleven sequences from Arkashin were 98-99% similar to unclassified sequences from YNP (Harris et al. 2004; Spear et al. 2005) and 27 sequences were 97% similar to an uncultured, unclassified sequence from Montserrat Island, in the Caribbean (Burton and Norris 2000). From Zavarzin, 15 sequences were 98% similar to an unclassified sequence from YNP (Spear et al. 2005). The physical and chemical conditions of the individual hot spring environments are expected to constrain the composition of communities therein, and the distribution of groups observed in this study compares well with other locations. For example, the genera *Hydrogenobaculum*, *Desulfurella*, *Sulfurihydrogenibium* and genera of the *Chloroflexi* can be considered groups commonly occuring in environments dominated by sulfur chemistry (Skirnisdottir et al. 2000; Jackson et al. 2001; Mathur et al. 2007). The higher temperature or arsenic concentrations in Arkashin may inhibit growth of *Chloroflexi*, but the constraints restricting *Sulfurihydrogenibium* and allowing *Hydrogenobaculum* are more likely a result of pH. Members of both genera have been identified that are able to cycle As species (Donahoe-Christiansen et al. 2004; O'Neill et al. 2008), but *Hydrogenobaculum* tolerates more acidic conditions. Recovery of sequences and isolates related to microorganisms in the classes "*Clostridia*" and "*Dictyoglomia*" has been observed previously (Skirnisdottir et al. 2000; Wagner et al. 2008; Kublanov et al. 2009). One group seen in other studies (Skirnisdottir et al. 2000; Blank et al. 2002), but not observed here is the *Thermus-Deinococcus* group. However, in the studies cited, sequences belonging to that group were recovered from more alkaline springs. The same is true of the *Euryarchaeota*, seen in generally more acidic pools (Spear 2005).

Conclusions

Arkashin is an As-rich thermal pool where *Hydrogenobaculum*-related species are responsible for primary production. Other inhabitants of this pool include *Desulfurella*-, *Variovorax*- and *Pedobacter*-related bacteria. These microorganisms tolerate high As concentrations and *Hydrogenobaculum* may participate in As cycling. Challenges with recovery of DNA from Arkashin indicate that microbiological communities in As-rich features are under-represented in sequence libraries. PLFA and GDGT recovery demonstrated this pool is not limited in biomass. Zavarzin is inhabited by bacteria related to *Rosiflexus* and *Chloroflexus* as well as obligately heterotrophic species and many uncultured representatives of the *Crenarchaea*. The community in Zavarzin was more diverse than that of Arkashin and more of the sequences from Zavarzin were related to microorganisms known only from environmental samples. Thus the objectives of describing the communities in each pool and determining if any novel microorganisms inhabited them were met. The combination of geochemical, lipid, sequence and stable isotope data allowed for a partial ecological description of Arkashin and Zavarzin. These data can be incorporated into an increasingly detailed body of knowledge regarding hydrothermal ecosystems. Further characterization will be required for a comprehensive ecological description through identification of the novel community members.

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Table 3.1 Summary of data

	Arkashin	Zavarzin
field measurements (range)		
Temp (°C)	26-99	26-74
pH	5.5-7.0	5.5-7.5
DOC (mM)	1.01	1.52
texture [% weight (standard dev.)]		
clay avg	1.92 (0.12)	3.82 (0.80)
grvl avg	22.02 (8.96)	54.07 (4.01)
snd&slt avg	76.06 (8.99)	42.11 (3.87)
sediment elemental compositon (mg/kg)*		
Mg	75.43	117.84
Al	3387.92	2888.43
Ca	1125.37	454.01
V	4.95	58.32
Mn	9.74	263.15
Co	0.76	20.55
Cu	5.80	17.14
Zn	7.21	44.75
As	4251.57	48.86
Rb	2.08	bd
Sr	11.17	35.23
Cs	6.24	0.52
Ba	43.00	125.86
Pb	0.78	1.77
Fe	2618.73	60502.13
S, N and C (% weight and SI δ-values)**		
S	1.03	9.75
N	0.02	0.03
C	0.18	0.09
Corg	0.20	0.13
δ34S	1.22	1.54
δ15N	-7.50	-3.87
δ13Ctot	-18.68	-25.70
δ13Corg	-19.35	-24.81
Archaeal sequence library		
N	nr	91
Avg. length (bp)	nr	817
No. of OTUs***	nr	31
Diversity****	nr	3.11
Coverage*****	nr	85%
PLFA (rep 1, rep 2)		
% recovery******	105, 83	124, 121
conc (μg/g)	183, 350	436, 455
Diversity	2.29, 2.46	2.54, 2.57
Bacterial sequence library		
N	206	300
Avg. length (bp)	822	711
No. of OTUs	19	59
Diversity	2.11	3.00
Coverage	98%	90%

*for QA/QC information, please see Methods

**SI δ-values - stable isotope δ-vales vs. standards as described in Methods

***OTUs were defined at 0.03 average distance among members

****Diversity of OTUs as Shannon-Weiner index (Schloss and Handelsman 2005)

*****Coverage at 0.03 average distance as per Good 1953 (in Park 2008)

******Determined in comparison to an added standard; see Methods

nr = insufficent recovery for analysis

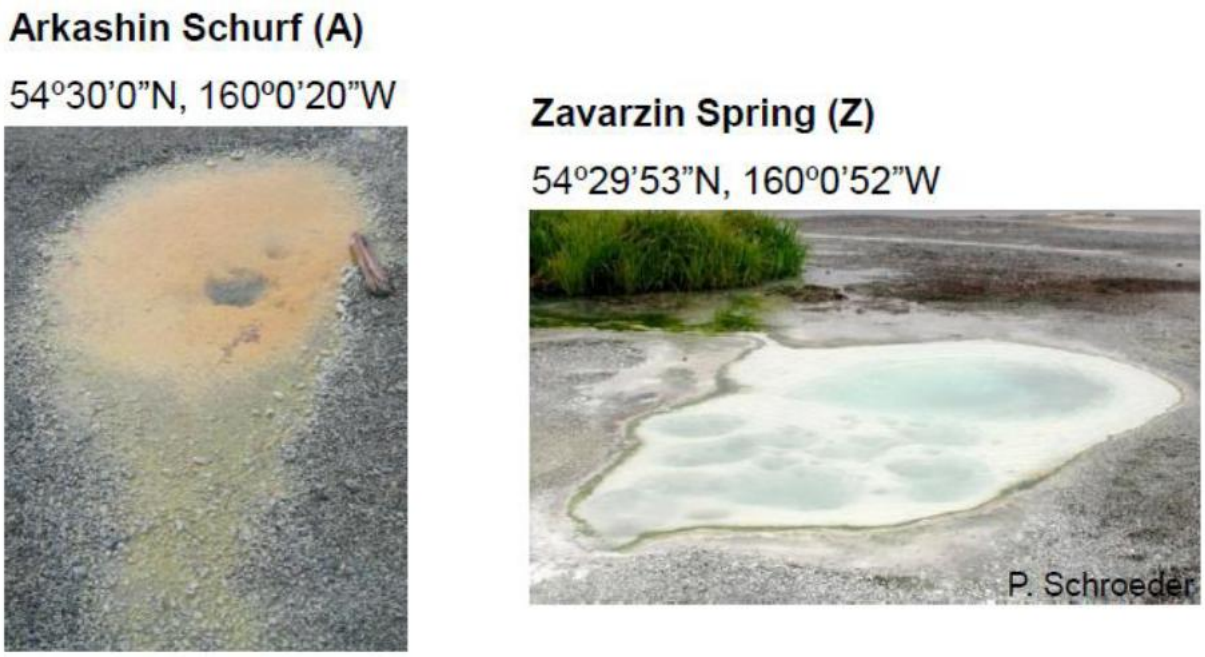


Figure 3.1. Arkashin Schurf (~1 m^2) and Zavarzin Spring (~10 m^2) along with geographic coordinates of latitude and longitude in degrees, minutes, seconds determined using the global positioning system. Photos were taken in 2004.

Figure 3.2 Operational taxonomic unit (OTU; grouped by 0.03 average distance among members) representatives from the Zavarzin archaeal 16S rRNA gene sequence library, highlighted in yellow, with closest relatives and type-strain sequences from public databases in a neighbor-joining tree based on a Jukes-Cantor distance matrix with 100 bootstrap replications. Type-strain sequences are indicated by (T) and sequences representing type-genera of taxonomic classes are indicated by (T*). Archaeal taxonomic groups, written in blue and green, are delineated by current definition rather than solely by clade in this tree.

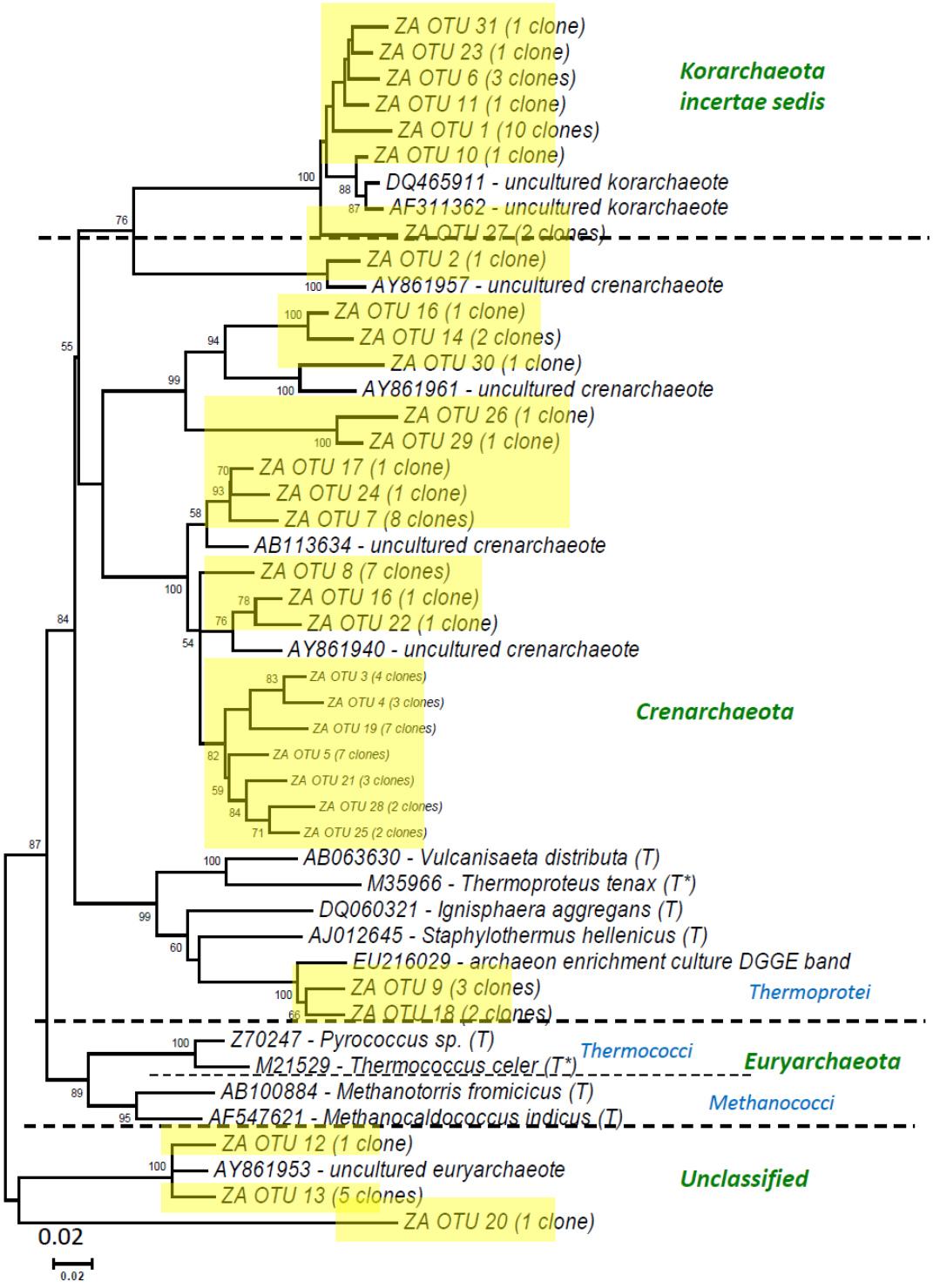
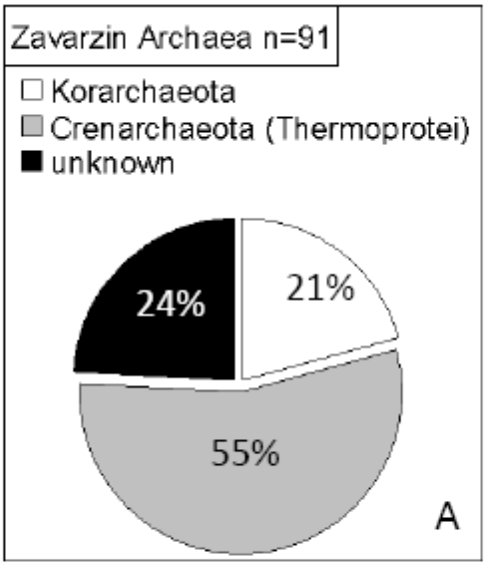

ZA OTU 31 (1 clone)
ZA OTU 23 (1 clone)
ZA OTU 6 (3 clones)
ZA OTU 11 (1 clone)
ZA OTU 1 (10 clones)
ZA OTU 10 (1 clone)
DQ465911 - uncultured korarchaeote
AF311362 - uncultured korarchaeote
ZA OTU 27 (2 clones)
Korarchaeota incertae sedis
ZA OTU 2 (1 clone)
AY861957 - uncultured crenarchaeote
ZA OTU 16 (1 clone)
ZA OTU 14 (2 clones)
ZA OTU 30 (1 clone)
AY861961 - uncultured crenarchaeote
ZA OTU 26 (1 clone)
ZA OTU 29 (1 clone)
ZA OTU 17 (1 clone)
ZA OTU 24 (1 clone)
ZA OTU 7 (8 clones)
AB113634 - uncultured crenarchaeote
ZA OTU 8 (7 clones)
ZA OTU 16 (1 clone)
ZA OTU 22 (1 clone)
AY861940 - uncultured crenarchaeote
ZA OTU 3 (4 clones)
ZA OTU 4 (3 clones)
ZA OTU 19 (7 clones)
ZA OTU 5 (7 clones)
ZA OTU 21 (3 clones)
ZA OTU 28 (2 clones)
ZA OTU 25 (2 clones)
Crenarchaeota
AB063630 - Vulcanisaeta distributa (T)
M35966 - Thermoproteus tenax (T*)
DQ060321 - Ignisphaera aggregans (T)
AJ012645 - Staphylothermus hellenicus (T)
EU216029 - archaeon enrichment culture DGGE band
ZA OTU 9 (3 clones)
ZA OTU 18 (2 clones)
Thermoprotei
Z70247 - Pyrococcus sp. (T)
M21529 - Thermococcus celer (T*)
Thermococci
Euryarchaeota
AB100884 - Methanotorris fromicicus (T)
AF547621 - Methanocaldococcus indicus (T)
Methanococci
ZA OTU 12 (1 clone)
AY861953 - uncultured euryarchaeote
ZA OTU 13 (5 clones)
Unclassified
ZA OTU 20 (1 clone)
0.02
0.02

Figure 3.3 A. Proportion of clones from the Zavarzin archaeal (ZA) 16S rRNA gene sequence library in archaeal taxonomic groups with >80% confidence by RDPClassifier (Wang 2007). B. Archaeal glycerol dialkyl glycerol tetraether (GDGT) relative distribution in duplicate aliquots from Arkashin (A) and Zavarzin (Z) sediment samples presented in elution order.


Zavarzin Archaea n=91
Korarchaeota
Crenarchaeota (Thermoprotei)
unknown
24%
21%
55%
A

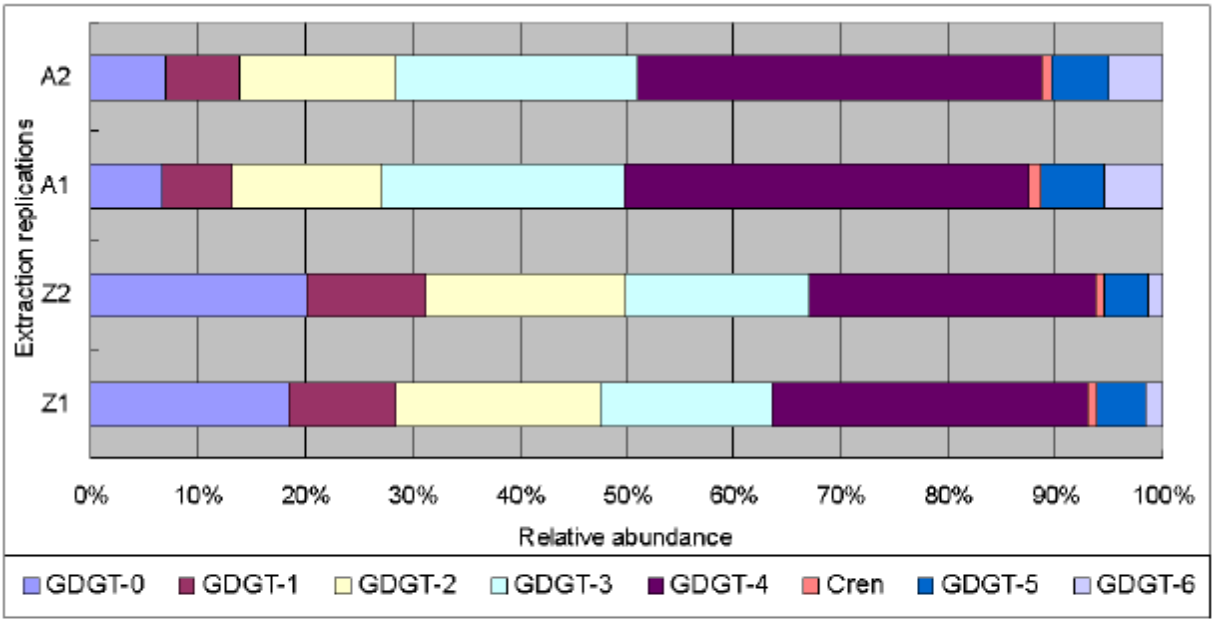

A2
A1
Z2
Z1
Extraction replications
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative abundance
GDGT-0
GDGT-1
GDGT-2
GDGT-3
GDGT-4
Cren
GDGT-5
GDGT-6
B

Figure 3.4. OTU representatives from the Arkashin bacterial (AB) 16S rRNA gene sequence library, highlighted in yellow, with sequences of closest relatives and type-strains from public databases in a neighbor-joining tree based on a Jukes-Cantor distance matrix with 100 bootstrap replications. AB sequences >95% identical to a type-strain are circled. Sequences from type-strains sequences are indicated by (T) and sequences representing type genera of taxonomic classes are indicated by (T*). Bacterial taxonomic groups, written in blue, are delineated by current definition rather than solely by clade in this tree.

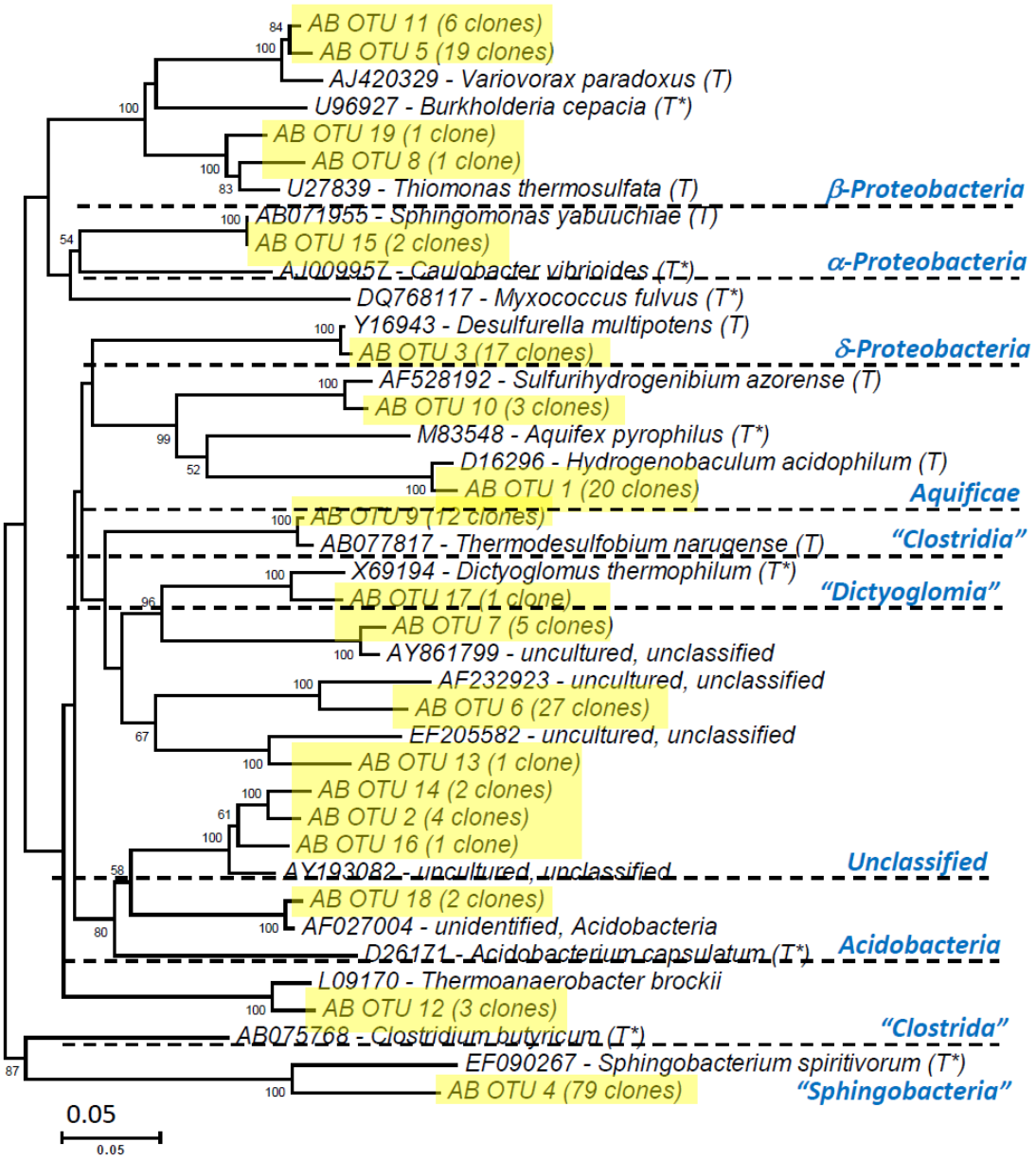

AB OTU 11 (6 clones)
AB OTU 5 (19 clones)
AJ420329 - Variovorax paradoxus (T)
U96927 - Burkholderia cepacia (T*)
AB OTU 19 (1 clone)
AB OTU 8 (1 clone)
U27839 - Thiomonas thermosulfata (T)
β-Proteobacteria
AB071955 - Sphingomonas yabuuchiae (T)
AB OTU 15 (2 clones)
AJ009957 - Caulobacter vibrioides (T*)
α-Proteobacteria
DQ768117 - Myxococcus fulvus (T*)
Y16943 - Desulfurella multipotens (T)
AB OTU 3 (17 clones)
δ-Proteobacteria
AF528192 - Sulfurihydrogenibium azorense (T)
AB OTU 10 (3 clones)
M83548 - Aquifex pyrophilus (T*)
D16296 - Hydrogenobaculum acidophilum (T)
AB OTU 1 (20 clones)
Aquificae
AB OTU 9 (12 clones)
AB077817 - Thermodesulfobium narugense (T)
"Clostridia"
X69194 - Dictyoglomus thermophilum (T*)
AB OTU 17 (1 clone)
"Dictyoglomia"
AB OTU 7 (5 clones)
AY861799 - uncultured, unclassified
AF232923 - uncultured, unclassified
AB OTU 6 (27 clones)
EF205582 - uncultured, unclassified
AB OTU 13 (1 clone)
AB OTU 14 (2 clones)
AB OTU 2 (4 clones)
AB OTU 16 (1 clone)
AY193082 - uncultured, unclassified
Unclassified
AB OTU 18 (2 clones)
AF027004 - unidentified, Acidobacteria
D26171 - Acidobacterium capsulatum (T*)
Acidobacteria
L09170 - Thermoanaerobacter brockii
AB OTU 12 (3 clones)
AB075768 - Clostridium butyricum (T*)
"Clostrida"
EF090267 - Sphingobacterium spiritivorum (T*)
AB OTU 4 (79 clones)
"Sphingobacteria"
0.05
0.05

Figure 3.5. OTU representatives from the Zavarzin bacterial (ZB) 16S rRNA gene sequence library, highlighted in yellow, >95% identical to sequences of type-strains from public databases in a neighbor-joining tree based on a Jukes-Cantor distance matrix with 100 bootstrap replications. Type-strain sequences are indicated by (T) and sequences representing type genera of taxonomic classes are indicated by (T*). Bacterial taxonomic groups, written in blue, are delineated by current definition rather than solely by clade in this tree.

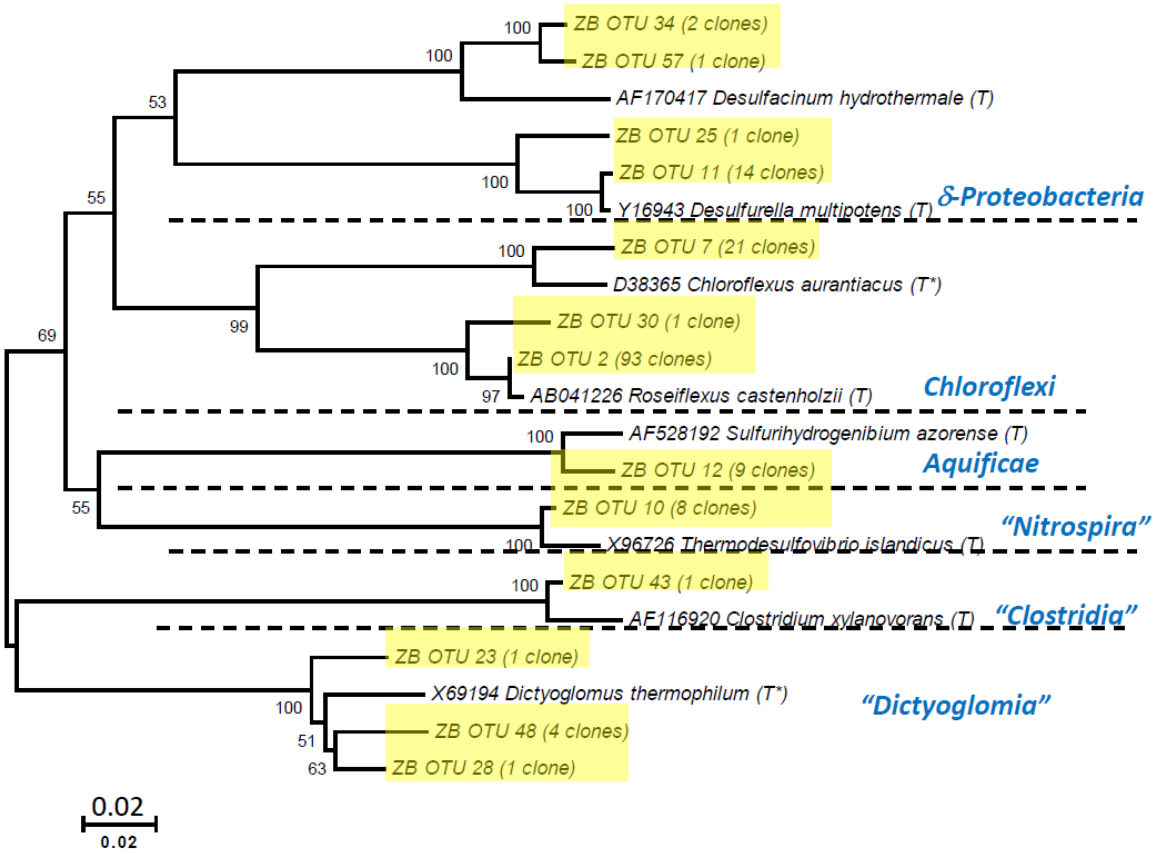
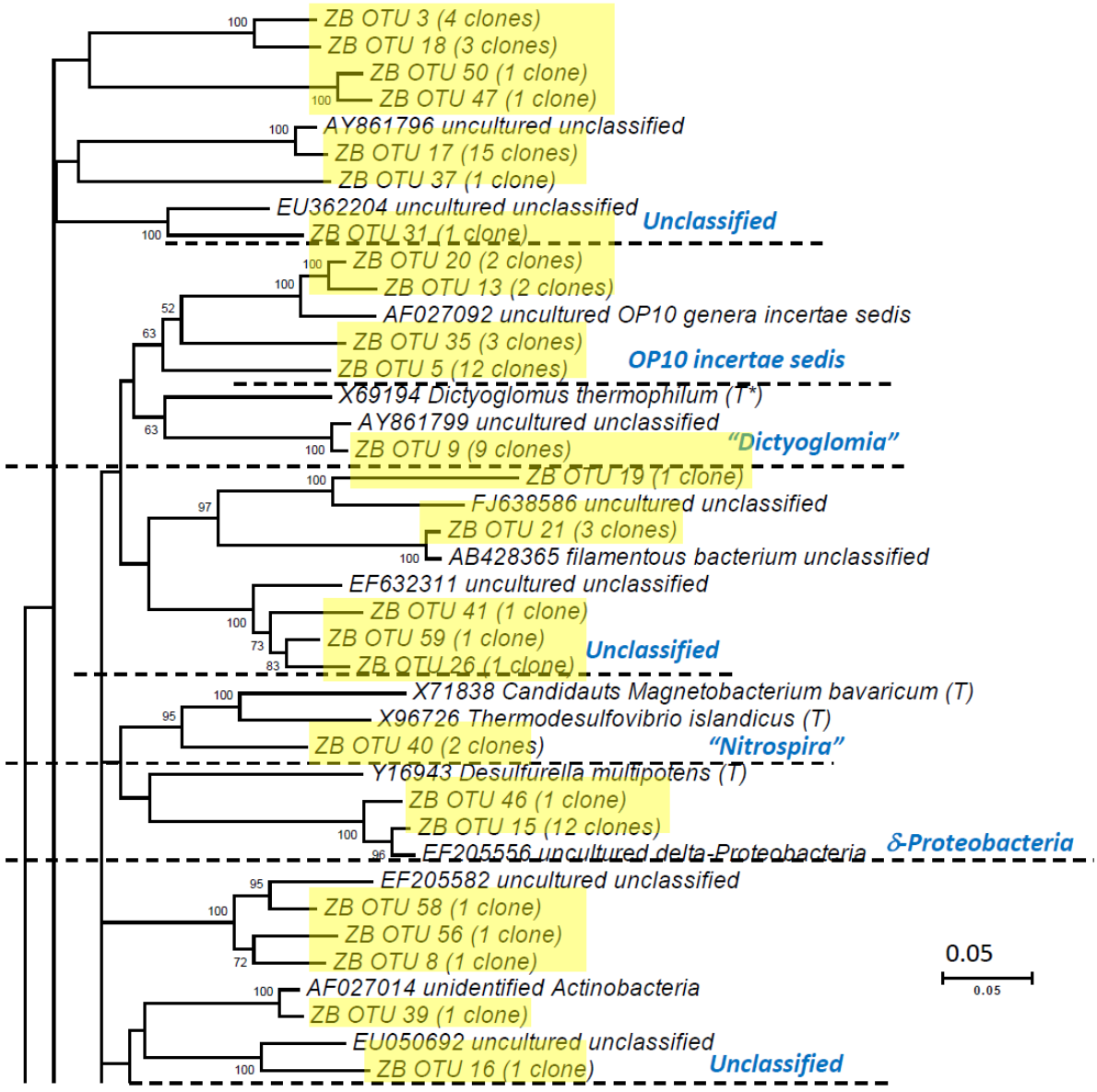

ZB OTU 34 (2 clones)
ZB OTU 57 (1 clone)
AF170417 Desulfacinum hydrothermale (T)
ZB OTU 25 (1 clone)
ZB OTU 11 (14 clones)
Y16943 Desulfurella multipotens (T)
δ-Proteobacteria
ZB OTU 7 (21 clones)
D38365 Chloroflexus aurantiacus (T*)
ZB OTU 30 (1 clone)
ZB OTU 2 (93 clones)
AB041226 Roseiflexus castenholzii (T)
Chloroflexi
AF528192 Sulfurihydrogenibium azorense (T)
ZB OTU 12 (9 clones)
Aquificae
ZB OTU 10 (8 clones)
X96726 Thermodesulfovibrio islandicus (T)
"Nitrospira"
ZB OTU 43 (1 clone)
AF116920 Clostridium xylanovorans (T)
"Clostridia"
ZB OTU 23 (1 clone)
X69194 Dictyoglomus thermophilum (T*)
ZB OTU 48 (4 clones)
ZB OTU 28 (1 clone)
"Dictyoglomia"
100
100
53
100
100
55
100
99
69
100
97
100
55
100
100
100
100
51
63
0.02
0.02

Figure 3.6. OTU representatives from the Zavarzin bacterial (ZB) 16S rRNA gene sequence library, highlighted in yellow, with sequences of closest relatives and type-strains from public databases in a neighbor-joining tree based on a Jukes-Cantor distance matrix with 100 bootstrap replications. Sequences of type-strains are indicated by (T) and sequences representing type genera of taxonomic classes are indicated by (T*). Bacterial taxonomic groups, written in blue, are delineated by current definition rather than solely by clade in this tree.


ZB OTU 3 (4 clones)
ZB OTU 18 (3 clones)
ZB OTU 50 (1 clone)
ZB OTU 47 (1 clone)
AY861796 uncultured unclassified
ZB OTU 17 (15 clones)
ZB OTU 37 (1 clone)
EU362204 uncultured unclassified
ZB OTU 31 (1 clone)
Unclassified
ZB OTU 20 (2 clones)
ZB OTU 13 (2 clones)
AF027092 uncultured OP10 genera incertae sedis
ZB OTU 35 (3 clones)
ZB OTU 5 (12 clones)
OP10 incertae sedis
X69194 Dictyoglomus thermophilum (T*)
AY861799 uncultured unclassified
ZB OTU 9 (9 clones)
"Dictyoglomia"
ZB OTU 19 (1 clone)
FJ638586 uncultured unclassified
ZB OTU 21 (3 clones)
AB428365 filamentous bacterium unclassified
EF632311 uncultured unclassified
ZB OTU 41 (1 clone)
ZB OTU 59 (1 clone)
ZB OTU 26 (1 clone)
Unclassified
X71838 Candidauts Magnetobacterium bavaricum (T)
X96726 Thermodesulfovibrio islandicus (T)
ZB OTU 40 (2 clones)
"Nitrospira"
Y16943 Desulfurella multipotens (T)
ZB OTU 46 (1 clone)
ZB OTU 15 (12 clones)
EF205556 uncultured delta-Proteobacteria
δ-Proteobacteria
EF205582 uncultured unclassified
ZB OTU 58 (1 clone)
ZB OTU 56 (1 clone)
ZB OTU 8 (1 clone)
AF027014 unidentified Actinobacteria
ZB OTU 39 (1 clone)
EU050692 uncultured unclassified
ZB OTU 16 (1 clone)
Unclassified
0.05
0.05

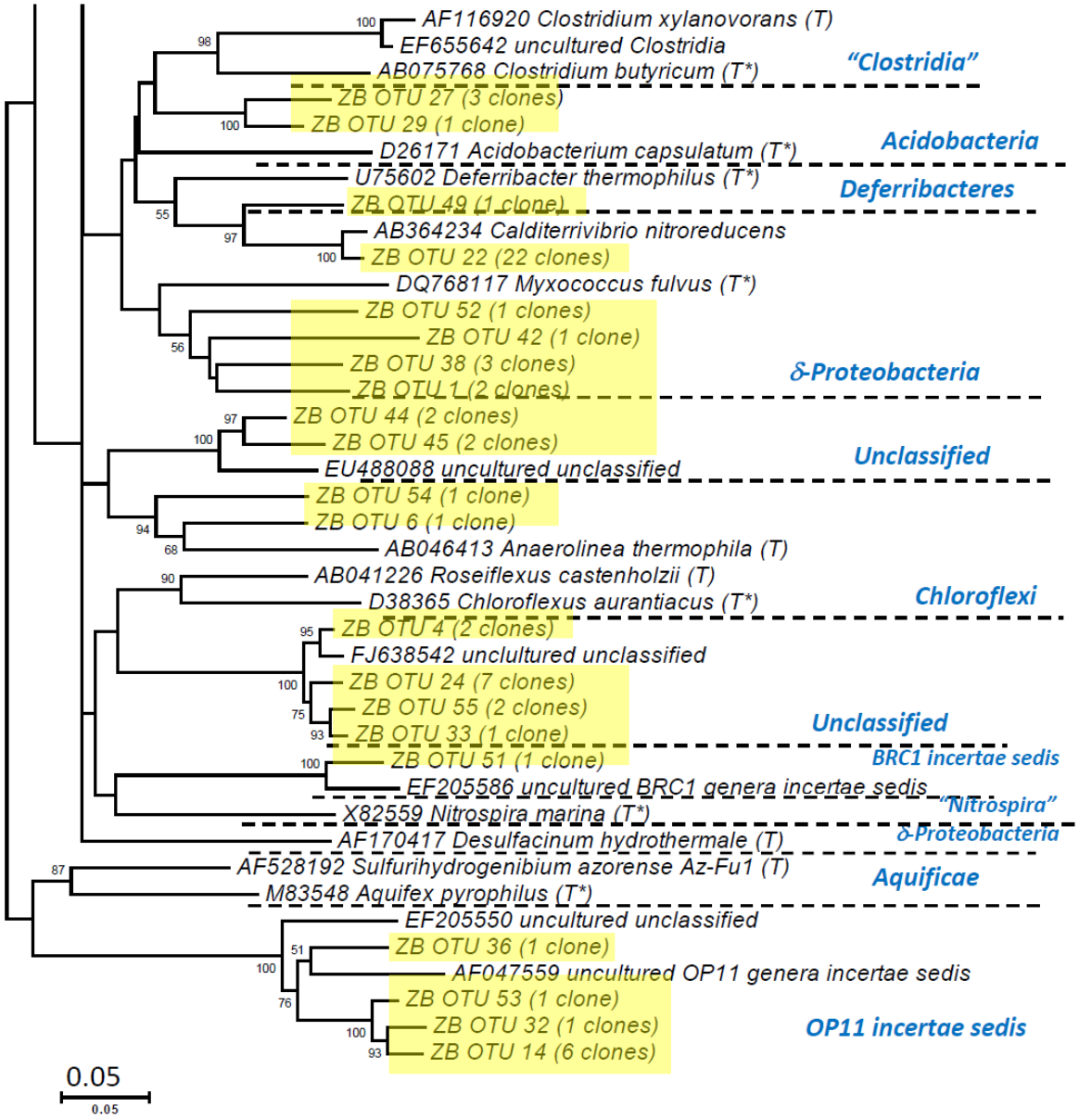
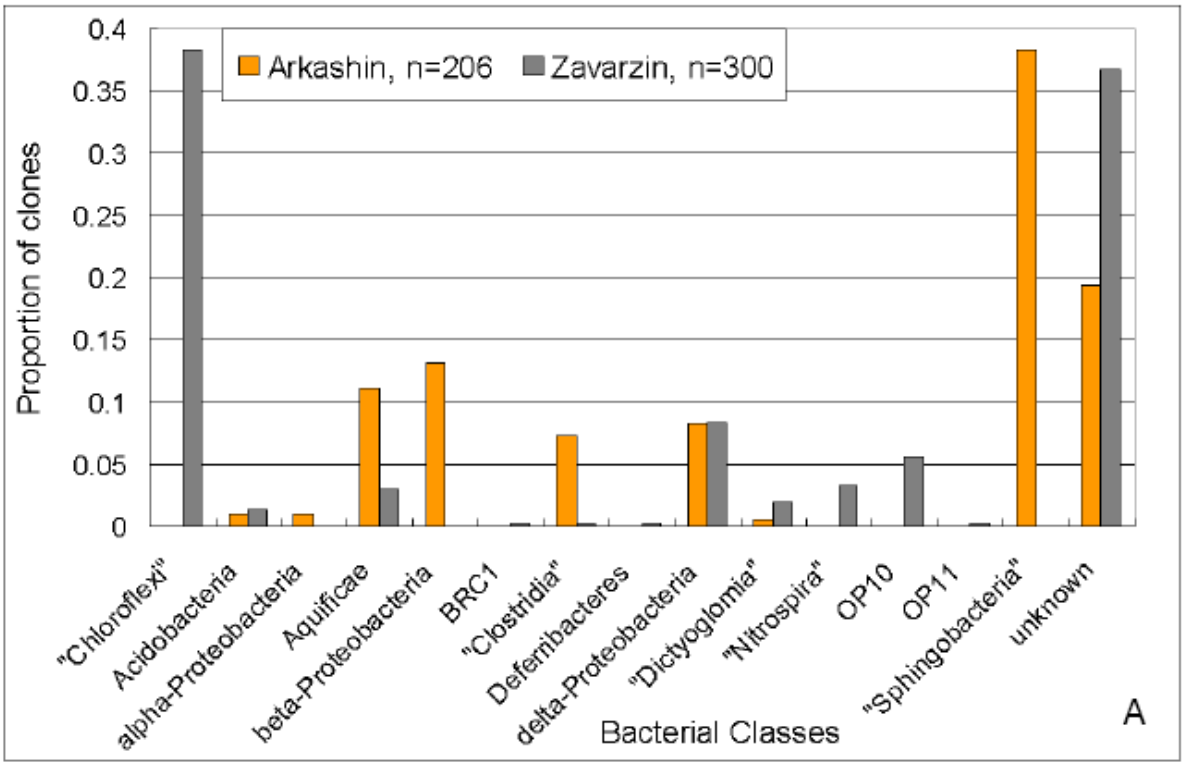
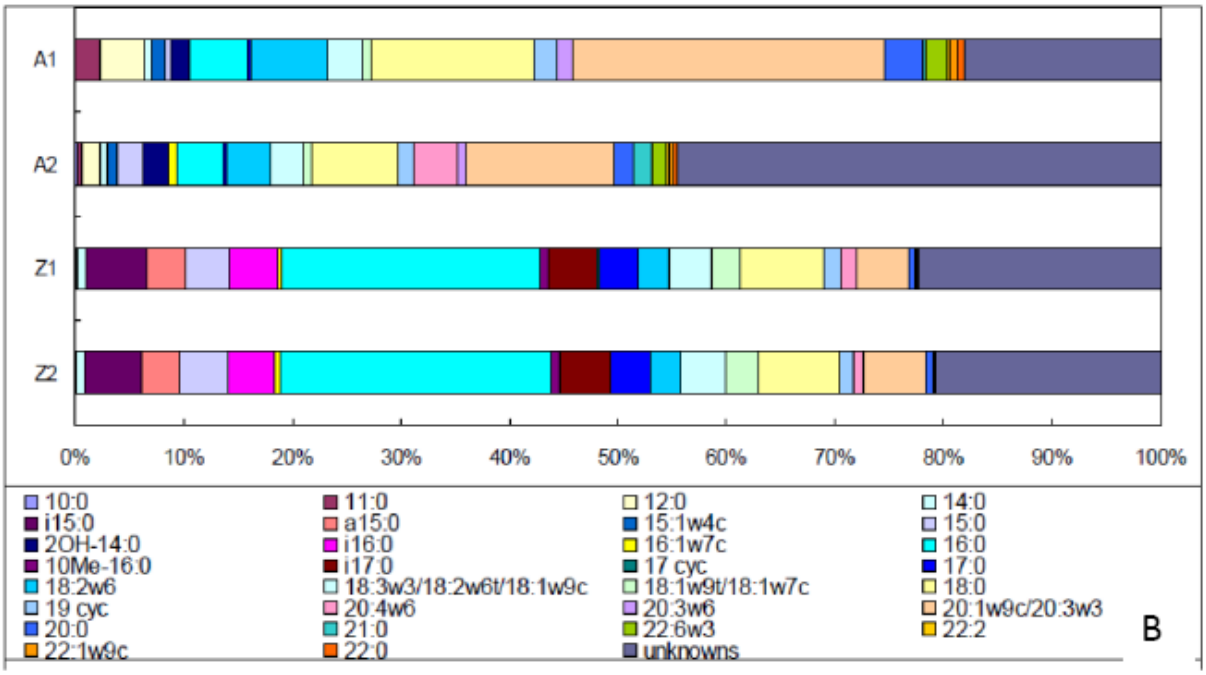


Figure 3.6 continued

Figure 3.7 A. Proportion of clones from the Zavarzin bacterial and Arkashin bacterial 16S rRNA gene sequence library in bacterial taxonomic groups with >80% confidence by RDPClassifier (Wang 2007). B. Bacterial phospholipid fatty acid (PLFA) relative mol% distribution in extraction replicates from Arkashin (A) and Zavarzin (Z) sediment samples presented in elution order; the mol% values of unknown peaks were estimated and summed.


Arkashin, n=206
Zavarzin, n=300
Proportion of clones
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
"Chloroflexi"
Acidobacteria
alpha-Proteobacteria
Aquificae
beta-Proteobacteria
BRC1
"Clostridia"
Defernibacteres
delta-Proteobacteria
"Dictyoglomia"
"Nitrospira"
OP10
OP11
"Sphingobacteria"
unknown
Bacterial Classes
A


A1
A2
Z1
Z2
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
10:0
11:0
12:0
14:0
i15:0
a15:0
15:1w4c
15:0
2OH-14:0
i16:0
16:1w7c
16:0
10Me-16:0
i17:0
17 cyc
17:0
18:2w6
18:3w3/18:2w6t/18:1w9c
18:1w9t/18:1w7c
18:0
19 cyc
20:4w6
20:3w6
20:1w9c/20:3w3
20:0
21:0
22:6w3
22:2
22:1w9c
22:0
unknowns
B

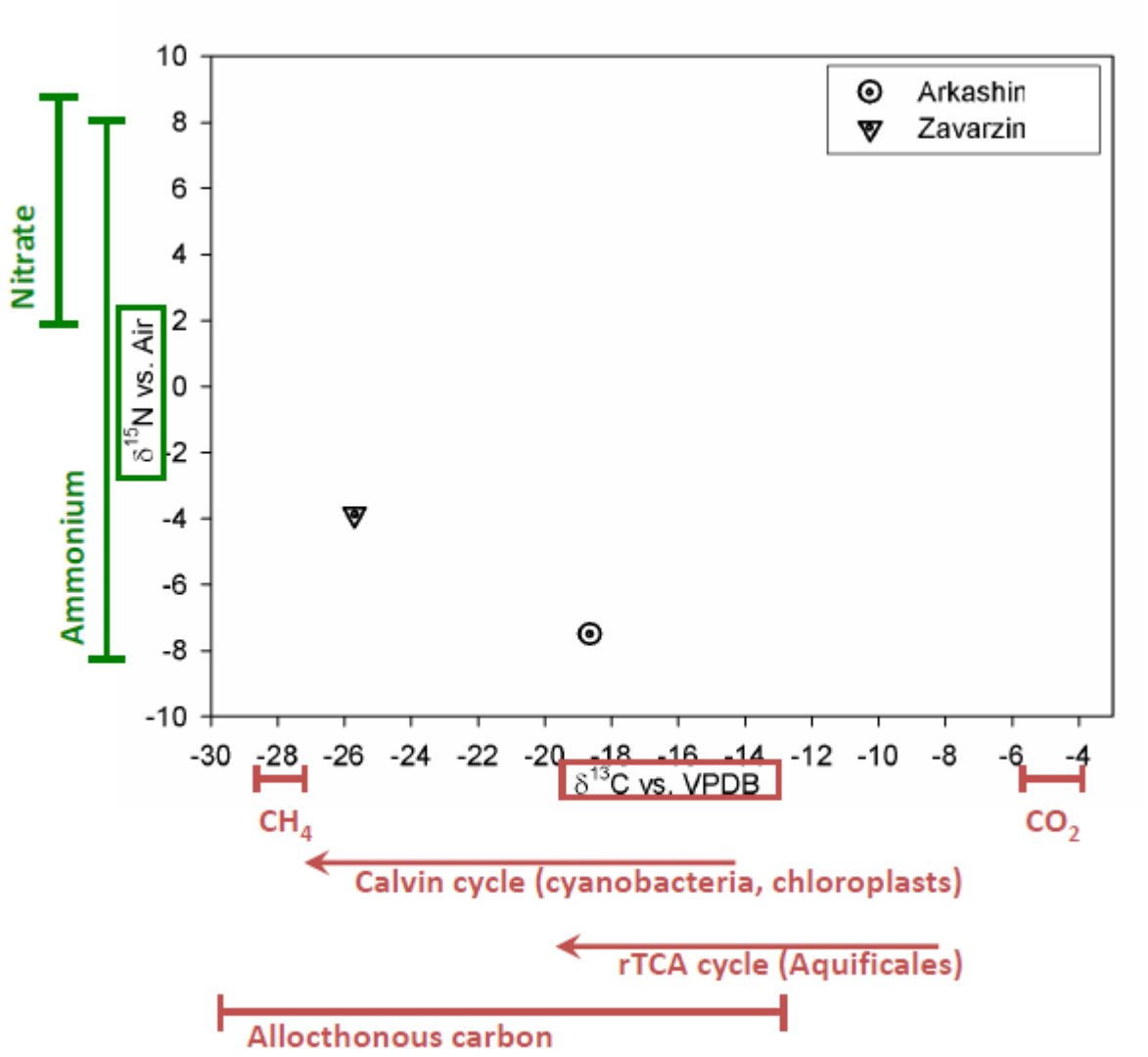


Figure 3.8 Stable isotope values measured in this study and reference values for potential components of the C and N cycles in Arkashin and Zavarzin. Ammonia and nitrate data are from C.S. Romanek, unpublished. The CO_2 and CH_4 data are from Zhao 2008. The nitrogen data shown in green reference only on the y-axis; correspondingly, the carbon data shown in red reference only on the x-axis.

CHAPTER 4

VARIATION WITH DEPTH IN ELEMENTAL COMPOSITION AND MICROBIAL COMMUNITIES IN CORE SAMPLES FROM TWO THERMAL POOLS IN THE UZON CALDERA, KAMCHATKA, RUSSIA[3]

[3]Burgess, E.A., J.M. Unrine, G.L. Mills, J. Wiegel. In preparation

ABSTRACT

Sediment core samples from two thermal pools in the East Thermal Field, Uzon Caldera, Kamchatka, Far East Russia, were examined in to characterize the changes with depth in geochemical and microbiological variables. Arkashin Shurf (Arkashin), a small, human-formed pool, is known for high concentrations of As. A peak in concentration of many As was associated with a low concentration, 10 μg g^{-1}, of phospholipid fatty acids (PLFA). The highest concentration of PLFA, 698 μg g^{-1}, was recovered from the surface sediments of Arkashin. Among PLFA biomarkers at the surface were those indicating the presence of *Aquificales*, and $\delta^{13}C$ values suggested the reverse TCA cycle was active in CO_2 fixation. A trend of increased ^{13}C depletion with depth in Arkashin indicated a change in carbon source or cycling process. In core samples from Zavarzin Spring (Zavarzin), a larger pool that was not human-formed, strata were delineated by white surface laminae and darker deep strata. PLFA concentrations in Zavarzin ranged from 29-168 μg g^{-1}. PLFA biomarkers in Zavarzin indicated *Chloroflexus* and cyanobacterial mats at the surface. Cyanobacteria, which use the Calvin cycle to fix CO_2, were implicated as primary producers by $\delta^{13}C$ values. Again, $\delta^{13}C$ were more depleted in deeper strata. Further investigation should reveal biomarkers for Archaea. The data presented indicate that the biomass and composition of microbial communities can shift considerably with macroscopically visible changes in geochemical conditions.

INTRODUCTION

The concept of scale in the ecology of hydrothermal features requires balance between macroscopically observable features and microbiologically relevant environments. Individual features with distinct physico- and geochemical conditions can be inhabited by distinct microbial communities (Skirnisdottir et al. 2000; Mathur et al. 2007; Pearson et al. 2008). These variables may change considerably over several meters along a hot spring outflow (Langner et al. 2001; Fouke et al. 2003; van der Meer et al. 2008). Dramatic changes also occur in millimeters of depth in microbial mats (Ward et al. 1998; Nubel et al. 2002; Dillon et al. 2007). The changes across centimeters with depth from the surface

have not been well-characterized in terrestrial thermal pools. The study of variation with centimenter scales of depth has been a useful approach to characterize microbial communities in marine sediments (Weber and Jorgensen 2002; Schouten et al. 2003; Joye et al. 2004), but has not been commonly applied in terrestrial hydrothermal environments.

The Uzon Caldera is a terrestrial hydrothermal environment in the Eastern volcanic zone of Kamchatka, Russia, where there are multiple geochemically distinct thermal fields (Karpov and Naboko 1990). For the present study, two pools from the East Thermal Field were sampled for analysis of multiple variables. The pools, Zavarzin Spring (Zavarzin) in the eastern sector and Arkashin Shurf (Arkashin) in the central sector, are approximately 600 m apart and are geochemically distinct due to heterogeneous transport of meteoric and magmatic inputs in the caldera (Karpov and Naboko 1990, Hollingsworth 2006). Arkashin and Zavarzin are temporally stable, and of similar pH (circumneutral to slightly acidic) and average surface temperature (~50°C), but geochemically quite distinct. The objective of this work was to describe the geochemical and microbiological changes with depth in core samples collected from these two thermal pools.

MATERIALS AND METHODS

Core collection and sub-sampling

Cores were collected in August 2005 and 2006 using a 10 cm diameter clear polycarbonate coring tube with a beveled end (Figure 4.1). Temperature was measured at the surface prior to sampling and the bottom of each core immediately after sampling. Cores were extruded and aseptically bisected longitudinally to observe visible changes in color and texture for identification and collection of sub-samples. Sub-sampling was done quickly and aseptically from the centers of the cores to limit oxidation and avoid collecting contaminated sediments from the cores' outer surfaces. Sub-samples were collected in wide-mouth glass jars closed with butyl rubber stoppers. Upon returning from the field, samples were homogenized in an anaerobic chamber and split for subsequent analyses. Sample storage is described for each analysis type below.

Cores are identified here with the letters A or Z for Arkashin and Zavarzin respectively, 05 or 06 for year collected, and -1 or -2 to indicated the duplicated cores in 2005. Sub-samples from each core are identified by depth in cm below the surface in parentheses. Thus, sub-sample Z05-2(3-6.5) is from Zavarzin 2005 core 2, 3-6.5 cm from the surface.

Cores from Arkashin were 12 to 41 cm long and cores from Zavarzin were 27 to 32 cm long. Sub-samples were collected from the cores as indicated in Table 4.1. Core Z05-2 contained a large, possibly pyroclastic, cobble in the last 6 cm, limiting sub-sampling to 24 cm. Nearly all sub-samples were analyzed for elemental composition and C and N stable isotope δ-values. TRFLP profiles were measured in A05-2 and Z05-2 sub-samples, and PLFA profiles in A06 and Z06 sub-samples.

Collection and analysis of sediment sub-samples

Sediment from each sub-sample was dried at 60°C and ground using an acid-washed mortar and pestle. Two ~0.25 g replicates from each were microwave digested with nitric acid then diluted to 50 mL with deionized, distilled H_2O. Analytical method blanks and standard reference materials were included in each digestion batch. Following digestion, samples, blanks and standards were analyzed for 24 elements (Li, Be, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Cd, Cs, Ba, Tl, Pb, U, Hg) using inductively coupled mass spectrometry (ICP-MS). Detection limits for 2005 sub-samples ranged from 0.01 $\mu g\ L^{-1}$ Co to 2.08 $\mu g\ L^{-1}$ Ca; relative percent difference (RPD) of replicate dilutions from 0.03% for Zn to 41% for Pb; average RPD of replicate digests from 5.45% for Cs to 50.7% for Cd; spike recovery from 26.55% for Zn to 120% for Ni; recovery of SRM was from 17.5% for Ca to 109% for Ni. Fe, As and Al in the 2005 samples required additional dilution for analysis. Detection limits in the higher dilution ranged from 0.02 $\mu g\ L^{-1}$ As to 9 $\mu g\ L^{-1}$ Fe; average RPD of replicate dilutions from 2.67% for Al to 14.3% for As; average RPD of replicate digests from 5.08% for Fe to 18.9% for Al; spike recovery from 99.8% for Fe to 109% for Al; recovery of SRM was from 28% for Al to 96% for As. Detection limits for the 2006 samples ranged from 0.014 $\mu g\ L^{-1}$ Cs to 12.4 $\mu g\ L^{-1}$ Al; average RPD of replicate

dilutions from 0.53% for Al to 7.41% for Pb; average RPD of replicate digests from 4.16% for Cs to 18.2% Mg; spike recovery from 95% for Zn to 131% for Ca; recovery of standard reference materials (SRM) was from 32% for Sr to 97% for Cu. Variables with QC values outside the ranges described were removed from analysis, except in the cases where were values below detection, which are reported as such. Fe was also analyzed by ICP-optical emission spectroscopy (OES). ICP-OES values only are reported for Fe. The method detection limit was 0.07 ppm Fe, the average RPD of replicate dilutions was 19%, 8.4 % of replicate digests. Values for sediment chemistry are presented in mg kg^{-1} of original wet sample weight.

Terminal restriction fragment length polymorphism analysis (TRFLP)

Within a week upon returning from the field in 2005, approximately 1 g of sediment stored at 4°C from each sub-sample was used for DNA extraction using the UltraClean Soil DNA Kit (MoBio Laboratories, Inc.; Carlsbad, CA, USA). Environmental DNA extraction products were stored at -20°C until analysis.

DNA was quantified by absorbance at 260 nm using a NanoDrop ND-1000 (ThermoScientific, Wilmington, DE) and all samples were diluted to the same concentration. An approximately 605 bp region of the 16S rRNA gene was amplified with either Bacterial-specific primers E334f, FAM-labeled, and E939r or Archaeal-specific primers A571f, HEX-labeled, and UA1204r (Baker et al. 2003) in a 50 μL PCR volume. PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen GmbH; Hilden, Germany) and visualized and quantified on 1% agarose gel via ethidium bromide staining and UV illumination using an AlphaImager™ 3400 (Alpha Innotech Corp.; San Leandro, CA, USA) and associated AlphaEase FC software for Windows v. 3.1.2. Cleaned PCR products for all samples were again diluted to the same concentration. Independent restriction digests were set up with three restriction enzymes *Eag* I, *Eco*O109 I and *Sma* I, following the manufacturer's instructions for 10-fold over-digestion (New England Biolabs, Inc.; Ipswich, MA, USA). Restriction enzymes were heat-inactivated and digest products were cleaned by ethanol precipitation followed by resuspension in 10 mM Tris, 0.1

mM EDTA, pH 8. For each enzyme-digest, 2 µL were combined with 13.3µL HiDi-formamide and 0.7µL size standard (DeWoody et al. 2004). Fragments were separated in duplicate on a 3130xl Genetic Analyzer (Applied Biosystems, Inc.; Foster City, CA, USA). Terminal restriction fragments were analyzed with GeneMapper v. 3.7 (ABI) using a modified AFLP method and a bin size of +/- 1 bp. Duplicate profiles were combined and transformed to binary matrices using T-Align (Smith et al. 2005) and a final bin size of +/- 2 bp.

Phospholipid Fatty Acid (PLFA) Profiling

Due to sample size limitations, analysis focused on the bacterial PLFA rather than both bacterial PLFA and archaeal GDGT; PLFA were chosen due to the higher taxonomic resolution they provide. Sediments for PLFA profiling were stored at -80°C. Before lipid extraction, sediments were freeze-dried, homogenized, and 5-10 g, depending on availability, weighed out into glass centrifuge tubes. A sample blank was processed along with the samples and a fatty acid methyl ester (FAME) internal standard was added to each tube. Based on White, et al., (1998), total lipids were extracted overnight in 1:2:0.8 chloroform:methanol:50 mM phosphate buffer. Polar lipids were recovered via silicic acid column chromatography. Phospholipid fatty acids (PLFA) were trans-esterfied with methanol to FAME (White and Ringelberg 1998). FAME were separated by gas chromatography on an Agilent HP 6890 Series GC system with a DB-5 column (30 m; 0.25 mm id) and flame ionization detector (Agilent Technologies, Inc.; Santa Clara, CA, USA). Individual chromatography peaks were identified by retention time as compared to that of standard reference components and sample profiles were blank corrected by comparison to the profile of the sample blank. In the instance where an individual PLFA could not be identified due to the tendency of multiple fatty acids to exhibit the same retention time possible acids are indicated as a co-eluting group and separated by forward slashes. Sub-samples not represented in the results had too little material available to recover adequate biomass for analysis. Identification focused on PLFA 14-22 carbons in length, which are presented in mol% composition.

C and N Concentration and Stable Isotope δ-values

Homogenized, dried, and ground samples were weighed into combustion tins and analyzed at the Ecology Soil Analysis Laboratory (University of Georgia, Athens, GA, USA). Samples were acidified for organic C (C_{org}) analysis (Ryba and Burgess 2002). Replicate analyses of standards indicated a resolution of <+/-0.3‰. Among repeated measures of the same samples, the highest variance was +/-1.3‰. Variation among repeated measures was greater for C_{org}, up to +/-3.4‰. However, comparison of C_{org} and C_{tot} data indicated C values before and after acidification were the same ($r^2 = 0.86$, $p < 0.05$ for ranked values of $\delta^{13}C_{org}$ vs. $\delta^{13}C_{tot}$; $r^2 = 1$, $p < 0.5$ for log transformed C_{org} vs. C_{tot} content). Thus, C_{tot} values only are used in figures. C_{tot}, C_{org} and N δ-values are presented as $\delta^{13}C_{tot}$ vs. Vienna Pee Dee Belemnite (VPDB), $\delta^{13}C_{org}$ vs. VPDB and $\delta^{15}N$ vs. Air respectively, and elemental composition is presented as a percent of original sample weight.

Data/Statistical analyses

Similarities among TRFLP profiles were calculated from presence-absence data using the Sorensen's coefficient of similarity:

$$S_{AB}=2j/(2a+b),$$

where j = the number of species present in both samples, a = the number of species present in site A, and b = the number of species present in site B (Magurran 1988; Osborn et al. 2000), using Estimate S (Colwell, R.K., http://purl.oclc.org/estimates). A distance matrix was calculated as $1-S_{AB}$ and used as input for construction of an unweighted pair group method with arithmetic mean (UPGMA) tree using Mega, v. 3.1 (Kumar et al. 2004).

Similarity among PLFA profiles was determined from relative abundance data using the Bray-Curtis similarity index (Rees et al. 2004), calculated as

$$S_{AB}=2jN/(aN+bN),$$

where aN = the number of individuals in site A, bN = the number of individuals in site B, and jN = the lowest sum from either site of abundances of species found in both sites, using Estimate S. An UPGMA tree was constructed using a $1\text{-}S_{AB}$ matrix (Mega, v. 3.1; Kumar et al. 2004). Diversity of the PLFA profiles was also determined using Estimate S. Diversity was represented by the reciprocal of Simpson index: $D=\sum p_i^2$, where p_i is the proportion of lipid mol% represented by the *i*th PLFA, again using Estimate S.

RESULTS

Sample Summary

Core samples were collected in 2005 and 2006 adjacent to each of the two thermal pools as indicated in Figure 4.1. Arkashin core sample temperatures ranged from 37-40°C at the surface, measured just before sampling, to 44-69°C at depth, measured at the bottom of the core just after sampling. Of note, the second 2005 core sampling at Arkashin resulted in the formation of a "mini" pool which had a surface temperature of 63°C but no yellow color 3 days later. The following year, the mini-pool was 26°C at the surface and was the same color as Arkashin. Visually, the cores from Arkashin were heterogeneous in texture and color (Table 4.1). Sediment cores from Zavarzin had fewer distinct strata; generally white, fine-grain surface layers and black, coarse-textured deeper layers, although laminae and intermediate color layers were also observed (Table 4.1). Temperatures in Zavarzin core samples ranged from 26-45°C at the surface and 47-64°C at depth.

Sediment Geochemistry

Sediment geochemistry is reported on an elemental basis after oven-drying each sub-sample; analytes are described in terms of total concentration (Table 4.1). Total concentrations of As, Ca, Al, Cs and Rb were higher in Arkashin than in Zavarzin, whereas Mn and Ba concentrations were consistently higher in Zavarzin. Concentrations of Fe, Mg, Sr, Pb, V, Co, Cu and Zn are similar in both pools. In particular, the concentration of Cu increases with depth in both pools. The range and standard deviation

were quite large for some analytes, such as As and Al, indicating a larger variation within the core than for other analytes.

Bacterial Community Structure

To examine how the predominant bacterial and archaeal community members varied with depth in the core samples, TRFLP was performed using 16S rRNA genes. Although Archaeal positive controls yielded amplicons of the appropriate size, no PCR products were obtained from Archaeal primers and Zavarzin samples. No PCR products were obtained from any primers with Arkashin samples. Amplification of archaeal targets from Zavarzin and all targets from Arkashin was unsuccessful; however, this may be the result of DNA extraction inefficiencies and PCR limitations rather than absence of Archaea or Bacteria. From core Z05-2, after independent digests with three restriction enzymes, 41 unique bacterial terminal restriction fragments were identified. The highest richness of fragments was 23 in sub-sample Z05-2(18.5-24). The lowest richness, 9 fragments, was in sample Z05-2(3-6.5). Eleven fragments were identified in each of the remaining sub-samples. TRFLP profiles in Zavarzin showed two major clusters, sub-samples from less than 7cm and sub-samples from greater than 7cm depth from the surface (Figure 4.2). The highest similarity was between sub-samples Z05-2(0-3) and Z05-2(6.5-7).

To corroborate the TRFLP results, total bacterial phospholipid fatty acids (PLFA) were extracted quantitatively from sub-samples of cores A06 and Z06 by addition of an internal standard (Table 4.2). Limited sample availability did not allow for analysis of archaeal lipids. Comparison of relative abundances of PLFA demonstrated that each pool was inhabited by distinct communities, and that communities changed with depth (Figure 4.3). In a UPGMA tree, one sub-sample from each site clustered with the other site and profiles from mid-depth sub-samples in Arkashin formed a highly similar group (Figure 4.3B).

The highest concentration of PLFA, 698 μg/g, was recovered from A06(1-2). A higher concentration of PLFA recovered was generally associated with a greater diversity of PLFA detected (Table 4.2). The lowest amount of PLFA, 10 μg/g, was recovered from A06(13-21). All A06 sub-samples

had a >10% portion of unsaturated 18 C PLFA. In particular, the co-eluting group 18:3ω3/18:2ω6t/18:1ω9c was a >20% component in sub-samples A06(13-21), (21-28), and (28-33). Other components at >5% to >10% or >20% proportions in A06 sub-samples included 16:0, 18:0, cyc19:0, 20:2, 2-OH14:0, 20:4ω6, and 16:1ω7c. A notable to dominant proportion of each profile was comprised of fatty acids that were not identifiable with the standards used in this study. One exception was sub-sample A06(13-21), from which there were no unidentifiable fatty acids recovered.

PLFA concentrations from Z06 samples ranged from 29 to 169 μg/g. These sub-samples also had >10% concentrations of unsaturated 18 C fatty acids, and Z06(6-13) and (13-32) were dominated by 18:1ω9t/18:1ω7c. Other fatty acid components at >10% to >5% proportions in Z06 sub-samples included 16:0, 18:0, 20:4w6, 20:2, 16:1w7c, cyc19:0, and unknown fatty acids.

Carbon, Nitrogen Concentration and Stable Isotope δ-values

Sediment total C (C_{tot}), organic C (C_{org}) and total N, and the δ-values of total C ($\delta^{13}C_{tot}$), organic C ($\delta^{13}C_{org}$) and total N ($\delta^{15}N$) are reported in Table 4.3. Comparison of total C and organic C concentrations and δ-values indicated they were equivalent (see Methods). At both sites, C_{tot} and N concentrations decreased with depth. Additionally, $\delta^{13}C_{tot}$ values were increasingly depleted with depth (Figure 4.4). An opposite trend was observed with respect to $\delta^{15}N$ values in both pools. Upper sub-samples from Zavarzin had the most depleted $\delta^{15}N$ values. In Arkashin, $\delta^{13}C_{tot}$ and $\delta^{15}N$ values in upper sub-samples (<13 cm) ranged from approximately -21 to -16‰ and -9 to -3‰ respectively, while values in deeper sub-samples were more variable. Zavarzin $\delta^{15}N$ values in surface sub-samples clustered between -4 to -2‰, but deeper sub-samples ranged from -10 to -4‰. Most $\delta^{13}C$ values in Zavarzin sub-samples from >5 cm depth clustered around -27 to -25‰ whereas sub-samples from <5 cm depth ranged between -21 to -28‰.

DISCUSSION

Although only ~600 m apart, Arkashin and Zavarzin are separated by geological barriers and represent two geochemically and microbiologically distinct thermal pools within the East Thermal Field of the Uzon Caldera. Each pool had distinct patterns of change across centimeters of depth.

Geochemistry

Current conditions in a given pool reflect a combination of factors including transport and mixing of meteoric and magmatic waters (Migdisov and Bychkov 1998; Cleverley et al. 2003). The differences between Arkashin and Zavarzin are consistent with the idea of heterogeneous hydrology in continental thermal fields like those in Uzon. Arkashin represents a pool with an alkali-chloride type hydrothermal fluid. Alternatively, Zavarzin has been described as a mixed water type (of magmatic and meteoric inputs), that may also be influenced by a localized sulfur plume (Hollingsworth 2006).

Microbiology

Arkashin and Zavarzin are microbiologically distinct. TRFLP profiles in Zavarzin showed two major clusters, samples <7 cm and samples >7 cm. The highest similarity, between samples Z05-2(0-3) and Z05-2(6.5-7), follows the observation of laminae in the upper sub-samples of Zavarzin cores. Though multiple methods were employed, DNA recovery was not adequate from Arkashin for this study. However, the highest PLFA concentrations were recovered from Arkashin surface sub-samples.

Identification of specific bacterial groups can be made by the co-occurrence of various PLFA biomarkers. Among the commonly accepted PLFA biomarkers in hot springs are those for heterotrophic bacteria (iso and anteiso unsaturated 15-17 C chains), cyanobacteria, (16:0, 18:0, 16:1 and 18:1) and *Chloroflexus*, (16, 17 and 18:0 and 16, 17, and 18:1; Summons et al. 1996). Biomarkers 20:1ω9/20:3ω3 (4.75 mol%), 18:0 (7.81 mol%) and 18:1ω9t/18:1ω7c (1.41 mol%), were found in A06(1-2) and are consistent with those described for *Aquificales* (Jahnke et al. 2001; Stohr et al. 2001). Predominant PLFA

identified in Zavarzin sub-sample Z06(2.5-4.0), 16:0 (17.4 mol%), 18:0 (12.0 mol%) and 18:1 (9.54 mol%), may be representative of cyanobacteria and *Chloroflexus* mats in this sample. A mat community dominated by high population numbers of a few species is consistent with the observation that Z06(2.5-4.0) had a relatively high concentration of PLFA, 63.0 μg/g, but the lowest diversity, 6.57 (Table 4.2). In all Zavarzin sub-samples biomarkers for heterotrophic bacteria were identified as a component (4.6 to 7.3 mol%). This includes the major PLFA that occur in *Thermoanaerobacter uzonensis* and *Caldalkalibacillus uzonensis*, both species have representatives isolated from Zavarzin (Wagner, et al. 2008; Zhao, et al. 2008).

Microbial sulfate-reduction has been suggested as a contributing factor to sulfide mineralization in Uzon (Karpov 1991; Bonch-Osmolovskaya 1994; Migdisov and Bychkov 1998). Generally accepted biomarkers for well-known mesophilic sulfate-reducers (Londry et al. 2004) were not abundant. For example, 10Me16:0, occurred in A06(1-2) only, at a relative abundance of 0.34 mol%. Major fatty acids in *Thermodesulfovibrio* species, a15:0, 16:0, i16:0, and i17:0 (Sekiguchi et al. 2008), occurred in all samples. However, 16:0 is common in many bacteria. Removing 16:0 from consideration, the fatty acids of *Thermodesulfovibrio* occurred at <5 mol% in only the three surface sub-samples from Arkashin and in all sub-samples analyzed from Zavarzin at approximately 4-7%. Thus, sulfate-reduction by *Thermodesulfovibrio* is not likely to play a major role in arsenic sulfide mineralization in Arkashin. Finally, all sub-samples except A06(13-21) contained a number of unknown PLFA (8.22-44.7 mol%). The abundance of unknown PLFA suggests relatively uncharacterized microorganisms inhabit these two pools.

The UPGMA clustering of sub-samples demonstrated that the strata in the two pools generally grouped by depth and by pool. Where sub-samples from different pools clustered together represents the magnitude of changes between surface samples and deeper samples within each pool.

Carbon and Nitrogen Stable Isotope δ-values

As mentioned above, PLFA biomarkers identified in Arkashin sub-samples included those for *Aquificales*. *Aquificales* can function as primary producers using the reverse TCA cycle, resulting in $\delta^{13}C$ values of -4 to -13‰ relative to the source CO_2 (Hayes 2001). Thus, $\delta^{13}C_{tot}$ values in upper Arkashin sub-samples were consistent with *Aquificales* fixing atmospheric or hydrothermal CO_2 ($\delta^{13}C_{atmCO2}$ 0 to -10‰, $\delta^{13}C_{thermCO2}$ +2 to -12‰; Des Marais 1996). Possible sources for the lower $\delta^{13}C$ values observed in deeper Arkashin sub-samples may include hydrothermal methane or allocthonous inputs of organic matter, perhaps in run-off from precipitation events (Des Marais 1996). A likely scenario is methane oxidation by thermophilic *Archaea* (Schouten et al. 2003).

Anaerobic oxidation of light, thermogenic methane or heterotrophy may be important in the deeper sediments in Zavarzin as well. At the surface, *Chloroflexus* and cyanobacteria were indicated by PLFA biomarkers as potential primary producers, though each uses a different C-fixation pathway. Cyanobacteria, using the Calvin cycle, are expected to cause a -10 to -22‰ change from source CO_2, whereas *Chloroflexus*, via the 3-hydroxyproprionate pathway, fix inorganic carbon with minimal fractionation (Hayes 2001). The $\delta^{13}C$ values in upper Zavarzin samples indicated the dominance of cyanobacterial primary production.

Coincidentally, $\delta^{15}N$ values in Zavarzin were not consistent with cyanobacteria fixing atmospheric nitrogen, where minimal fractionation from 0‰ is expected (Estep and Macko 1984). The $\delta^{15}N$ values in both pools are consistent with hydrothermal ammonia (Kamchatka MO, unpublished data). That the values are more depleted as they reach the surface may indicate *in situ* N cycling (Estep and Macko 1984).

Conclusions

The objective of the described study was to characterize the geochemical and microbiological changes across visible gradients with depth from the surface in two terrestrial thermal pools, Arkashin and

Zavarzin. The data observed represent phenomena localized by site and by depth, such as the higher concentrations of As in Arkashin or the higher abundance of known heterotrophic PLFA in Zavarzin. Additionally, microorganisms may remain to be characterized in these hydrothermal features, as represented by unidentified PLFA in both pools. Shifts in $\delta^{13}C$ values in Arkashin may represent a shift from surface rTCA carbon fixation to another pathway or a different carbon source. The scale of this investigation, centimeters, is a reasonable approach to transition from macroscopic observations to microbial communities and to demonstrate the magnitude of variations within individual thermal pools.

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Table 4.1 Sub-sample depths, color, water content, and sediment chemistry for core samples.

Core	Sub-sample depth (cm from surface)	Color	Water (%)	Total concentrations of elements, determined by ICPMS or OES (Fe only) of sediment digests (mg kg^{-1})														
				Mg	Al	Ca	V	Mn	Co	Cu	Zn	As	Rb	Sr	Cs	Ba	Pb	Fe
A05-1	1.5-2.5	red-brown	30.0	128	3443	1328	6.35	7.97	0.323	5.59	27.8	1392	4.43	12.9	12.1	144	0.744	965
	2.5-5	green, black stripe	36.0	101	2567	767	5.03	7.68	0.737	8.97	14.3	5814	3.83	8.89	9.83	157	0.881	1665
	5-6.5	yellow-orange	35.2	130	2687	1007	6.56	9.10	0.423	7.16	14.7	5791	2.58	9.02	6.75	19.6	1.11	1308
	6.5-10	black	41.4	117	3999	1097	13.8	10.6	1.26	16.3	27.4	5221	5.48	12.7	15.1	16.5	0.789	2492
	10-12	brown-orange	39.6	142	5329	2047	23.2	9.79	1.67	10.8	23.8	6159	9.19	16.3	28.9	18.3	0.681	1941
A05-2	0-5	brown-grey	30.7	27.2	687	bd	3.29	1.87	0.057	4.18	19.0	1846	3.17	3.88	8.28	52.3	0.523	1365
	5-10	green-yellow	31.3	36.9	1129	230	5.78	1.92	bd	6.03	bd	27238	2.14	3.69	12.5	7.61	bd	1701
	10-13	yellow, black stripe	33.1	39.5	1168	326	13.2	3.33	1.12	24.9	25.8	39366	3.72	4.73	26.4	4.82	0.305	2571
	16-17	bright orange	20.0	157	9271	947	44.8	12.3	7.14	11.1	39.0	58954	6.66	8.52	31.2	10.6	1.10	14828
	17-19.5	dark purplish	29.7	122	17851	777	66.5	10.1	12.3	16.8	59.1	102445	4.76	7.51	19.8	9.02	2.50	19038
	19.5-23	red	12.9	132	14034	1000	75.6	13.1	14.5	20.4	69.3	123744	4.63	9.68	16.8	12.6	2.88	22000
	23-30	grey-black	26.0	197	8303	1667	55.8	19.4	12.1	18.8	58.6	3904	4.02	14.8	15.9	18.0	1.29	17604
	33-40	black	24.5	279	13284	2840	69.1	25.5	13.2	18.8	53.4	3085	4.59	20.2	18.1	18.3	1.49	37887
A06	1-2	yellow	23.4	510	6464	3235	15.0	38.9	0.910	bd	14.4	1052	2.08	23.2	6.68	112	0.651	4498
	2-5	greenish	38.6	128	2549	892	bd	9.72	0.395	6.69	14.6	5130	2.96	9.68	7.01	35.8	0.491	1226
	5-8.5	yellow-brown	33.1	147	3740	1562	8.79	12.6	1.10	12.4	64.0	9552	2.73	13.3	7.33	29.3	0.695	2398
	8.5-13	yellow-grey	30.1	193	9593	3225	40.7	15.2	5.39	11.3	24.3	8304	5.75	23.5	22.7	20.2	0.736	3827
	13-21	orange	24.9	272	16309	3977	75.5	20.4	10.3	15.9	54.7	53939	6.07	26.2	29.1	22.4	1.55	11894
	21-28	yellow	22.4	198	15791	4952	54.1	18.0	11.7	12.5	38.5	21056	5.98	33.4	31.7	24.0	0.680	8088
	28-33	purple-red	23.3	353	14249	5735	55.3	30.8	9.26	22.7	57.7	10333	4.10	35.1	14.9	24.6	bd	5865
	33-41	black	36.0	248	16732	5840	53.1	24.9	14.4	15.7	64.2	829	3.83	35.4	19.0	23.1	1.86	7014
Z05-1	0-2.5	yellow-white	31.8	170	1482	467	3.20	39.8	1.34	6.93	23.2	11.2	bd	4.53	bd	71.9	0.719	3143
	2.5-6	yellow-grey	31.8	650	4574	495	11.6	95.9	3.73	7.75	27.1	21.8	bd	5.81	0.240	91.2	1.17	8433
	6-25	dark grey	34.0	119	1210	232	4.60	81.6	5.19	13.2	44.1	32.2	bd	6.64	0.204	67.6	1.30	7749
	25-37	black	29.9	127	2804	698	30.2	379	20.6	17.0	57.0	34.9	0.42	9.36	0.638	53.8	1.51	40487
Z05-2	0-3	yellow	38.9	420	3130	623	8.87	74.0	2.52	7.14	53.6	23.2	0.38	7.66	0.250	44.9	1.20	9143
	3-6.5	grey	52.4	76.8	940	55.9	2.64	20.4	0.754	2.31	8.27	7.52	0.08	2.13	0.059	23.9	0.623	2406
	6.5-7	dark grey	32.1	624	4633	658	13.5	109	7.67	14.5	28.3	44.7	0.50	11.7	0.347	73.4	2.45	15908
	7-14	grey	30.0	48.6	592	bd	2.73	17.8	0.550	2.03	7.00	11.9	0.26	3.32	0.200	17.9	1.10	2076
	14-18.5	dark grey, mottled	34.1	42.3	827	79.1	3.44	27.6	1.53	14.6	19.2	11.8	0.38	10.5	0.298	143	0.962	2591
	18.5-24	black	41.4	41.5	662	79.2	6.14	65.6	9.23	24.7	9.70	58.1	0.29	5.76	0.250	47.9	2.44	8652
Z06	0-2.5	yellow-white	48.6	220	2473	471	4.59	54.1	1.35	bd	15.1	17.7	bd	5.49	bd	46.9	0.523	4183
	2.5-4	grey	37.4	200	2953	477	7.02	57.9	1.23	bd	13.0	66.9	bd	20.9	bd	126	0.890	5431
	4-6	brown	35.8	71.6	1340	bd	bd	17.1	0.757	bd	22.2	9.57	bd	14.1	bd	107	0.768	1068
	6-13	yellow-green	25.6	29.3	450	bd	bd	13.2	0.846	bd	13.5	6.37	bd	3.29	bd	80.4	bd	1141
	13-32	black	37.1	120	1174	bd	36.1	217	27.8	23.3	38.9	68.7	bd	5.90	0.439	99.1	2.23	51570

bd = below detection
nm = not measured

Table 4.2. PLFA concentration, recovery and diversity in 2006 core samples

Core	Sub-sample depth (cm from surface)	Concentration* (ug/g)	Recovery (% IS)	Simpson diversity**
A06	1-2	698	99	9.20
	5-8.5	128	44	9.39
	8.5-13	82	91	7.86
	13-21	10	85	8.35
	21-28	15	76	8.91
	28-33	20	105	9.26
	33-41	30	97	9.32
Z06	2.5-4	63	72	6.57
	6-13	168	81	9.51
	13-32	29	87	9.52

*determined based on internal standard added prior to extraction

**as per EstimateS (Colwell, etc.)

Table 4.3. Sub-sample depths, percent composition and stable isotope δ-values of C & N.

Core	Sub-sample depth (cm from surface)	Carbon (%)	$\delta^{13}C$ (‰)	Nitrogen (%)	$\delta^{15}N$ (‰)
A05-1	1.5-2.5	0.283	-18.8	0.0403	-5.64
	2.5-5	0.469	-17.9	0.0433	-8.54
	5-6.5	0.397	-18.4	0.0396	-7.02
	6.5-10	0.385	-18.1	0.0314	-8.36
	10-12	0.313	-19.3	0.0342	-3.27
A05-2	0-5	0.348	-17.7	0.0477	-7.48
	5-10	0.294	-16.2	0.0404	-6.66
	10-13	0.223	-18.4	0.0304	-5.42
	16-17	0.111	-24.3	0.0215	-1.65
	17-19.5	0.0551	-26.2	0.0124	-3.01
	19.5-23	0.0620	-27.9	0.0096	-8.84
	23-30	0.0395	-25.5	0.0113	-0.154
	33-40	0.0336	-25.3	bd	bd
A06	1-2	0.128	-17.6	0.0232	-5.97
	2-5	0.370	-18.5	0.0391	-7.38
	5-8.5	0.350	-19.1	0.0353	-6.63
	8.5-13	0.168	-20.6	0.0164	-3.26
	13-21	0.0697	-24.4	0.0129	0.835
	21-28	0.0538	-21.9	0.0137	-0.751
	28-33	0.0331	-25.5	bd	bd
	33-41	0.0273	-24.0	0.0095	1.60
Z05-1	0-2.5	1.02	-21.0	0.141	-12.5
	2.5-6	0.579	-22.0	0.0710	-9.29
	6-25	0.207	-24.5	0.0247	-5.78
	25-37	0.0789	-25.2	0.0261	-2.45
Z05-2	0-3	0.676	-23.0	0.0887	-9.77
	3-6.5	0.837	-22.1	0.108	-13.1
	6.5-7	0.649	-22.3	0.0787	-6.79
	7-14	0.161	-24.8	0.0374	-5.09
	14-18.5	0.233	-25.7	0.0378	-3.45
	18.5-24	0.274	-26.4	0.0396	-2.74
Z06	0-2.5	0.364	-27.1	0.0538	-8.80
	2.5-4	0.228	-25.8	0.0409	-5.06
	4-6	0.281	-25.4	0.0372	-3.09
	6-13	0.191	-25.8	0.0230	-3.31
	13-32	0.097	-25.5	0.0234	-3.56

*stable isotope ratios determined vs. PDB and Air for C and N respectively
nm = not measured

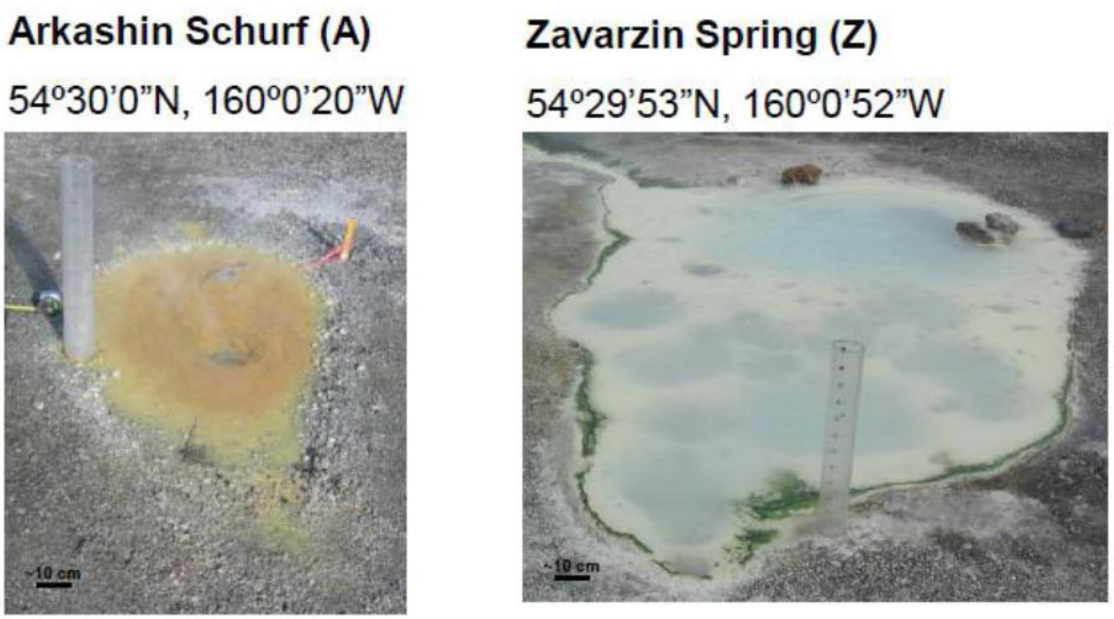


Figure 4.1. Geographic coordinates obtained by global positioning system and photographs of the pools showing the locations of two of the six core samples collected.

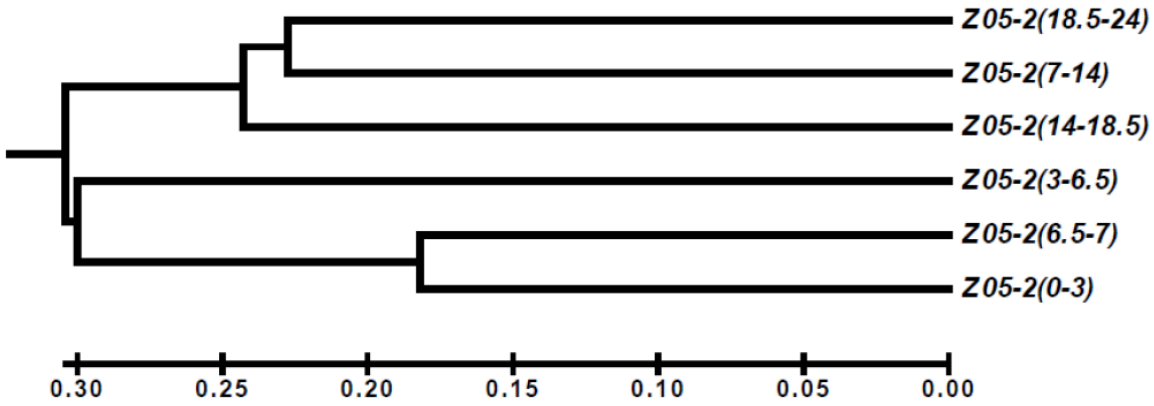
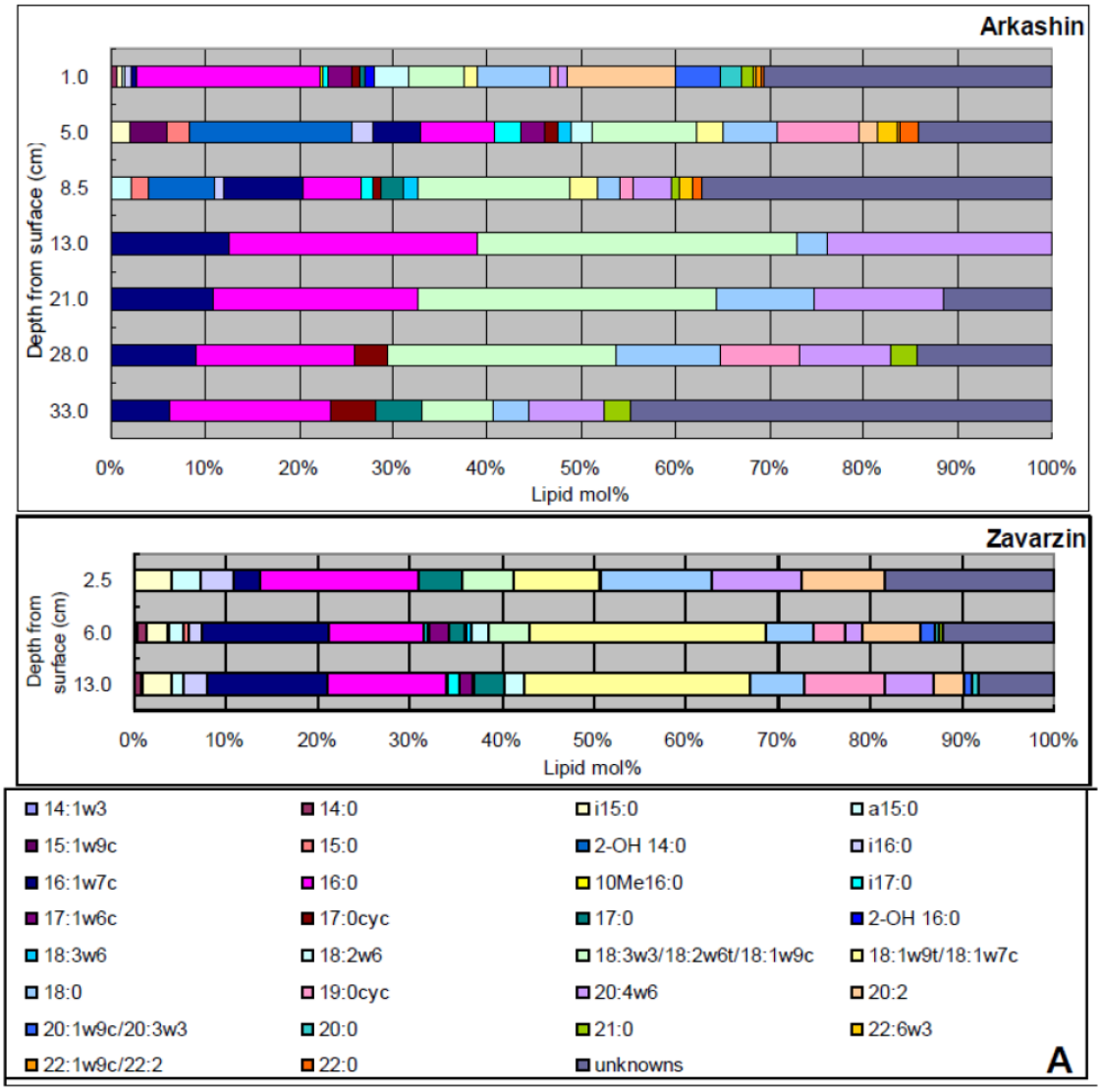
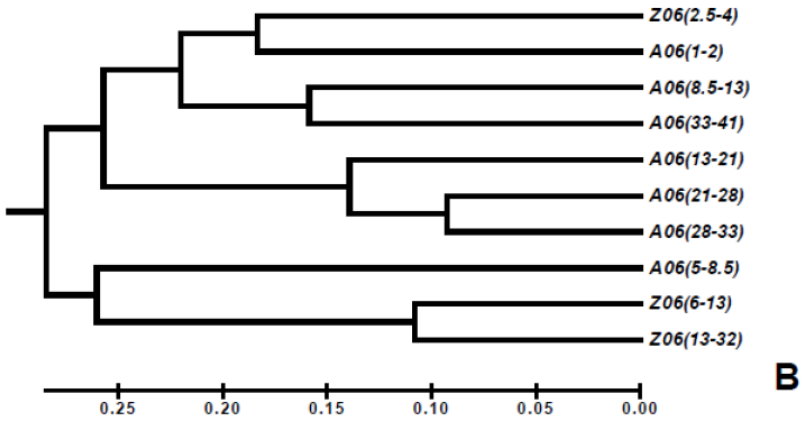


Figure 4.2. UPGMA tree of 1-Sorensen similarity matrix of terminal restriction fragment length polymorphism profiles from Z05-2 sub-samples.

Figure 4.3. Relative abundance composition profiles (A) and UPGMA tree from a 1-Bray-Curtis similarity matrix (B) of Bacterial phospholipids fatty acids from A06 and Z06 sub-samples.


Arkashin
Depth from surface (cm)
1.0
5.0
8.5
13.0
21.0
28.0
33.0
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Lipid mol%
Zavarzin
Depth from surface (cm)
2.5
6.0
13.0
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Lipid mol%
14:1w3
14:0
i15:0
a15:0
15:1w9c
15:0
2-OH 14:0
i16:0
16:1w7c
16:0
10Me16:0
i17:0
17:1w6c
17:0cyc
17:0
2-OH 16:0
18:3w6
18:2w6
18:3w3/18:2w6t/18:1w9c
18:1w9t/18:1w7c
18:0
19:0cyc
20:4w6
20:2
20:1w9c/20:3w3
20:0
21:0
22:6w3
22:1w9c/22:2
22:0
unknowns
A


Z06(2.5-4)
A06(1-2)
A06(8.5-13)
A06(33-41)
A06(13-21)
A06(21-28)
A06(28-33)
A06(5-8.5)
Z06(6-13)
Z06(13-32)
0.25
0.20
0.15
0.10
0.05
0.00
B

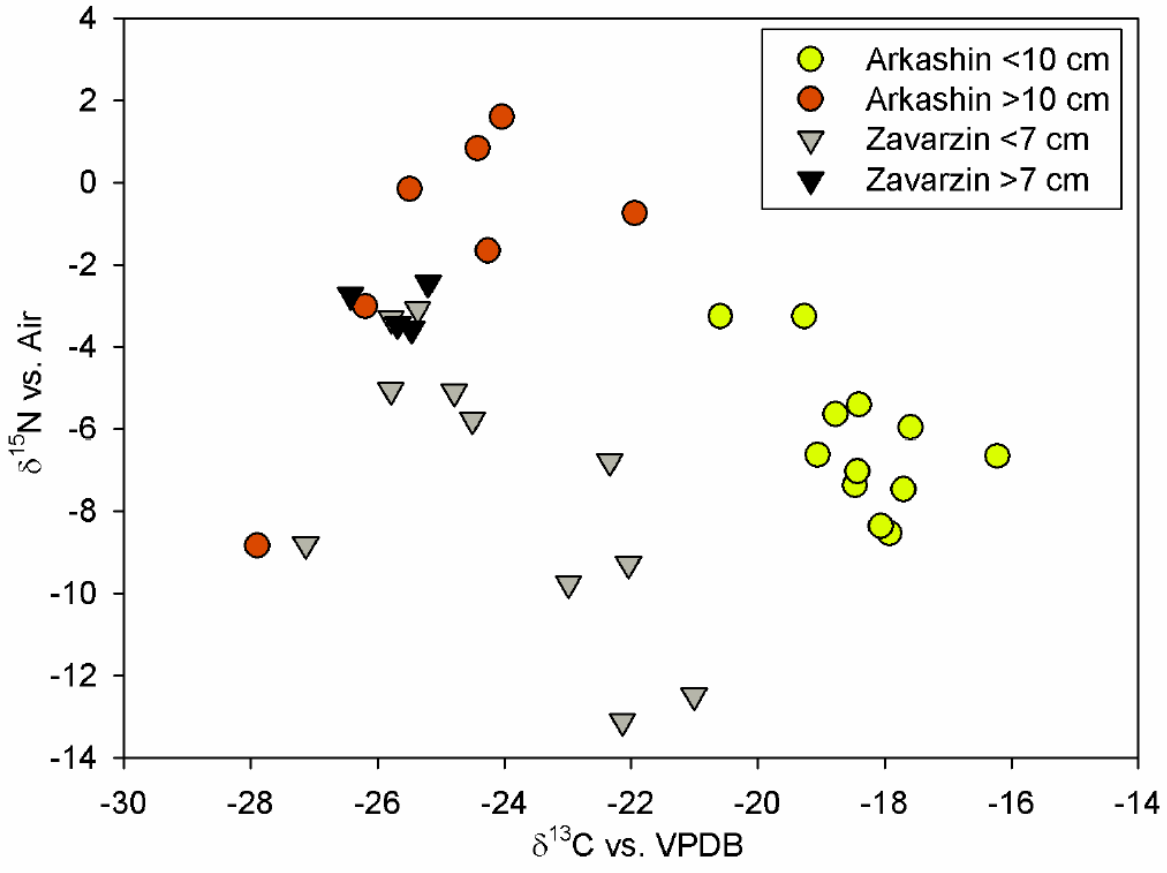


Figure 4.4. Relationship among N and C stable isotope δ-values in sub-samples from all cores in both pools.

CHAPTER 5

EVIDENCE FOR SULFATE-REDUCTION AND BIOGENIC SULFIDE MINERALIZATION IN CORE SAMPLES FROM TWO THERMAL POOLS IN UZON CALDERA, KAMCHATKA, RUSSIA[4]

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ABSTRACT

Geochemical and microbiological variables were examined in core samples from two thermal pools in the Uzon Caldera, Kamchatka, Russia to characterize the contribution of sulfate reduction to sulfide mineralization in a contemporary hydrothermal system. In Arkashin Shurf, a small, human-formed pool, color variation in soil strata was associated with varying concentrations of As and S. A zone of active arsenic-sulfur cycling was suggested by a sulfate-reduction rate of 50.6 nmol cm^{-3} day^{-1} at 19.5-23 cm below the surface, where the sulfate concentration was 1.93 mM. This zone was also associated with relatively high concentrations of chalcophilic elements (Cu, Zn, As, Pb, Fe) and enriched in the heavy-isotope of sulfur (more positive $\delta^{34}S$ values). Core samples from Zavarzin Spring were characterized by fine-textured surface layers, light in color, with abundant elemental S, and coarse-textured, darker layers with increasing depth. Concentrations of chalcophilic elements and $\delta^{34}S$ values were highest in the transition zone between surface and deeper layers in Zavarzin Spring. The highest sulfate-reduction rate in Zavarzin Spring, 3.96 nmol cm^{-3} day^{-1}, was measured in the deepest layers, where the suflate concentration was 1.93 mM and concentrations of chalcophilic elements were high and $\delta^{34}S$ values were more positive relative to other sub-samples. These data indicate that sulfate-reduction is relatively low in Akrashin and Zavarzin relative to other hydrothermal features and that the $\delta^{34}S$ signal of sulfide minerals derived from sulfate-reduction may be obscured by sulfur cycling *in situ* and mixing of various S species with different $\delta^{34}S$ values

INTRODUCTION

The prevalence of hydrothermal environments throughout Earth's history and the possibility of their occurrence other planets or moons justifies the investigation of contemporary hydrothermal environments as interpretive analogs (Wacey et al. 2009; Reysenbach and Cady 2001; Bock and Goode 1996; Westall 2005). Understanding the role of microorganisms in such systems should provide insight to biogeochemical cycling, early life on Earth, and astrobiology. For example, microorganisms imprint their

signature in sulfide minerals through metabolic reactions such as sulfate-reduction. The activity of sulfate-reducing prokaryotes can play an important role in determine the $\delta^{34}S$ signatures of contemporary and ancient sulfur-containing minerals (Habicht and Canfield 1996; Kakegawa and Ohmoto 1999; Bradshaw et al. 2008).

The Uzon Caldera is a contemporary hydrothermal system situated in the Eastern volcanic zone of Kamchatka, Far East Russia (Figure 5.1). The hydrology in Uzon is heterogeneous, and there are multiple geochemically distinct thermal fields within the caldera as well as active mineralization, especially of arsenic sulfides (Migdisov and Bychkov 1998). Microbial metabolism has been suggested as a contributing factor to mineralization in Uzon (Bonch-Osmolovskaya, 1994; Karpov 1991; Migdisov and Bychkov 1998). However, the contribution of sulfate-reduction to sulfide mineralization has not been examined directly or in detail.

Two pools from the eastern and central sectors of East Thermal Field were sampled for analysis of multiple variables (Figure 5.1). The pools selected, Zavarzin Spring (Zavarzin) in the eastern sector (54°29’53”N, 160°0’52”W) and Arkashin Shurf (Arkashin) in the central sector (54°30’0”N, 160°0’20”N), are temporally stable and of similar pH (circumneutral to slightly acidic) and surface temperatures (32-65°C in Arkashin and 26-74°C in Zavarzin). The objective was to characterize and compare the geochemical and microbiological changes with depth in core samples collected from two geochemically distinct pools. This study examined the hypothesis that biogenic sulfide from sulfate-reduction contributes to sulfide mineralization in Uzon.

RESULTS AND DISCUSSION

Arkashin and Zavarzin are two distinct pools

Arkashin, in the central sector of the East Thermal Field, is a pool dug by a prospector at the site of a small vent more than 20 years ago that almost immediately filled with fluid and, at 1 m^2, has remained essentially the same in size and shape since that time (Karpov et al. 1988; T. Sokolova, pers. comm.). Arkashin is characterized by flocculation of particles variably colored in shades of orange to

yellow. Surface water temperature and pH in various locations at Arkashin range from 65°C near the vent to 32°C at the edge of the pool, with temperatures as high as 99°C only ~10 cm into the vent sediments. The pH ranges from 3.7 to 7.0. Zavarzin, in the east sector of the East Thermal Field, is approximately 10 m^2 and has abundant microbial mat formations dusted with a surface layer of clay and elemental sulfur. Zavarzin is a naturally formed pool that has varied somewhat in size and shape since its initial designation and receives seasonal inputs of meteoric water from snowmelt flooding of a nearby stream (Karpov 1998). Surface temperature ranges in Zavarzin from 74°C to 26°C and pH from 5.5 to 7.5.

Sample Summary

Core samples were collected in 2005 and 2006 adjacent to each of the two thermal pools. Cores were sub-sampled based on visual changes in color and/or textures (Figure 5.2). Sub-samples were assessed for S concentration and stable isotope δ-values and particle size characterization. Additionally, sulfate reduction rates (SRR), pore-water chemistry, and mineralogy were measured in A05-2 and Z05-2 sub-samples. (For additional details, see Experimental Procedures.) Cores from Arkashin were heterogeneous in texture and color; cores from Zavarzin had white, fine-grain surface layers and black, coarse-textured deeper layers (Figure 5.2, Table 5.1). Arkashin core temperatures ranged from 37-40°C at the surface, measured just before sampling, to 44-69°C, measured at the bottom of the core just after sampling. Temperatures in Zavarzin cores ranged from 26-45°C at the surface and 47-64°C at the bottom of the cores. The A05-1 core was ~12 cm long and split into 5 sub-samples. The A05-2 core was 40 cm long and 8 sub-samples were collected. Core A06 was 41 cm long and also yielded 8 sub-samples. Core Z05-1 was ~27 cm long and yielded 4 sub-samples, and core Z05-2 was 30 cm long, yielding six sub-samples. Five sub-samples were collected from the 32 cm long Z06 core.

Principal Components Analysis

To reduce the complexity of the data and identify relationships among sub-samples, variables were ordinated using principal component analysis (PCA; Gotelli and Ellison 2004). Three PCAs were

performed: one for Arkashin data only (24 variables in 19 sub-samples), one for Zavarzin data only (23 variables in 14 sub-samples) and one that included data from both sites (23 variables in 33 sub-samples, Figure 5.3).

In the Arkashin-only analysis, sub-samples grouped by depth (Figure 5.3A). Generally, upper samples (<13 cm from the surface) were positive and deeper samples (>13 cm) were negative along the first component axis which explained nearly 50% of the variance. Seventy-five percent of variance was explained within three calculated components. Samples A05-2(17-19.5) and (19.5-23) were of special interest for their high content of alkali indicators and elements known to form sulfide minerals: V, Co, Cu, Zn, As, Cs, Pb, Fe, S, and Rb.

More than 80% of the variance among Zavarzin sub-samples was explained in four component axes. Sub-samples from Zavarzin-only analysis also grouped by depth on the first component, although not as tightly as in Arkashin (Figure 5.3B). Clay content and sulfur content had positive loading on both of the first two components, indicating the importance of these variables.

When analyzed together, 36% of the variance among sub-samples from both sites was explained in the first component axis (Figure 5.3C). Almost all sub-samples were separated by site on the second component. The first and second components cumulatively explained 51% of the variance among the sub-samples. No variables in particular dominated either of the first two component axes and >75% of the variance was not explained until the fifth component calculated.

Previous studies have indicated both in Uzon (Karpov 1991; Migdisov and Bychkov 1998) and elsewhere (Pope et al. 2004) that conditions in a hydrothermal system vary on large and small spatial and temporal scales. In the east sector, the underlying strata are alternating layers of coarse- and fine-grained tuffs, *i.e.*, consolidated volcanic ejecta in an ashy matrix. The chemical composition of the waters is dominated by mixing of meteoric and magmatic waters (Karpov and Naboko 1990; Karpov 1991). In the central sector of the East Thermal Field, deep-source, alkali metal enriched, neutral-chloride thermal waters reach the surface with limited mixing and are enriched in magmatic-associated elements, especially As, and active As-S mineralization has been described in the central sector (Karpov and

Naboko 1990; Migdisov and Bychkov 1998; Cleverley et al. 2003). In the context of this study, based on PCA, the pools were statistically different from one another, and sub-samples of the similar depth and/or color within each pool individually were relatively consistent, even between sampling years. The geochemical distinction between Arkashin and Zavarzin relates back to their positions on either side of faults delineating the central and east sectors of the East Thermal Field (Karpov and Naboko 1990)

Texture Analysis

Sediment texture in sub-samples was analyzed as percent weight in each of three particle size fractions; clay, sand and silt, and gravel (Table 5.1). Locations of active mineralization zones have been described as migrating in Uzon, generally attributed to temperature and redox conditions (Karpov 1991; Karpov 1992; Migdisov and Bychkov 1998). Changes in texture with depth result from heterogenous flow through the sediments, which will translate into variations in residence time of water and the analytes therein. Additionally, microbial activity contributing to mineralization may be the result or cause of textural differences. Core samples from both pools were generally coarser with depth; however a separation between surface and deep textures was especially distinct in Zavarzin. Above approximately 6 cm the average clay content was 4.94%. Below 6 cm the average clay content was 1.60%. As expected from visual observation, sediment texture in Arkashin was quite variable. However, distinct strata, were observed in both pools.

Mineral Identification

Represented by the variable colors of strata near Arkashin, the As-S mineralogy of the central sector to approximately one meter depth typically includes X-ray amorphous As-sulfides orpiment (As_2S_3), uzonite (As_4S_5), alacranite (As_8S_9), and realgar (As_4S_4; Cleverley et al. 2003; Karpov 1991; Migdisov and Bychkov 1998). Stibnite (Sb_2S_3), occurs deeper in the sediments where temperatures are higher (Migdisov and Bychkov 1998). In addition, Fe and Hg are also present in minerals such as pyrite and cinnabar in trace amounts (Karpov 1991). Two mineral phases were identified with Fourier

Transform Raman spectroscopy in this study (Figure 5.4). Peaks in the spectra from A05-2 sub-samples were consistent with realgar (As_4S_4) (Muniz-Miranda et al. 1996). Spectra from white Z05-2 sub-sample spectra were consistent with elemental sulfur.

Aqueous Geochemistry

Water content in sub-samples ranged from 12.9% to 41.4% in Arkashin and from 25.6% to 52.4% in Zavarzin (Table 5.1). These values were used to determine *in situ* concentrations of pore-water analytes in 05-2 cores (Table 5.2). Total aqueous concentrations of Cl^-, As and V were higher in Arkashin than in Zavarzin. The elements Rb, Sb, and Cs, present in Arkashin pore-water, were generally below detection in Zavarzin pore-water. Sulfate concentrations were lowest in Arkashin. In Zavarzin, aqueous Mn was higher than in Arkashin. Aqueous concentrations of F^-, Fe, Cu, Sr and Ba were similar in both pools.

The high concentration of As oxyanions in Arkashin (up to 2650 μM) was distinctive compared to Zavarzin (79.6 μM). Other As-rich environments include hot springs in Yellowstone National Park, USA (40 μM); Mono Lake, a saline lake in California (200 μM); and Champagne Pool, New Zealand (70.7 μM; Langner et al. 2001; Hoeft et al. 2002; Phoenix et al. 2005). Average concentrations of the aqueous arsenate and arsenite oxyanions respectively were 35.7 and 594 μM in Arkashin and 11.4 and 40.1 μM in Zavarzin. In both pools, arsenite predominated. Redox conditions in the mineralizing zones of the central sector of East Thermal Field are defined by fluid composition and temperature (Migdisov and Bychkov 1998). Additionally, the differences between the average concentrations of total aqueous As oxyanions (as determined by IC-ICPMS) and aqueous As (as determined by ICPMS) were 1604 μM in Arkashin and 341 μM in Zavarzin, indicating the abundance of aqueous As species other than oxyanions (e.g., thio-arsenic species; Table 5.2).

The difference between As in Arkashin and Zavarzin reflects the heterogeneous hydrology in continental thermal fields like those in Uzon. The higher concentrations of Cl^- and the alkali metals Cs and Rb in Arkashin suggest that this pool is dominated by alkali-chloride type waters (Hollingsworth

2006). High chloride concentrations allow for magmatic-associated elements to remain as soluble chloride complexes until they reach the surface, where temperatures are lower, conditions become more oxidizing, and metoric inputs are more immediate (Reed and Palandri 2006). Alternatively, in Zavarzin, lower concentrations of As and other magmatic elements, except sulfur, were observed. Notably, this pool, has been described as a mixed water type (of magmatic and meteoric inputs), that may also be influenced by a localized sulfur plume (Hollingsworth 2006).

Chalcophilic elements, sulfate-reduction and evidence for biomineralization

Chalcophilic Elements

The term chalcophilic (literally, "copper-loving") from the Goldschmidt classification is used here to describe the group of elements characterized by their occurrence in relatively stable sulfide minerals and in the current context includes Fe. To elucidate relationships among chalcophilic elements in each pool, concentrations of Cu, Zn, As, Pb, Fe, and S were standardized by dividing the concentrations in sub-samples by the sum concentration of each element in the cores. The values therefore are on a scale proportional among elements and represent the differences among depths by core (Figure 5.5A). In Arkashin, the chacophilic elements, especially S and As, co-vary with depth. The highest concentrations of both As and S co-occur in the mid-depth sub-samples A05-2(17-19.5) and (19.5-23), and A06(13-21) and (21-28). In Zavarzin, S concentrations were highest in the surface sub-samples Z05-1(0-2.5), Z05-2(3-6.5) and Z06(0-2.5). Highest concentrations of the other chacophilic elements occurred in the transition zone (e.g. Z05-2(6.5-7) and Z06(2.5-4) had peaks in Pb and As respectively) or the deeper, dark sub-samples (e.g. Z06(13-32), Z05-2(18.5-24) and Z05-1(25-37) had peaks in Fe, Cu and Zn respectively).

The variations in concentrations of chalcophilic elements were compared to the concentrations of S, to infer if they were involved in sulfide mineralization (Figure 5.5B). The standardized concentrations in Arkashin were normally distributed and regression was performed for each element with S. As, Zn, Fe, and Pb all had a significant amount of their variance attributable to a linear relationship with S (*p*-values

<0.0001, 0.0350, 0.0027, and 0.0063, respectively; for Cu p=0.098). The coefficients of variation (r^2) with S were 0.78, 0.21, 0.39, and 0.33 for As, Zn, Fe and Pb respectively. Thus, chalcophilic elements, especially As, actively participate in sulfur cycling in this pool. The magnitude of r^2-values among sulfur and chalcophilic elements in Arkashin correspond to sulfide mineral solubility constants, as well as variations in the abundance of constituent elements and their affinities for sulfide as compared to other ligands (e.g. chloride) as conditions change in surfacing magmatic fluids (Reed and Palandri 2006). In Zavarzin, the chalcophilic elements had an inverse relationship with S concentrations. The S data from Zavarzin sub-samples were not normally distributed, prohibiting parametric regression analysis. Sulfur predominantly occurs at the surface in Zavarzin and is in elemental form; however, the high concentrations of chalcophilic elements in the deeper layers may occur in sulfide complexes or minerals.

Sulfate-Reduction, Sulfur Concentration, and Stable Isotope δ-values

Sulfate-reduction rates (SRR) were determined in sub-samples in the A05-2 and the Z05-2 cores based on *in situ* sulfate concentrations from porewater analysis (Table 5.1). Due to sampling limitations, SRR for 05-1 sub-samples were calculated based on the average sulfate concentrations from 05-2 cores. Sulfate-reduction was detected in three sub-samples from A05-1, ranging from 0.240 to 0.432 nmol cm^{-3} day^{-1}. A05-2(19.5-23) was the only sub-sample in which sulfate-reduction was above detection limits in A05-2; the measured rates were 27.1 to 50.6 nmol cm^{-3} day^{-1}. Sulfate-reduction in core Z05-1, above detection limits in two sub-samples, ranged from 0.528 to 8.832 nmol cm^{-3} day^{-1}. Sulfate-reduction in the Z05-2 core was detectable in three sub-samples and ranged from 0.216 to3.96 nmol cm^{-3} day^{-1}, increasing with depth (Table 5.1).

SRR in Arkashin and Zavarzin were lower than expected. A sulfate-reduction rate of 2,250 nmol cm^{-3} day^{-1} occurs in a cyanobacterial mat of Termofil'nyi Spring in the Uzon Caldera (Slobodkin et al. 1999). Although there were abundant mat formations and relatively high sulfate concentrations in Zavarzin, SRR in surface sub-samples were below detection. SRR in Zavarzin and Arkashin are also much lower than observed in mat samples (212-11,100 nmol cm^{-3} day^{-1}) from various pools in

Yellowstone National Park (Fishbain et al. 2003). SRR measured in Arkashin and Zavarzin are in the range of rates determined in Yellowstone National Park sediment sites, 1.44-703 nmol cm^{-3} day^{-1} with some samples below detection. Sulfate concentrations in the Yellowstone National Park study ranged from approximately 0.31 to 87.5 mM (Fishbain et al. 2003). In Arkashin, sulfate ranged from 0.484 to 2.46 mM and in Zavarzin from 1.37 to 6.84 mM (Table 5.2)

Sulfate-reduction measured in sediments incubated at multiple temperatures indicate different groups of microorganisms (meso-, thermo-, hyperthermophilic) are present in sediment samples and are active at the appropriate temperatures (Weber and Jorgensen 2002).The results from this study indicated that, although sulfate-reduction occurs at lower rates at lower than the *in situ* temperatures, sulfate-reduction is more likely to be inhibited by temperatures that are too high. Yellowstone National Park pools at temperatures similar to Arkashin and Zavarzin had SRR of 266 to 483 nmol cm^{-3} day^{-1} (Roychoudhury 2004).

Another explanation for low SRR is that sulfate reduction may not be the most energetically favorable pathway in either pool. For example, sulfate reduction might have been limited due to the presence of arsenate as an alternative terminal electron acceptor (Amend and Shock 2001). As would be predicted thermodynamically, *Desulfotomaculum auripigmentum,* an isolate able to reduce both arsenate and sulfate, utilizes arsenate as a primary pathway, even in medium containing sulfate at ten times the concentration of arsenate (Newman et al. 1997). Sulfate and arsenate reduction rates compared in sediment core samples from Mono Lake and hypersaline Searles Lake, CA, revealed that SRR were highest in the top 2 cm of Mono Lake sediments at 1280 nmol cm^{-3} day^{-1} and no sulfate reduction was detectable in Searles Lake – hypothetically due to the higher energy requirements in the more saline lake (Kulp et al. 2006). At a sulfate concentration nearly 1,000 times that of inorganic As, arsenate reduction was responsible for oxidation of 8-14% of primary production and sulfate reduction up to 41% in the Mono Lake water column (Oremland et al. 2000). In Arkashin, arsenate concentration was 14 mM, relatively low for Arkashin, in the only A05-2 sub-sample with sulfate reduction rates above detection limits. Alternatively, sulfate reduction was highest in Zavarzin in the presence of 27 mM arsenate, a

relatively high concentration in that pool. Thus, the presence of arsenate was not a definitive factor restricting the occurrence of sulfate reduction in Zavarzin, but may play some role in Arkashin. Another alternative terminal electron acceptor available, especially in Zavarzin, was elemental sulfur. Although sulfidogenic sulfur-reducing prokaryotes have been isolated from Uzon (e.g. (Miroshnichenko et al. 1998), the importance of sulfur reduction or disproprotionation cannot be determined from these data (Philippot et al. 2007). Ultimately, the metabolic plasticity of the microbial community will influence the use of various electron sources and acceptors and complex environmental conditions influencing these processes may be expected to obscure thermodynamic predictions.

Examination of $\delta^{34}S$ values in conjunction with the sulfate-reduction rate data provided evidence for a biogenic contribution to sulfide mineralization as discussed below. Overall, sulfur concentration was generally an order of magnitude higher in Zavarzin (1.52–52.6%) than in Arksahin (0.355–7.87%), but total sulfur $\delta^{34}S$ values ranged over 13.3‰ in Arkashin and only 5.97‰ in Zavarzin (Table 5.1). Sulfate concentration in Arkashin peaked (2.46 mM) in sub-sample A05-2(5-10), but also exhibited a relatively high concentration (1.93 mM) in A05-2(19.5-23), where sulfate-reduction was detectable (Figure 5.6). The variations in sulfate concentrations increased with increasing total S concentration and coincided with variations in $\delta^{34}S$ values, especially in sub-samples A05-2(16-17) through (23-30). In Zavarzin, high concentrations of sulfate and total S were observed in sub-sample Z05-2(0-3). Sulfate concentration in Zavarzin was highest (5.93 mM) in Z05-2(6.5-7); sulfate and total S (1.49 mM and 1.54% respectively) were lowest as $\delta^{34}S$ peaked in sub-samples Z05-2(7-14).

The $\delta^{34}S$ values of sediment samples collected for this study are in agreement with $\delta^{34}S$ of S-bearing minerals of another recent Uzon study in which they ranged from -0.4 to 5.7‰ in a Zavarzin core sample and 1.3 to 3.2‰ in samples collected with depth from a spot close to Arkashin (Hollingsworth 2006). The measured $\delta^{34}S$ values of aqueous sulfate were reported as 7.6‰ in Arkashin and 8.0‰ in Zavarzin and the predicted $\delta^{34}S$ value for the reservoir contribution of sulfide in Uzon was -1.5 to +1.6‰ in that study. Microorganisms are often invoked to explain low $\delta^{34}S$ values of sulfide minerals relative to

coeval sulfate observed in the rock record (Bradshaw et al. 2008). A sulfate-sulfide fractionation in excess of 20‰ was observed at sulfate concentrations from 20-65 mM and reduction rates of at least 10 μmol cm^{-3} d^{-1} (Habicht and Canfield 1996). Sulfate concentrations were only as high as 2.46 and 6.84 mM in Arkashin and Zavarzin respectively and, as discussed, sulfate-reduction rates in both pools were relatively low. Observations from Arkashin and Zavarzin demonstrated that $\delta^{34}S$ values, concentrations of chalcophilic elements and rates of sulfate-reduction were variable (Figure 5.6). The presence of sulfide mineralizing elements can act as sulfide ligands and counteract end-product inhibition during sulfate-reduction (Labrenz et al. 2000). An appropriately poised redox potential may allow for microbial cycling of aqueous S and As species. The microbial activity may either enhance or inhibit precipitation of minerals allowing for the observation of high aqueous concentrations of metals in hydrothermal minerals (Wilkinson et al. 2009). Enrichment of biogenic sulfide in mineral phases also might be enhanced as concentrations of sulfide increase around cells, which can serve as nucleation sites for mineral formation (Douglas and Beveridge 1998). The higher range of $\delta^{34}S$ observed in Arkashin may represent a more complex network of sulfur-cycling, associated also with the high As concentrations. The greatest shift in $\delta^{34}S$ values occurred in the interface between the light and dark sub-samples in Zavarzin. At this interface, elemental sulfur disproprotionation (Canfield and Thamdrup 1996) may further influence $\delta^{34}S$ in the soil.

Arkashin and Zavarzin are two useful models for sulfur in hydrothermal environments

Among many of the variables analyzed, a pattern of dynamic variation in sub-samples was observed in Arkashin cores. Possible reasons include the time required for layers in Arkashin to form (Migdisov and Bychkov 1998), various minerals' stabilities vs. chloride complexes (Reed and Palandri 2006), a tipping point in redox or temperature (Karpov and Naboko 1990), texture variations or microbial activity (this study). Sulfate-reduction in Arkashin was associated with a peak in total concentration of chalcophilic elements. Relative contributions of biogenic and abiogenic sulfide to mineralization must

also be considered, because high concentrations of chalcophilic elements in Arkashin sub-samples were associated with peaks in total sulfur concentration. The microbial contribution interpretation is not supported in part by the $\delta^{34}S$ data, in part because the biological contribution of sulfide is only a minor component of the total sulfur measured. At present, active microbial As-S cycling in mid-depth Arkashin sub-samples may be preventing mineralization or dissolving minerals, contributing to textural heterogeneity. (Douglas and Beveridge 1998; Kakegawa and Ohmoto 1999; O'Day et al. 2004; Pope et al. 2004; Phoenix et al. 2005).

In Zavarzin, there was a distinct transition between the upper, white, clay-rich sub-samples and the deeper, black, coarser sub-samples of cores collected. Periodic eruptions of hydrothermal inputs and precipitation of fine sulfur particles causing sedimentation and burial of mats with subsequent diagenesis is a possible mechanism for this observation. Mats especially abundant the 2004 field season were obscured by fine sediment in the subsequent field season (Wiegel, pers. obsv.). There was much less layering observed compared with the assemblage of elements in Arkashin. Possible explanations include homogenization of layers because hot spring flow and seasonal flooding from a nearby freshwater stream might mixing or washing out forming horizons. The lack of colorful strata may also be from a less diverse chemistry. Lower concentrations of chalcophilic elements did occur deeper in Zavarzin, however, where they were associated with sulfate-reduction.

Further implications

Mineralization zones in volcanic areas have been described such that at temperatures below 100°C, surfical reactions are expected to involve microbial activity (Bradshaw et al. 2008) (Migdisov and Bychkov 1998) (Des Marais and Walter 1999). However, without accounting for abiotic sulfide inputs and alternative metabolisms, $\delta^{34}S$ values of sulfide minerals relative to sulfate may not fully represent the microbial metabolic dynamics, and sulfate-reduction may be over-interpreted (Philippot et al. 2007; this study). Although the importance of *in situ* texture remains unclear, covariance of texture as a variable

with other variables of interest in biomineralization, such as $\delta^{34}S$ values and high aqueous concentrations of mineralizing elements, indicates soil particle size deserves further investigation (Kakegawa and Ohmoto 1999; Wilkinson et al. 2009) this study). Current geochemical models characterizing the fluid chemistry in cores from Uzon identified temperature and initial fluid composition as the deciding factors describing As transport and mineral phase formation in Uzon (Cleverley et al. 2003) (Migdisov and Bychkov 1998). Future modeling efforts should benefit from incorporation of terms for *in situ* microbial activity and matrix texture.

EXPERIMENTAL PROCEDURESs

Core collection and sub-sampling

Cores were collected in August 2005 and 2006 adjacent to Zavarzin and Arkashin using a 10 cm diameter clear polycarbonate coring tube with a beveled end. Temperature was measured at the surface before sampling and at the bottom of each core immediately upon recovery. Cores were extruded and split aseptically to observe visible changes in color and texture for identification of sub-samples (Figure 5.1). Sub-sampling was done quickly and aseptically from the centers of the cores, to limit oxidation and avoid collecting contaminated sediments from the cores' outer surfaces. Sub-samples were collected into wide-mouth glass jars closed with butyl rubber stoppers. Upon returning from the field, samples were homogenized and split for storage until analysis.

Collection and analysis of sediment samples

Sediment from each sub-sample was dried at 60°C and ground with an acid-washed mortar and pestle. Two ~0.25 g replicates from each were microwave digested with nitric acid then diluted to 50 mL with deionized, distilled H_2O. Analytical method blanks and standard reference materials were included in each digestion batch. Following digestion, samples, blanks and standards were analyzed for 24 elements (Li, Be, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Cd, Cs, Ba, Tl, Pb, U, Hg) using inductively coupled mass spectrometry (ICP-MS). Detection limits for 2005 sub-samples ranged

from 0.01 μg L^{-1} Co to 2.08 μg L^{-1} Ca; RPD of replicate dilutions from 0.03% for Zn to 41% for Pb; average RPD of replicate digests from 5.45% for Cs to 50.7% for Cd; spike recovery from 26.55% for Zn to 120% for Ni; recovery of SRM was from 17.5% for Ca to 109% for Ni. Fe, As and Al in the 2005 samples required additional dilution for analysis. Detection limits in the higher dilution ranged from 0.02 μg L^{-1} As to 9 μg L^{-1} Fe; average RPD of replicate dilutions from 2.67% for Al to 14.3% for As; average RPD of replicate digests from 5.08% for Fe to 18.9% for Al; spike recovery from 99.8% for Fe to 109% for Al; recovery of SRM was from 28% for Al to 96% for As. Detection limits for the 2006 samples ranged from 0.014 μg L^{-1} Cs to 12.4 μg L^{-1} Al; average RPD of replicate dilutions from 0.53% for Al to 7.41% for Pb; average RPD of replicate digests from 4.16% for Cs to 18.2% Mg; spike recovery from 95% for Zn to 131% for Ca; recovery of SRM was from 32% for Sr to 97% for Cu. Variables with QC values outside the ranges described were removed from analysis, except in the cases where values below detection are reported as such. Fe was also by ICP-optical emission spectroscopy (OES). ICP-OES values only are reported for Fe. The method detection limit was 0.07 ppm Fe, the average rpd of replicate dilutions was 19%, 8.4 % of replicate digests. Values for sediment chemistry are presented in mg kg^{-1} of original sample weight.

Sediments were analyzed for the identification of minerals, as possible, using FT-Raman spectroscopy. A small portion of dried samples were excited at 1064 nm and sampled at 4 cm^{-1} resolution using a FT-Raman 960 Spectrometer E.S.P (Thermo Nicolet) with an In-Ga-As detector.

Particle Size Characterization

A spatula was used to mix sub-samples in glass jars and collect approximately 2-5 g of sediment for particle size characterization by a modification of the micro-pipette method (Miller, et al., 1987). Sediment weights were recorded and sediment combined with 40 mL of dispersant solution (0.05% sodium metaphosphate, 0.01 M NaOH) in 50 mL centrifuge tubes and shaken horizontally for approximately 96 hours. Samples were removed from shaker, shaken thoroughly by hand and left to settle

for 110 minutes. From each tube, 2.5 mL was collected from a depth of 2.5 cm, dispensed into a pre-weighed Al tin, dried overnight and weighed to determine percent clay content. These values were corrected by the weight of salts in the dispersing solution. The remaining sediment was poured through a 2 mm sieve and the >2mm fraction weighed to determine percent gravel content. The sand and silt content, considered a single fraction, was determined as the remaining percent of original sample weight.

S Concentration and Stable Isotope δ-values

The $\delta^{34}S$ values were determined by oxidation of all sulfur in total from sediment sub-samples to SO_2 for analysis (Ueda and Krouse 1986). Oven-dried, ground and homogenized samples were further ground together with V_2O_5, SiO_2, and Cu powders, packed with quartz wool into quartz glass tubes, and reacted under vacuum at 1050°C. SO_2 was extracted using a conventional vacuum line equipped with a variable temperature trap. Purified SO_2 was analyzed on a Finnigan MAT 252 mass spectrometer. Standard reference materials IAEA-S1 ($\delta^{34}S$ = -0.3 ‰) and NBS-123 ($\delta^{34}S$ = +17.1 ‰) were prepared and analyzed with each batch of samples. Sample isotopic results are normalized to these standards using a two-point scale, thus $\delta^{34}S$ values are reported relative to Vienna Cañon Diablo Troilite (VCDT). Replicate analyses of standard reference materials indicated a resolution of the method of +/-0.4‰ Total sulfur is presented in percent of original sample weight.

Collection and analysis for pore water chemistry

Approximately one gram of sediment from each sub-sample was collected, weighed, and combined with 5 mL of sterile, anaerobic distilled, de-ionized H_2O in a glass serum bottle under an anaerobic headspace and shaken vigorously. The dilution water was collected from the serum bottles with needle and syringe and filter sterilized into fresh autoclave steril, anaerobic bottles. After returning from the field these samples were stored at 4°C. Seven anions (fluoride, nitrate, nitrite, bromide, phosphate, sulfate, and chloride) were analyzed via ion chromatography (IC) with electrical conductivity detection

(ECD) on a Dionex system. Relative percent difference (RPD) of replicate injections of the same sample ranged from 8.6% for chloride to 15% for bromide (outlier, 142% for nitrate); spike recovery ranged from 81% for chloride to 111% for fluoride. Twenty-two elements (Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, Sb, Cs, Ba, Hg, Tl, Pb, and U) were analyzed via inductively coupled plasma mass spectrometry (ICPMS) on an Elan DRC Plus (PerkinElmer SCIEX). Average RPD of replicate diultions ranged from 4.4% for As to 40% for Cu; spike recovery from 92% for As to 119% for Mn (with one outlier, 32% for Fe); detection limits from replicate instrument blanks ranged from 0.01 ppb for Ba to 4.02 ppb Fe. Arsenate and arsenite oxyanions were measured via IC-ICPMS. Average RPD of replicate injections of the same sample were 28, 29 and 33% for As(III), sumAs and As(V) respectively. Variables with QC values outside the ranges described were removed from analysis, except in the cases where values below detection are reported as such. Values are rounded to three significant figures. Additional sediment from sub-samples was oven-dried for other analyses (see below) and water content in all sub-samples (%W) was determined as %W = 100[(wet weight – dry weight)/wet weight]. Thus, *in situ* pore water concentrations were calculable from method dilution = [(%W * field wet weight) + 5 mL] / (%W * field wet weight). Data are presented with three significant figures

Sulfate-Reduction Rates

Methods for the measurement of sulfate-reduction are well-established (Fossing and Jorgensen 1989). *In situ* sulfate-reduction rates (SRR) were determined from core sub-samples using five end-cut 5 mL syringes per sub-sample representing one control and two replicates for two time points. The syringes were filled to the 2 mL line and closed with black butyl-rubber stoppers. One control syringe for each depth was immediately fixed by the addition of 1 mL 20% wt/vol zinc acetate. All syringes were injected with 0.1 mL $^{35}SO_4^{2-}$ (activity $33x10^6$ counts per minute) through the stoppers. Syringes were enclosed in linen bags and incubated at the surface in the individual pools. Results are reported for two syringes from each depth fixed after six hours with the addition of 1 mL 20% zinc acetate. After fixation, syringes were emptied into 50 mL centrifuge tubes with an additional 20 mL volume of 20% zinc acetate. Upon

returning from the field, the 50 mL centrifuge tubes were stored at -20°C until distillation of total reduced inorganic sulfur (TRIS) using the single-step hot, acidic chromium method (Fossing, et al., 1989). Both sulfate and TRIS were measured for ^{35}S activity via scintillation counting in ScintiSafe Gel scintillation cocktail (Fisher). Detection limits were determined by site as [3 x (standard deviation of radioactivity of TRIS in controls)] + (average radioactivity of TRIS in controls) or 919 cpm for Zavarzin and 687 cpm for Arkashin (Kallmeyer et al. 2004). The average radioactivity of TRIS in controls was subtracted from radioactivity in TRIS in syringes fixed at 6 hours for calculation of SRR. SRR was calculated as SRR = 1.02[(r*S)/(R*T)], where 1.02 is the isotope fractionation factor, r is radioactivity in TRIS, S is pore-water sulfate concentration, R is radioactivity in sulfate and T is time of incubation (Fossing and Jorgensen 1989), and SRR are presented as the range of duplicates.

Data/Statistical analyses

The relationships among chalcophilic elements in Arkashin were examined with regression analysis. These variables were represented as the concentration of an element in a sub-sample from a core sample proportional to the total concentration of the element in all sub-samples from that core sample. After they were standardized as described, the data for Arkashin were normally distributed (Anderson-Darling test for normality, p-values all > 0.05). No other transformation attempts normalized all variables. No amount of transformation normalized all variables in Zavarzin; more specifically, it was not possible to normalize S data at all in Zavarzin.

Ordination of 23 and 24 (Arkashin only) geochemical variables from up to 33 samples was accomplished with a Pearson correlation matrix principal components analysis (PCA) using Minitab (Release 14, Minitab Inc.). PCA was chosen as an appropriate method for data reduction and cluster identification and since it does not make assumptions about the underlying distributions of the variables. Use of a correlation matrix serves to standardize variables by their variances prior to calculation of principal components. Additional data were included from Burgess, E.A., et al.; in preparation (C_{tot} and N concentrations and stable isotope δ-values). For variables with values in five or less sub-samples below

detection, one-half the detection limit was used in principal components analysis. Variables with five or more values below detection were not included in analysis.

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Table 5.1 Sub-sample depths, color, water content, texture, sediment chemistry, and S content and δ-values for core samples.

Core	Sub-sample depth (cm from surface)	Color	Water (%)	Particle size fractions (%, standard deviation)			Sulfur (%)	$d^{34}S$* (‰)	SRR (nmol cm-3 day-1)**	
				Clay (<2 mm)	Sand and Silt	Gravel (>2 mm)			low	high
A05-1	1.5-2.5	red-brown	30.0	4.42(0.45)	82.4(1.65)	13.2(2.11)	0.812	7.60	0.240	0.432
	2.5-5	green, black stripe	36.0	9.59(0.35)	76.4(0.89)	14.0(1.03)	1.09	1.54	bd	bd
	5-6.5	yellow-orange	35.2	6.36(0.04)	70.7(3.22)	22.9(3.26)	0.811	2.03	bd	bd
	6.5-10	black	41.4	7.36(0.72)	86.3(1.74)	6.32(2.45)	0.755	1.66	0.240	0.240
	10-12	brown-orange	39.6	5.14(0.47)	78.7(6.41)	16.1(5.94)	1.51	12.6	bd	0.264
A05-2	0-5	brown-grey	30.7	5.12(0.78)	77.3(8.03)	17.6(8.81)	0.398	2.93	bd	bd
	5-10	green-yellow	31.3	3.95(0.97)	40.0(7.19)	56.1(8.14)	2.16	1.26	bd	bd
	10-13	yellow, black stripe	33.1	2.80(0.16)	71.4(8.07)	25.8(7.95)	2.45	1.41	bd	bd
	16-17	bright orange	20.0	1.71(0.29)	41.7(3.44)	56.6(3.20)	4.78	5.01	nm	nm
	17-19.5	dark purplish	29.7	3.16(0.27)	89.3(0.04)	7.52(0.23)	6.02	2.69	nm	nm
	19.5-23	red	12.9	2.54(0.42)	50.4(8.64)	47.1(8.92)	7.87	3.39	27.1	50.6
	23-30	grey-black	26.0	1.91(0.43)	56.3(15.0)	41.8(15.3)	1.26	0.0120	bd	bd
	33-40	black	24.5	2.56(1.56)	39.0(21.7)	58.5(23.2)	3.59	0.472	nm	nm
A06	1-2	yellow	23.4	1.73(0.00)	45.2(3.70)	53.1(3.71)	0.355	2.22	nm	nm
	2-5	greenish	38.6	5.33(0.70)	56.7(16.0)	38.0(16.7)	0.549	1.87	nm	nm
	5-8.5	yellow-brown	33.1	5.87(1.02)	68.6(7.49)	25.6(8.51)	1.03	1.60	nm	nm
	8.5-13	yellow-grey	30.1	4.70(0.70)	67.6(8.70)	27.7(9.40)	0.805	1.33	nm	nm
	13-21	orange	24.9	2.48(0.38)	43.9(1.67)	53.7(2.05)	3.55	0.475	nm	nm
	21-28	yellow	22.4	3.11(0.64)	51.0(4.44)	45.9(5.08)	2.79	-0.711	nm	nm
	28-33	purple-red	23.3	1.18(0.02)	40.7(0.12)	58.2(0.14)	0.607	-0.365	nm	nm
	33-41	black	36.0	2.49(0.93)	60.5(3.52)	37.0(4.45)	0.789	0.0155	nm	nm
Z05-1	0-2.5	yellow-white	31.8	12.4(7.21)	86.0(6.59)	1.54(1.35)	52.6	-0.488	bd	bd
	2.5-6	yellow-grey	31.8	4.31(2.10)	90.5(5.71)	5.22(3.60)	29.7	-0.0414	bd	bd
	6-25	dark grey	34.0	2.15(1.03)	47.6(21.4)	50.2(22.3)	1.93	1.21	0.600	8.832
	25-37	black	29.9	1.28(0.30)	53.1(16.1)	45.6(16.3)	8.40	2.04	0.528	4.104
Z05-2	0-3	yellow	38.9	4.49(0.13)	87.2(0.80)	8.28(0.88)	32.1	-0.0541	bd	bd
	3-6.5	grey	52.4	9.30(10.7)	83.8(11.8)	6.91(1.11)	31.5	-0.304	bd	0.696
	6.5-7	dark grey	32.1	nm	nm	nm	4.79	0.456	nm	nm
	7-14	grey	30.0	1.97(0.10)	67.3(6.16)	30.7(6.21)	1.54	5.93	0.216	0.912
	14-18.5	dark grey, mottled	34.1	1.49(0.12)	61.1(8.64)	37.4(8.55)	1.83	-0.880	nm	nm
	18.5-24	black	41.4	1.88(0.30)	96.8(0.69)	1.28(0.39)	1.29	0.431	2.54	3.96
Z06	0-2.5	yellow-white	48.6	1.17(0.04)	86.4(3.42)	12.4(3.46)	31.7	-0.714	nm	nm
	2.5-4	grey	37.4	1.68(0.19)	83.5(7.52)	14.8(7.33)	2.06	-1.86	nm	nm
	4-6	brown	35.8	1.20(0.16)	48.1(1.59)	50.8(1.75)	4.86	-2.66	nm	nm
	6-13	yellow-green	25.6	0.96(0.34)	33.3(12.7)	65.7(13.0)	36.5	-1.49	nm	nm
	13-32	black	37.1	1.50(0.42)	68.4(10.4)	30.1(10.0)	6.77	-0.159	nm	nm

bd = below detection
nm = not measured
*stable isotope δ-values determined vs. VCDT
**SRR = sulfate reduction rates, from two replicates, designated low and high; 05-1 values estimated from sulfate concentrations in 05-2 cores

Table 5.2 Sub-sample depths and pore water chemistry for core samples.

	Sub-sample depth	Anions, determined by IC-ECD (mM)				As species, by IC-ICPMS (mM)			Aqueous concentrations of elements, determined by ICPMS (mM)									
Core	(cm from surface)	Fluoride	Bromide	Sulfate	Chloride	Arsenite	Arsenate	ΣAsOx*	V	Mn	Fe	Cu	As	Rb	Sr	Sb	Cs	Ba
A05-2	0-5	0.674	0.128	1.56	98.3	44.0	48.1	92.1	8.06	8.35	814	22.1	385	8.51	2.19	3.12	4.05	5.34
	5-10	1.32	2.28	2.46	96.2	350	53.8	404	8.70	6.32	368	7.84	1285	7.60	3.85	6.87	4.01	7.18
	10-13	0.436	0.121	1.35	97.0	455	24.1	479	14.1	12.9	746	6.14	1304	6.78	3.03	bd	3.09	11.1
	16-17	0.937	bd	1.23	120	2571	83.3	2654	9.62	7.03	891	3.14	9505	5.34	2.73	bd	2.19	7.92
	17-19.5	1.71	0.297	0.595	85.4	862	31.9	894	5.73	3.29	360	1.76	3417	4.23	4.07	2.00	1.77	6.13
	19.5-23	2.73	1.79	1.93	158	68.4	14.3	82.8	15.8	15.2	731	10.4	274	8.76	11.5	38.9	3.22	12.9
	23-30	0.540	0.043	0.484	74.1	170	18.0	188	7.20	3.53	613	8.83	718	3.35	1.24	10.0	1.22	4.12
	33-40	1.12	0.115	0.812	110	235	12.3	248	4.58	2.43	346	5.82	989	3.11	0.85	4.07	0.92	5.66
Z05-2	0-3	0.580	0.009	6.84	29.7	31.8	0.000	31.8	4.58	89.0	422	3.79	52.0	bd	6.67	bd	bd	7.10
	3-6.5	2.05	bd	4.82	25.3	2.89	3.76	6.64	4.72	95.0	254	8.08	9.84	bd	17.5	bd	bd	1.55
	6.5-7	0.743	bd	5.93	46.3	36.2	11.6	47.7	6.82	73.2	634	7.53	65.5	bd	5.46	bd	bd	7.82
	7-14	0.467	bd	1.49	46.3	14.9	7.23	22.2	7.45	29.4	504	16.1	34.4	1.88	2.68	bd	bd	4.14
	14-18.5	0.400	0.180	1.37	30.8	60.4	19.2	79.6	4.67	2.96	230	2.69	1764	3.63	2.20	2.49	1.28	3.80
	18.5-24	0.319	bd	1.93	27.4	94.3	26.9	121	4.43	77.6	256	5.53	430	bd	3.48	bd	bd	4.49

bd = below detection *sum of arsenic oxyanions arsenate and arsenite (the previous two columns)
nm = not measured

Figure 5.1. Image of the Kamchatka Peninsula showing the location of the Uzon Caldera along with Quickbird satellite images of Uzon and photographs showing the location and appearance of Arkashin and Zavarzin. Geographic coordinates for the pools are provided in degrees minutes seconds obtained using the global positioning system.

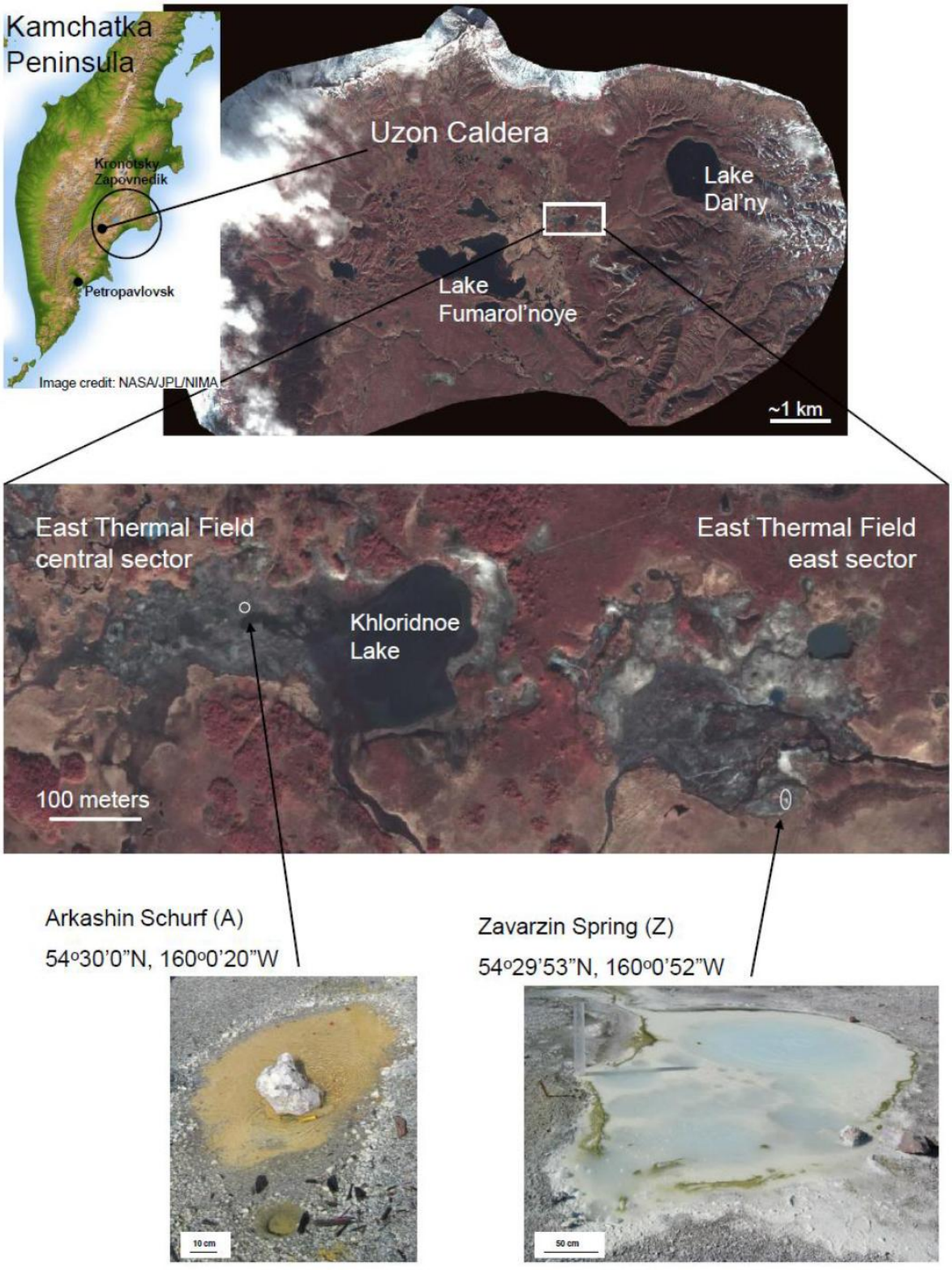

Kamchatka Peninsula
Kronotsky Zapovnedik
Petropavlovsk
Image credit: NASA/JPL/NIMA
Uzon Caldera
Lake Dal'ny
Lake Fumarol'noye
~1 km
East Thermal Field central sector
East Thermal Field east sector
Khloridnoe Lake
100 meters
Arkashin Schurf (A)
54°30'0"N, 160°0'20"W
Zavarzin Spring (Z)
54°29'53"N, 160°0'52"W
10 cm
50 cm

Figure 5.2. Core samples collected from each pool. Sub-samples from core samples are identified in the text according to the key provided. The scale applies to all six cores

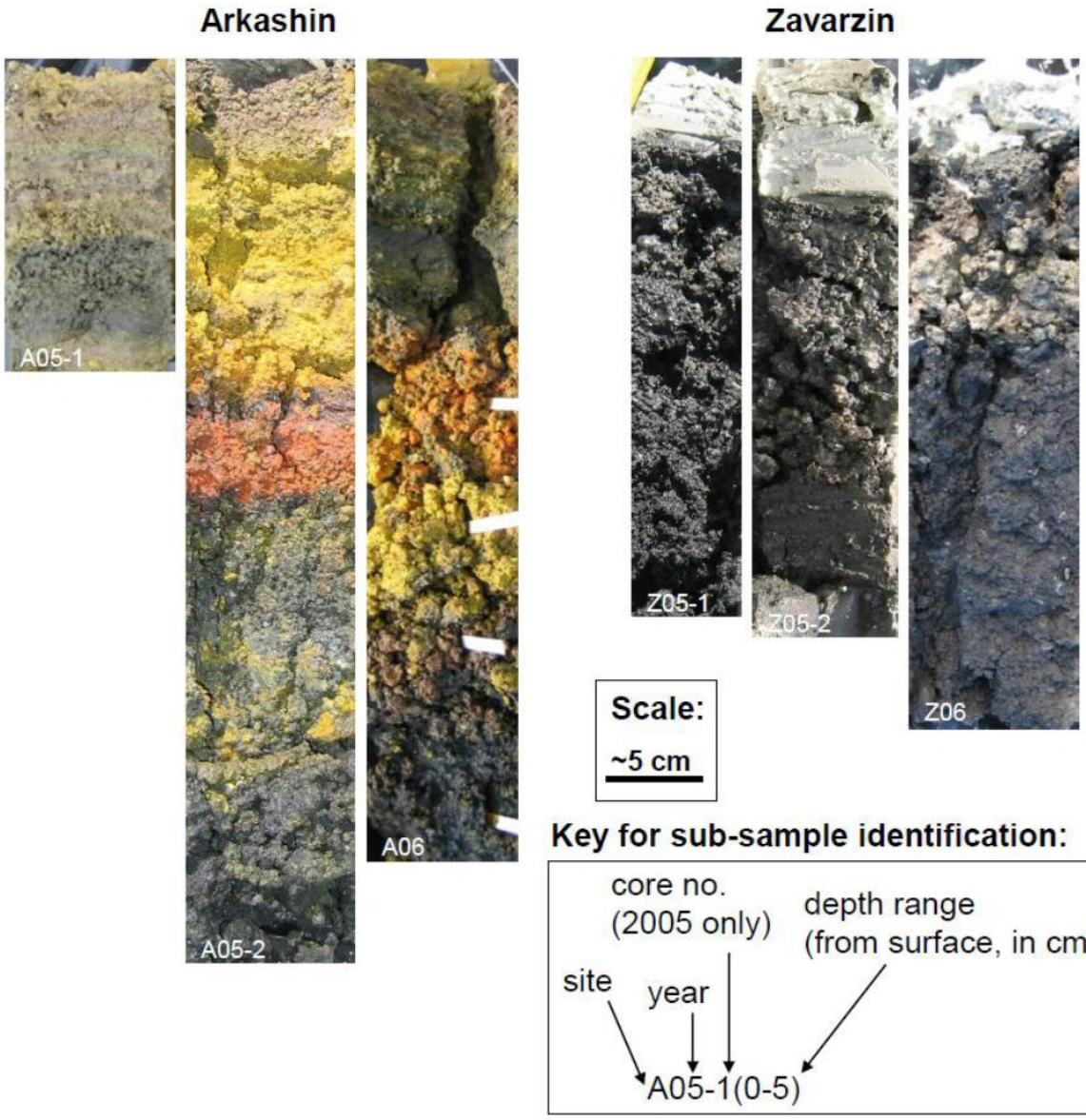
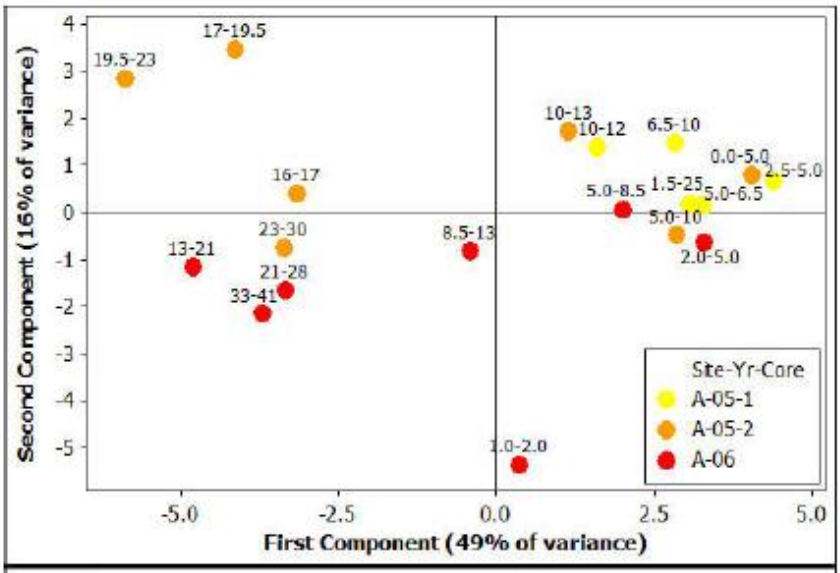
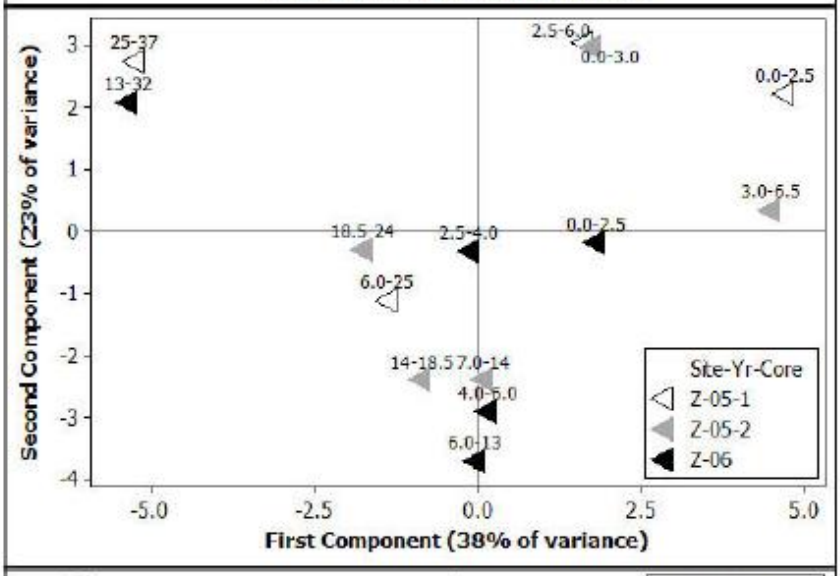

Arkashin
Zavarzin
A05-1
A05-2
A06
Z05-1
Z05-2
Z06
Scale:
~5 cm
Key for sub-sample identification:
core no.
(2005 only)
depth range
(from surface, in cm)
site
year
A05-1(0-5)

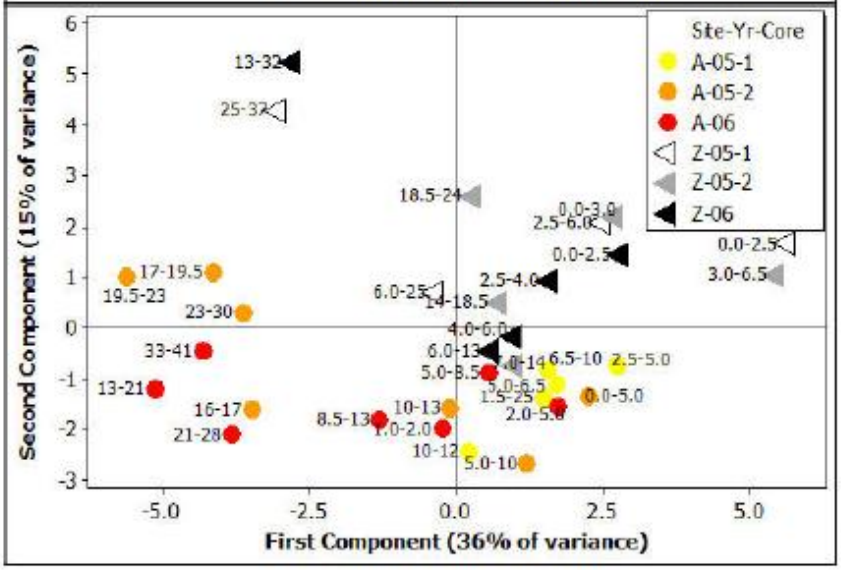
Figure 5.3. Principal components analysis of multiple variables in cores from (A) Akrashin only, (B) Zavarzin only and (C) both sites. Details of variables analyzed are presented in the text. Points represent sub-samples and are labeled with depth range in cm from the surface.


Second Component (16% of variance)
First Component (49% of variance)
17-19.5
19.5-23
10-13
10-12
6.5-10
0.0-5.0
2.5-5.0
16-17
5.0-8.5
1.5-25
5.0-6.5
5.0-10
23-30
8.5-13
13-21
2.0-5.0
21-28
33-41
1.0-2.0
Site-Yr-Core
A-05-1
A-05-2
A-06

A


Second Component (23% of variance)
First Component (38% of variance)
25-37
13-32
2.5-6.0
0.0-3.0
0.0-2.5
3.0-6.5
18.5-24
2.5-4.0
0.0-2.5
6.0-25
14-18.5
7.0-14
4.0-5.0
6.0-13
Site-Yr-Core
Z-05-1
Z-05-2
Z-06

B


Second Component (15% of variance)
First Component (36% of variance)
13-32
25-32
18.5-24
0.0-3.0
2.5-6.0
0.0-2.5
3.0-6.5
17-19.5
19.5-23
2.5-4.0
6.0-25
14-18.5
23-30
4.0-6.0
33-41
6.0-13
7.0-14
6.5-10
2.5-5.0
5.0-8.5
13-21
5.0-6.5
1.5-25
0.0-5.0
16-17
10-13
8.5-13
2.0-5.0
1.0-2.0
21-28
10-12
5.0-10
Site-Yr-Core
A-05-1
A-05-2
A-06
Z-05-1
Z-05-2
Z-06

C

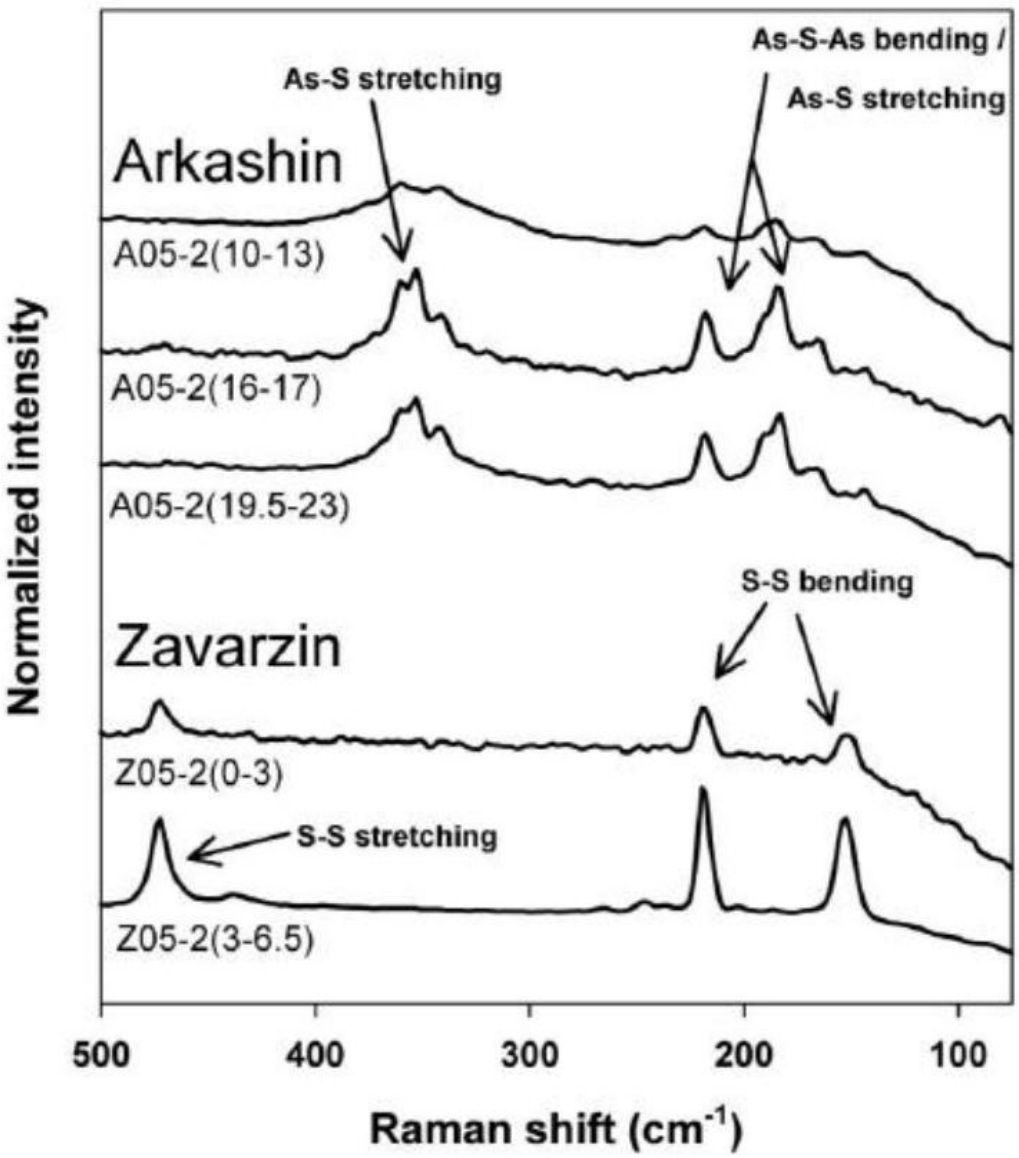


Figure 5.4. FT-Raman spectra of sub-samples from 05-2 cores. Sub-sample ids are indicated at the left of each spectrum. Peaks in the spectra are indicated as to the source of the observed signals.

Figure 5.5. Relationship among chalcophilic elements with depth (A) and versus total S (B). Changes in sediment concentration among elements were standardized by dividing the concentration of each element in sub-samples by the sum concentration of that element in cores. The legend is the same for both plots in both panels. S is plotted on both axes in panel B to provide an r^2=1 relationship

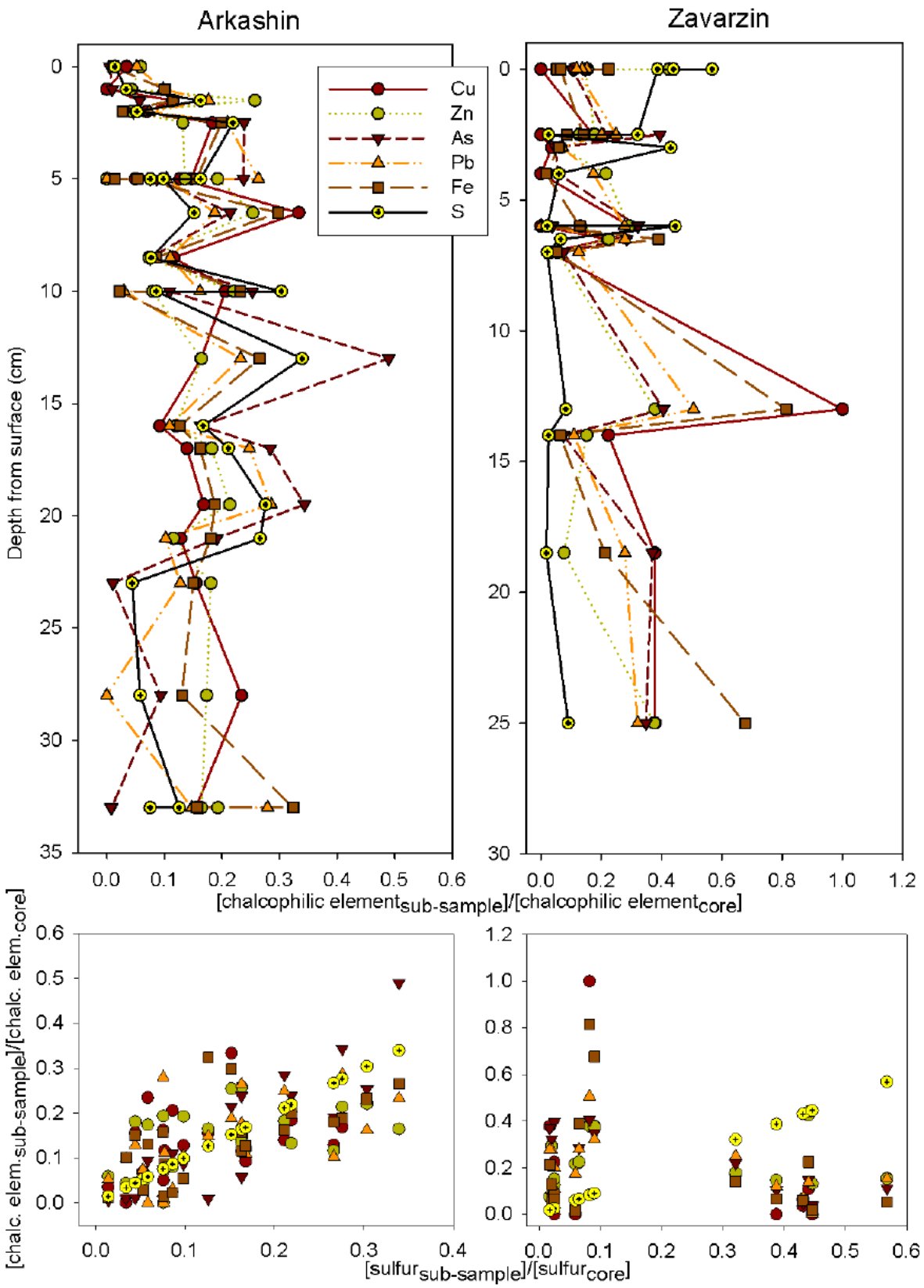
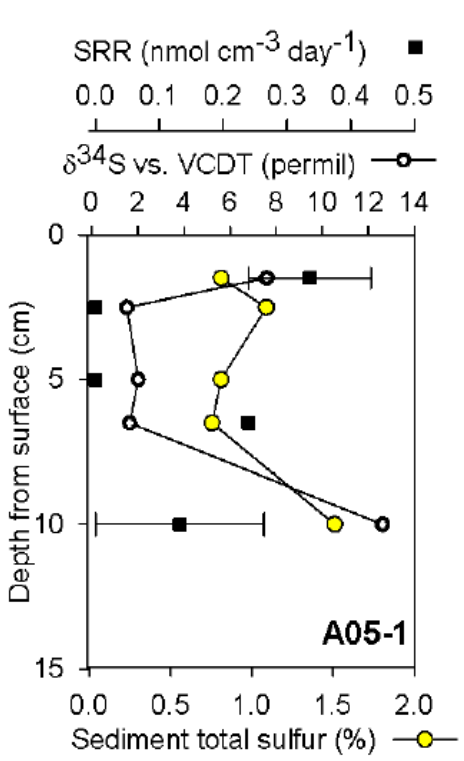
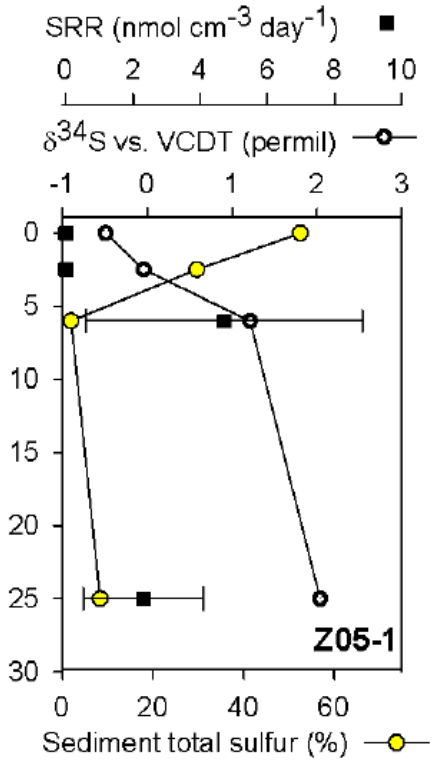
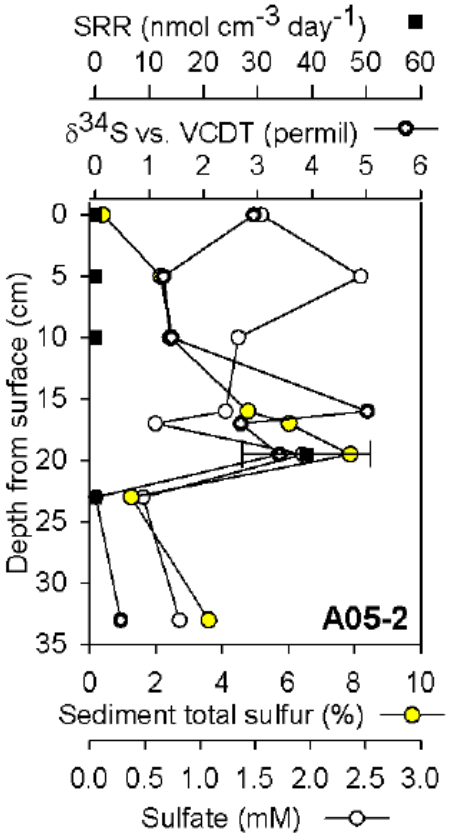
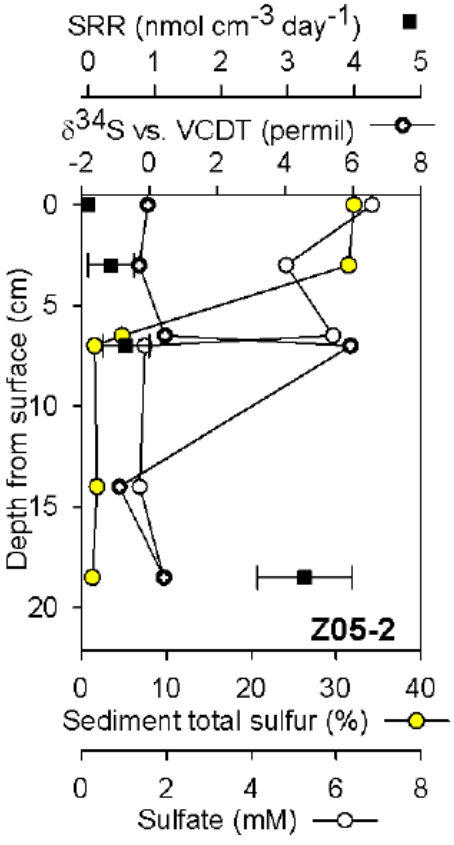

Arkashin
Zavarzin
Cu
Zn
As
Pb
Fe
S
Depth from surface (cm)
[chalcophilic element sub-sample]/[chalcophilic element core]
[chalc. elem.sub-sample]/[chalc. elem.core]
[sulfur sub-sample]/[sulfur core]

Figure 5.6. Comparison of sulfate-reduction rates (SRR), sulfate, sediment total S and $\delta^{34}S$ vs. VCDT Cores are identified in the lower right corner of each graph. SRR are represented at each sampling depth as the midpoint of the range from duplicate measurements at that depth. Note that the y-axes are adjusted for the different lengths of cores recovered.


SRR (nmol cm-3 day-1)
0.0 0.1 0.2 0.3 0.4 0.5
δ34S vs. VCDT (permil)
0 2 4 6 8 10 12 14
Depth from surface (cm)
0 5 10 15
A05-1
0.0 0.5 1.0 1.5 2.0
Sediment total sulfur (%)


SRR (nmol cm-3 day-1)
0 2 4 6 8 10
δ34S vs. VCDT (permil)
-1 0 1 2 3
0 5 10 15 20 25 30
Z05-1
0 20 40 60
Sediment total sulfur (%)


SRR (nmol cm-3 day-1)
0 10 20 30 40 50 60
δ34S vs. VCDT (permil)
0 1 2 3 4 5 6
Depth from surface (cm)
0 5 10 15 20 25 30 35
A05-2
0 2 4 6 8 10
Sediment total sulfur (%)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Sulfate (mM)


SRR (nmol cm-3 day-1)
0 1 2 3 4 5
δ34S vs. VCDT (permil)
-2 0 2 4 6 8
Depth from surface (cm)
0 5 10 15 20
Z05-2
0 10 20 30 40
Sediment total sulfur (%)
0 2 4 6 8
Sulfate (mM)

CONCLUSIONS

The objectives of this research were to catalog the communities in each pool in order toprovide clues about the pools' ecology and enable comparisons to other locations, determine if geochemistry and microbiology co-vary across a visible scale with depth, and examine the relationship between biological sulfate-reduction and As-S mineralization. The research to meet these objectives has led to the following conclusions.

- Each pool was geochemically distinct. Arkashin had very high arsenic concentrations and Zavarzin was characterized by abundant elemental sulfur.
- The geochemical distinctions in each pool leads to distinct and different microbiological communities.
- Arkashin was dominated by *Hydrogenobaculum*-related primary producters. Other community members included *Desulfurella*-, "*Sphingobacteria*"- and *Variovorax*- related bacteria.
- Zavarzin was dominated by Chloroflexus and heterotrophic microorganisms, including Crenarchaeota.
- Each pool contained at least 20% of the sequences recovered were related to uncultured and unclassified microorganisms (<80% confidence in *Class* designation).
- The microbiological communities changed with depth and changing geochemical conditions. Changes in Arkashin coincided with a possible shift in carbon source and were paralleled by visible changes in color and texture due to changes in As and S concentrations
- Sulfur and arsenic cycling are closely linked in Arkashin. Sulfur and chalcophilic elements are not tightly associated in Zavarin.

- Sulfate-reduction rates were relatively low; however, sulfate-reducing organisms have a role in sulfur-cycling in both pools.

Future research directions suggested by these conclusions include additional molecular sampling, isolation of novel microorganisms, characterization of arsenic metabolism in Arkashin, and elemental sulfur metabolism in Zavarzin, microscopy of microorganism and mineral interactions.

APPENDIX A

A SUMMARY OF ENRICHMENT AND ISOLATION EFFORTS

Arsenic and sulfur occur in multiple oxidation states and in various minerals associated with hydrothermal systems. Redox-active elements such as arsenic and sulfur are useful to bacteria as terminal electron acceptors in anaerobic respiration. Thermodynamically, arsenate is a better terminal electron acceptor than sulfate. The change in Gibb's free energy for the conversion of lactate to acetate is -242 kJ/mol when coupled to arsenate reduction versus -134 kJ/mol when coupled to sulfate reduction as determined for reactions at 55^{o}C (Amend and Shock 2001). In Mono Lake, CA, where sulfate concentration is nearly 1,000 times that of inorganic arsenic, arsenate reduction is responsible for mineralization of 8-14% of primary production and sulfate reduction mineralizes up to 41% (Hoeft, et al. 2002; Oremland, et al. 2004).

A number of microorganisms capable of dissimilatory arsenate- and/or sulfate-reduction have been cultured in isolation (Table A.1). Some isolates have been identified that are capable of both (Macy, et al. 2000; Newman, et al. 1997b). One isolate able to reduce both arsenate and sulfate has been shown to utilize arsenate first, even in medium containing sulfate at ten times the concentration of arsenate (Newman, et al. 1997b). A hyperthermophilic isolate able to reduce arsenate was also shown to use thiosulfate and elemental sulfur as electron acceptors, but the utility of sulfate was not reported (Huber, et al. 2000). Two other arsenate-reducing strains are either unable to reduce sulfate or demonstrate reduced growth on sulfate versus arsenate (Blum, *et al*. 1998). Finally of interest is one isolate of the genus *Thermus* that has been shown to both reduce arsenate during heterotrophic growth and oxidize arsenite under aerobic conditions (Gihring and Banfield 2001).

Addressing specifically the microbiology of Kamchatka, sulfur-reducing prokaryotes of the genus *Desulfurella* have been isolated from local hydrothermal features (Miroshnichenko, et al. 1998).

Additionally, isolates from the order *Aquaficales*, known for the deposition of elemental sulfur, have been isolated from Kamchatka (A.-L. Ryesenbach, pers. comm.). To date, however, neither sulfate- nor arsenate-reducing prokaryotes have been isolated from Uzon.

One relevant aspect of the mineralogy in Uzon is the contemporary deposition of arsenic- and sulfur-containing ores including red realgar (As_4S_4), yellow-orange orpiment (As_2S_3), and dark green scorodite ($Fe^{3+}AsO_4 \bullet 2H_2O$; Karpov 1998). These minerals are colorfully visible in the caldera landscape.

Desulfotomaculum auripigmentum, an isolate from arsenic-contaminated lake sediment has been shown to precipitate yellow arsenic trisulfide (As_2S_3) as a by-product of the reduction of both arsenate and sulfate (Newman, et al. 1997a). Realgar precipitated in the presence of *Pyrobaculum arsenaticum*, a hot spring isolate from Italy, when cultures were provided with arsenate and sodium thiosulfate or L-cysteine as electron acceptors (Huber, et al. 2000). In addition to the precipitation of minerals, dissolution of minerals has also been observed. *D. auripigmentum* was able to grow on scorodite, reducing the arsenate in this mineral (Newman, et al. 1997). Thus, prokaryote reduction of arsenate and sulfate in Uzon hot springs is expected to influence the deposition and dissolution of arsenic and sulfur containing minerals. No isolation attempts have been described specifically to examine this possibility.

Additionally, one may expect that arsenate- and sulfate-reduction pathways are competing for carbon and energy in Arkashin and Zavarzin food webs and that the utility of each metabolism is influenced by the presence of the other. Such biogeochemical dynamics will have implications in the long-term dynamics of the sulfur, arsenic and carbon cycles, the composition of the microbial communities and the mineralogy of Uzon.

Multiple rounds of enrichment/isolation were attempted (see Appendices B and C). Fresh enrichment cultures for isolation of arsenate- and sulfate-reducing prokaryotes were inoculated in the field or from samples stored at 4°C. The general approach used anaerobic, high-temperature culturing and isolation techniques (Ljungdahl and Wiegel 1986, Wiegel 1986). For independent rounds of isolation, different media were used. Culture observations were made using phase-contrast or fluorescence microscopy. Isolates were identified by 16S rRNA gene sequence.

During the 2004 field season, 0.5 mL sediment samples, in triplicate for Zavarzin, were used to inoculate 3.5 mL of anaerobic medium containing 11 mM sulfate as a terminal electron acceptor and 1 mM ferrous iron as an indicator of sulfate reduction. Acetate, lactate, benzoate (10 mM each), and pyruvate (5 mM) were added as carbon and electron sources. The remaining medium constituents were used as recommended by J. Wiegel (pers. comm.). These cultures were decimally diluted to 10^{-5} under two temperature (60 and 80^{o}C) and two pH (6 and 8) regimes. Results indicate that all samples contained sulfate reducing bacteria (Table A.2). Additionally, cultures were inoculated for enumeration of arsenate-reducing prokaryotes. The medium used was the same as that used to enrich and enumerate sulfate-reducing prokaryotes, except 10 mM arsenate was provided as a terminal electron acceptor instead of sulfate. These cultures were not assessed for evidence of arsenate reduction. Enrichments from Arkashin generated a yellow-orange precipitant similar to that observed in the thermal pool, which is not an experimental result, but is interesting in light of the investigation at hand.

Arkashin enrichments inoculated in 2005 also contained flocs of similar character to those osbserved in the field. Fluorescence microscopy of these cultures revealed microcolonies (Figure A.1). Additionally, IC-ICPMS analysis demonstrated higher As(III) concentrations in cultures relative to uninoculated media. Isolation on agar containing elemental sulfur from 2005 field samples was successful and four strains of *Thermoanaerobacter uzonensis* were obtained (Figure A.2). These strains may be helpful in current biogeographical analyses of the species (I. Wagner, pers. comm.).

Although no further isolates were obtained from the work described, the results described do support the interpretation that sulfate-reducing and arsenate-reducing prokaryotes inhabit the thermal pools Arkashin and Zavarzin. The conditions that make Uzon ideal for investigation also mandate meticulous development of protocols and alternatives. The development of *in situ* methods and field techniques is a crucial component of analysis of environmental phenomena. The methods developed and the skills and knowledge gained from this research will be useful in subsequent field investigations.

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Table A.1 Some examples of arsenate and/or sulfate reducing prokaryotes in isolation.

Isolate	Source environment	e- acceptors*		e- donors*		Citation
		As (V)	S (VI)	acetate	lactate	
Pyrobaculum arsenaticum	Hot spring, Italy	+	nr	nr	nr	Huber 2000
Bacillus selenitireducens	Mono Lake, CA, USA	+	--	+	--	Blum 1998
B. arsenicoselenatis	Mono Lake, CA, USA	+	~	+	--	Blum 1998
Thermus HR13	Hot spring drainage, CA, USA	+	nr	+	nr	Gihring 2001
Desulfotomaculum (OREX-4)	As contaminated lake sediments, MA, USA	+	+	+	+	Newman 1997b.
Desulfitobacterium GBFH	As contaminated lake sediments, ID, USA	+	--	+	--	Niggemyer 2001
Desulfovibrio Ben-RA	mud sample, Bendigo, Australia	+	+	+	nr	Macy 2000
Desulfomicrobium Ben-RB	mud sample, Bendigo, Australia	+	+	+	nr	Macy 2000

Table A.2 Ranges of estimated numbers of sulfate-reducing prokaryotes in Arkashin and Zavarzin by medium treatment (values are per 0.5 mL of sample).

	Temperature (^{o}C)/pH^{25oC} regime			
Sample	60/6	60/8	80/6	80/8
Zavarzin*				
-upper	10^{3}-10^{4}	10^{2}-10^{3}	<1	10^{3}-10^{4}
-interface	10^{2}-10^{3}	10^{3}-10^{5+}	10^{1}-10^{3}	10^{1}-10^{3}
-bottom	10^{1}-10^{2}	10^{2}-10^{4}	<1	10^{1}-10^{2}
Arkashin	10^{1}-10^{2}	10^{4}-10^{5}	lost	10^{2}-10^{3}

*Zavarzin samples represent sections of a core

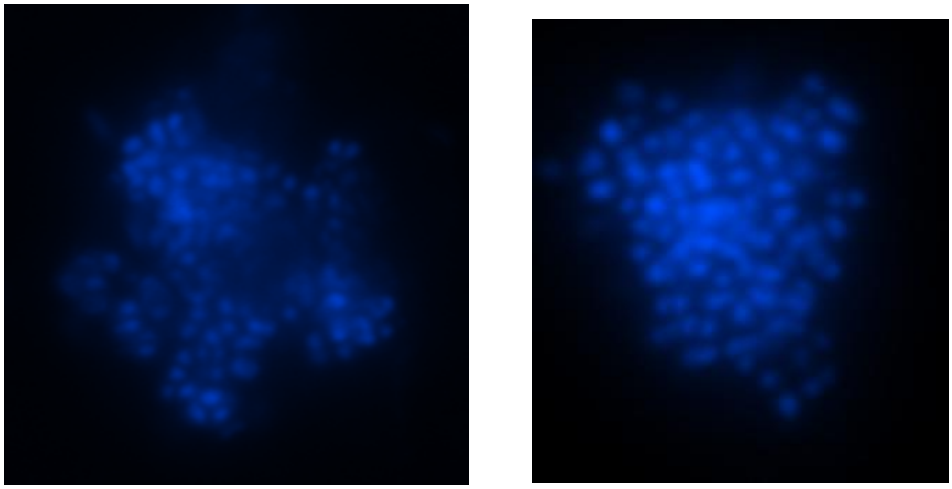

Figure A.1 Photomicrographs of DAPI-stained “microcolonies” from Arkashin enrichment cultures.

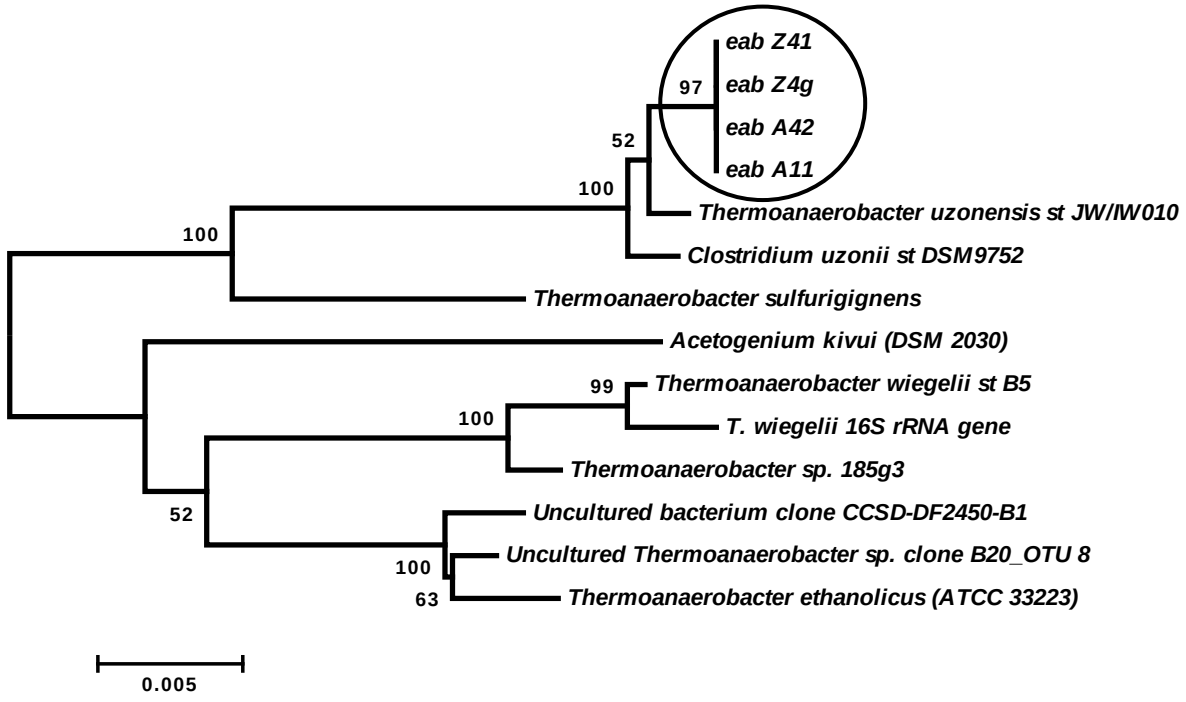

Figure A.2. Jukes-Cantor distance, neighbor-joining 16S rRNA tree depicting the relationship of isolates from this research (circled) to other representatives of the Clostridia. Bootstrap values are for 100 replicates.

APPENDIX B

A TIMELINE OF ENRICHMENT AND ISOLATION EFFORTS

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
11/05/03	making medium (phosphate solutions, anaerobic carbon sources)	n/a	Carbon sources 1	recipes and enrichment overview including culture id system
11/21/03	vitamin solution recipe	n/a	Vitamin solution 1	from Weidong Zhao
11/25/03	making medum and enrichment dilution scheme	n/a	Sulfate-reducer 1	21 samples x 2 pH (6 and 8) x 2 incubation temperatures (62 and 73°C) = 84 bottles
11/26/03	propagating cultures	NTF* samples: JW03-002	Sulfate-reducer 1, no pyruvate	from Kevin Lee's field enrichments
12/1/03	microscopy for contamination	n/a	Sulfate-reducer 1	black ppt** in uninnoculated medium is from abiotic reactions
12/12/03	innoculation of NTF samples	NTF samples: JW03-001, JW03-004 through 006	Sulfate-reducer 1, pH 6 and pH8, incubation temperatures 62 and 73°C	samples were grey and silty with some white pieces, see also sulfate-reducers enrichment spreadsheet.xls
12/15/03	microscopy of NTF samples	NTF samples: JW03-001, JW03-004 through 006	Sulfate-reducer 1	not much black ppt, long rods, short rods, rods in chains, cocci
12/15/03	C-free medium	n/a	C-free	to compare with precipitate in the presence of C sources
12/15/03	Arkashin sample innoculation	Arkashin samples: JW03-022 through JW03-024	Sulfate-reducer 1, pH 6 and pH8, incubation temperatures 62 and 73°C	Samples yellow-orange, JW03-022 and 024 fine, 023 more coarse, took photos
12/16/03	transfer/subculture NTF enrichments, pH check	NTF samples: JW03-001, JW03-004 through 006	Sulfate-reducer 1, C-free	pH decreasing, transfers went fine
12/18/03	microscopy of Arkashin, pH check	Arkashin samples: JW03-022 through JW03-024	Sulfate-reducer 1, C-free	black ppt, rods of various sizes, some sporulating, also eukaryotes; pH 6 the same, pH 8 decrease <1 pH unit

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
12/19/03	Arkashin transfer medium	n/a	C-free, Sulfate-reducer 1	~30 tubes each medium
12/20/03	Arkashin transfering	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	black ppt was settled in the 62°C/pH 8 cultures
12/22/03	microscopy of NTF transfers and medium pH check	NTF samples: JW03-001, JW03-004 through 006	C-free, Sulfate-reducer 1	see enrichment spreadsheet hardcopy, pH 6 to circumneutral, pH 8 changed <1 pH unit, also wrote out transfer timetable for cultures
12/23/03	making transfer medium	n/a	C-free, Sulfate-reducer 1, both pH 6 and 8	~100 tubes each medium
12/26/03	propagate NTF transfers, make glycerol aliquots	NTF samples: JW03-001, JW03-004 through 006	C-free, Sulfate-reducer 1, Glycerol aliquots	still not much black ppt, varying degrees of turbidity, made 65 ~2 mL aliquots
12/29/03	Arkashin transfer and microscopy, pH check, 62°C incubation	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	black ppt in 62°C, pH slight increase, see also enrichment spreadsheet
12/31/03	Arkashin transfer and microscopy, pH check, 73°C incubation	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	less ppt in 73°C, still more than controls, again, slight pH increases, need to be wary of introducing air…
12/31/03 - 1/04/04	making glycerol stocks of enrichments NTF and Arkashin, culture observations	Arkashin samples: JW03-022 through JW03-024, NTF samples: JW03-001, JW03-004 through 006	Glycerol aliquots	some NTF cultures released bubbles from settled turbidity upon slight shaking, smelled sulfide in some Arkashin enrichments
1/6/04	NTF microscopy, pH check and cell counting	NTF samples: JW03-001, JW03-004 through 006	n/a	slight pH increases, cell counts ~2 to 15 (average of 3 counts for 15s each in random spots on slides)
1/7/08	making transfer medium	n/a	C-free, Sulfate-reducer 1	~100 tubes each medium
1/8/04	transfer of NTF cultures	NTF samples: JW03-001, JW03-004 through 006	C-free, Sulfate-reducer 1	some cultures, esp. from 001 with black ppt and silvery sheen on top, one 006 culture with pressure (gas production)

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
1/12/04	microscopy, cell count and pH of Arkashin cultures	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	variations in pH changes, cell counts 0 to 19 cells, multiple morphologies
1/28/04	transfer of Arkashin cultures	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	old cultures to storage at room temp
1/31/04	making agar medium	n/a	C-free agar, Sulfate-reducer 1 agar	~80 tubes each medium
2/1/04	making agar roll tubes, culture transfers	Arkashin samples: JW03-022 through JW03-024	C-free agar, Sulfate-reducer 1 agar	transferred all cultures, agar-rolled 62°C cultures
5/1/04	making agar medium	n/a	C-free agar, Sulfate-reducer 1 agar	~40 tubes each medium
5/4/04	make colony picking vials	n/a	n/a	~30 30-mL glass vials, gassed with nitrogen, closed with Hungate caps and autoclaved
5/13-20/04	picking colonies, microscopy of syeneresis water	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	multiple colony morhphologies, including some dark, microscopy of many rods, occassional cocci
6/9/04	Observation/microscopy of cultures from colonies	Arkashin samples: JW03-022 through JW03-024	n/a	some black ppt, occasional cells of multiple morphologies
7/23/04	picking colonies	Arkashin samples: JW03-022 through JW03-024	C-free, Sulfate-reducer 1	two colony morphologies; black and round, amorphous and dirty white
7/26/04	microscopy of liquid cultures inoculated 7/23/04	Arkashin samples: JW03-022 through JW03-025	C-free, Sulfate-reducer 1	black ppt, fat rods or dancing rods and debris
8/4/04	Making arsenate-reducer and sulfate-reducer MPN medium for 2004 field season	n/a	MPN*** media	2 pHs x 6 dilutions x 3 replicates x 8 planned samples x 2 terminal electron acceptors = 576 bottles (with color coordination of stoppers and crimps)
8/8/04	Making arsenate-reducer and sulfate-reducer MPN media - results	n/a	MPN media	lost some bottles with black stoppers (do not stop air well), all arsenate medium was pink (aerobic?)

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
8/9/04	Re-make sulfate-reducer and arsenate-reducer MPN media	n/a	MPN media	arsenate medium pink/purple
8/19/04	Winding Stream, sample collection and ennumeration inoculations	JW04-005 through JW04-008	Lee/Wiegel SRB media	multiple pHs, temperature and dilution combinations
8/20-21/04	Central Thermal Field, including Arkashin, sample collection and inoculations	EAB04-005 through EAB-007	MPN media	attempted to reduce the pink in arsenate medium with addition of yeast extract
8/22/04	Yellow-Blue [Zavarzin], sample collection, ennumeration inoculation	core sample into EAB04-008 through EAB04-010	MPN media	had to make some samples into slurries with filtered water
8/23/04	Summary of enumeration inoculations	see results	MPN media	EAB04-001, 006, 008-010 and JW04-001-003, 005-009, 019-021, 023
9/8-15/04	Initial results of enumerations, culture observations, etc.	as described above	MPN media	created an excel file
9/10/04	Make sulfate-reducer transfer medium - same as sulfate-reducer MPN medium	n/a	MPN medium (for sulfate-reducers)	~150 bottles
9/24/04	Culture observations, microscopy and transfers	listed in notebook	MPN media	mix of healty and sickly cultures, mix of morphologies in healthy cultures
10/8/04	Culture observations and transfers	listed in notebook	MPN media	transfers from both field enrichments and 9/24/04 cultures
10/14/04	Making MPN agar medium (sulfate-reducer)	n/a	MPN media agar	~60 tubes
10/15/04	Culture obsv, microscopy, transfers, agar tubes and glycerol stocks	EAB04-005, cultures at 60°C, pH 6 and pH 8, multiple dilutions	MPN media	orange ppt in arsenate-reducer medium, no black ppt in sufate-reducer medium, clumps of filaments, spores, rods
10/20/04	Culture obsv, transfers, agar roll tubes and glycerol stocks	EAB04-008 through 010, cultures at 60°C, pH 6 and pH 8, multiple dilutions	MPN media	yellow ppt in arsenate-reducer medium, black ppt in sulfate-reducer medium, some with no ppt, varying among dilutions
10/26/04	Culture observations and transfers	EAB04-005, multiple dilutions	MPN media	many with no apparent growth
11/5-12/04	Culture observations, microscopy and transfers, agar roll tubes	EAB004-005, 008 through 010	MPN media	ppt in some tubes of both media, various morphologies

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
11/15/04	Make colony-picking medium and vials	n/a	Minimal medium	~84 small volume tubes, one larger volume
12/14/04	Culture observations, pick colonies, microscopy	EAB04-009	Minimal, MPN and MPN agar media (for sulfate-reducers)	picked 19 colonies
12/16/04	Making sulfate-reducer medium, liquid and agar	n/a	MPN and MPN agar media (for sulfate-reducers)	~100 bottles
11/7/05	Making modified Wolfe's mineral solution	n/a	Wolfe's mineral solution	2 500-mL bottles
11/11/05	Making modified Wolfe's vitamin solution	n/a	Wolfe's vitamin solution	~8 125-mL bottles
11/14/05	Making YP87 Medium with Acetate	n/a	YP87 Medium	medium, made on new gassing station, still pink after autoclave, checked gassing lines
11/15/05	Re-making YP87 Medium with Acetate	n/a	YP87 Medium	medium clear, but cloudy
11/17/05	Making YP87 Medium adding bicarbonate after autoclaving	n/a	YP87 Medium	addition of bicarbonate disrupted pH/redox potential (medium turned pink)
11/17/05	Re-making 1 M bicarbonate	n/a	n/a	addition of bicarbonate turned medium pink, but cleared, clearing took 1-2 weeks
12/1/05	Making more YP87 medium, playing with adding carbonate	n/a	YP87 Medium	medium turns cloudy and/or pink if bicarbonate added before autoclaving
12/2/05	Adding bicarbonate after autoclaving, checking redox and pH of YP87 medium	n/a	YP87 Medium	preparation and equilibration takes too long
12/28/05	Making modified M1 medium	n/a	M1 Sulfate medium	~25 bottles
1/2-17/06	Inoculate enrichment cultures for sulfate-reducers	EAB05-001W, 001Y, 002Y, 001Blu, 001Blk, 002B, 002G (stored at 15°C)	M1 Sulfate medium, YP87 medium	incubation at 60°C, 8 incoulations in M1 x 2 dilutions + 2 inocluations in YP87 x 2 dilutions = 20 inoculations, none with observed growth
1/5/06	Making M1 medium	n/a	M1 Sulfate medium	~25 bottles

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
1/13/06	Glycerol stocks from Kam 2004 for fresh isolation efforts	EAB04-005, EAB04-010	n/a	sub-sampled stocks for inoculating
8/25/06	Making enrichment medium for 2006 field season	n/a	M1 Sulfate, M1 Sulfur, M1 Arsenate media	~25 bottles each medium and ~25 bottles M1 Sulfur and M1 Arsenate at 1/2 dilution
9/6/06	Sampling for enrichments and casting off prokaryote traps	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	n/a	drawings of Arkashin and Zavarzin with locations marked, temperature and pH data
9/7/06	Enrichment inoculation and incubation	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfate, M1 Sulfur, M1 Arsenate media	2 sites x 3 media x 2 media dilutions x 4 or 5 enrichment dilutions ~ 50 inoculations
9/17/06	Collect and assess enrichments	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfate, M1 Sulfur, M1 Arsenate media	Arkashin: no growth apparent, Zavarzin: Possible growth in low dilutions
9/18/06	Final sampling and trap collection	EAB06-016g, 016s, and 017 (Arkashin); EAB06-018g, 018s, and 019 (Zavarzin)	n/a	collected traps in thermoses filled with water from pools, collected sediments in rubber-stoppered, screw-capped bottles
9/25/06	Checking sulfur and sulfate cultures for smell of sulfide in the highest dilutions	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfate, M1 Sulfur, M1 Arsenate media	all sulfur cultures positive, sulfate cultures stronger in lower dilutions
926/06	Microscopy of samples from prokaryote traps	EAB06-016g and 016s (Arkashin); EAB06-018g and 018s (Zavarzin)	n/a	very little growth on glass wool; on sulfur, cells more abundant in Zavarzin
9/26/06	Making medium for transfers	n/a	M1 Sulfur and M1 NoTEA media	half sulfur, half no terminal electron acceptor (to be added later from stock solutions)
9/26/06	Transferring cultures to fresh medium	EAB06-001 (Zavarzin) & EAB06-002 (Arkashin)	M1 Sulfate, M1 Sulfur, M1 Arsenate media	transferred all cultures, also made glycerol stocks

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
9/26-10/17/06	Culture observations	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfate, M1 Sulfur, M1 Arsenate media	clumpy growth by eye and microcolonies by microscopy in Arkashin cultures in M1 Arsenate medium, abundant and diverse rods in Zavarzin M1 Sulfur cultures, sulfide smell
10/5/06	Making alternative terminal electron acceptor stock solutions	n/a	n/a	arsenate, sulfate, polysulfide
10/5/06	Adding polysulfide to stock solution	n/a	M1 NoTEA medium	elemental sulfur precipitation
10/5/06	Make glycerol aliquots	n/a	Glycerol aliquots	made aliquots and 50 mL stock
10/10/06	Method testing glycerol stocking of cultures	EAB06-001 (Zavarzin)	M1 Sulfur, 1/2 dilution	testing two methods, turbidity in both cultures, cells under microscope
10/11/06	Medium for dilution to extinction	n/a	M1 Sulfur, 1/2 dilution	assumed ~10^8 cells/mL, dilution scheme down to 0.05 cells/mL
10/11/06	Inoculation for dilution to extinction	EAB06-001 (Zavarzin)	M1 Sulfur, 1/2 dilution	mixed success, clear dilutions between turbid, variety of rods under microscope
10/13-16/06	A second dilutions series and observations	EAB06-001 (Zavarzin)	M1 Sulfur, 1/2 dilution	looks better, inocluated highest dilution with growth into fresh medium
10/16/06	Microscopy of possible pure cultures	EAB06-001 (Zavarzin)	M1 Sulfur, 1/2 dilution	diverse rods
10/19/06	Microscopy of cultures	EAB06-002 (Arkashin)	M1 Sulfate, M1 Arsenate	variety of rods
10/20/06	Medium for roll tubing	n/a	M1 NoTEA, M1 Agar	prepared to roll
10/26/06	Agar roll tubes of M1 Arsenate cultures	EAB06-002 (Arkashin)	M1 Arseante, M1 Agar	no growth
10/27/06	Making M1 Sulfur Agar plates for isolation of S reducing organisms	EAB06-001 (Zavarzin)	M1 Sulfur Agar	well-dispersed elemental sulfur in plates
10/30/06	Make M1 Arsenate medium, 1/2 dilution	n/a	M1 Arsenate, M1 Agar	10 small volume vials, 24 bottles, 24 agar tubes
10/31/06	Streaking M1 Sulfur Agar plates for isolation	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur Agar	possible colony morphology must have been mineralization, no cells observed in microscopy
11/1/06	Microscopy and roll-tubing of 1/2 As cultures	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arsenate, M1 Agar	possible growth in liquid observed 11/6/06
11/3/06	Making M1 Sulfur Agar plates	n/a	M1 Sulfur Agar	well-dispersed elemental sulfur in plates, in anaerobic chamber

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
11/3/06	Re-streaking M1 Sulfur Agar plates for isolates	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur Agar	growth, no visibly isolated colonies
11/6/06	Re-re-streak M1 Sulfur Agar plates for isolates	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur Agar	isolated colonies
11/8/06	Microscopy of M1 Sulfur Agar plates and M1 Arsenate cultures	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arsenate, M1 Sulfur Agar	various rods
11/8/06	Pick M1 Sulfur Agar colonies, re-streak, inoculate cultures	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur, M1 Sulfur Agar	opaque white clumps, eggy smell
11/13/06	Pick M1 Sulfur Agar colonies, re-streak, inoculate cultures, glycerol stocks, cultures for DNA extraction	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur, M1 Sulfur Agar	opaque white clumps, eggy smell
11/13-16/06	Picking M1 Arsenate colonies from roll-tubes	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arsenate, M1 Agar	colony descriptions, picked ~23 colonies, growth observed in liquid and agar
11/20/06	Roll-tube observations	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arsenate, M1 Agar	growth in all cultures
11/17/06	Isolate DNA from M1 Sulfur putative isolates, amplify 16S rRNA genes	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur	successful PCR
11/17/06	Glycerol stock M1 Arsenate cultures	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	Glycerol aliquots	glycerol stocked cultures from 11/1/06
11/20/06	Make M1 Arsenate medium, 1/2 dilution	n/a	M1 Arsenate, M1 Agar	25 vials, 22 bottles, 17 tubes
11/21/06	IC-ICPMS of M1 Arsenate cultures	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arsenate, M1 Agar	little to no evidence for arsenate reduction as compared to uninoculated medium

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
11/27/06	Sequencing M1 Sulfur putative isolates	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	n/a	five putative isolates, all *Thermoanaerobacter uzonensis*
12/1/06	Picking M1 Arsenate colonies from roll-tubes, second round	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arsenate, M1 Agar	growth observed in agar 12/13/06
12/24/06	Inoculate M1 Arsenate cultures, glycerol stocks	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Arseante, Glycerol aliquots	little to no growth
1/9/07	Microscopy of M1 Arsenate cultures	EAB06-001 (Zavarzin)	M1 Arsenate	little to no cells observed
1/30/07	Grow strains of *T. uzonensis* for Isaac	EAB06-001 (Zavarzin) and EAB06-002 (Arkashin)	M1 Sulfur Agar	for use in biogeographical studies
3/13/07	Make AZ Mineral medium	n/a	AZ Mineral Medium	for use to dilute sediments in inoculations
3/21-5/7/07	AZ Arsenate reducer medium	n/a	AZ Mineral medium, AZ Stock Solutions	carbon and electron source combinations included acetate-only, acetate and hydrogen, acetate and hydrogen sulfide, carbon dioxide and hydrogen, carbon dioxide and hydrogen sulfide
4/09/07	Elemental sulfur agar plates	n/a	AZ Sulfur Agar	storing 1/2 the plates in anaerobic chamber
4/10-5/8/07	AZ Sulfate reducer medium	n/a	AZ Mineral medium, AZ Stock Solutions	carbon and electron source combinations included acetate-only, acetate and hydrogen, acetate and hydrogen sulfide, carbon dioxide and hydrogen, carbon dioxide and hydrogen sulfide
5/07/07	Addition of H2S gas to media	n/a	AZ Arsenate and Sulfate reducer media	added as a gas because addition from sodium sulfide stock solution changed pH and caused precipitates to form
5/8/07	Check pH of medium,	n/a	AZ Arsenate and AZ Sulfate reducer media	only slight preciptates, all pH 7-7.5 (original attempts with sodium sulfide were pH ~9)

Date	Entry title/objective	Field sample ids	Recipe name (as per Appx B)	Notes/results summary
5/9-6/5/07	Time zero measurements	n/a	AZ Arsenate and Sulfate reducer media	measured, with help, arsenate, arsenite, sulfate, acetate, hydrogen, and carbon dioxide
6/7/07	Inoculation; microscopy, sample dilution	EAB06-017 (Arkashin) and EAB06-019 (Zavarzin)	AZ Arsenate and AZ Sulfate reducer media, AZ Sulfur Agar	~40 cultures, plus killed and uninoculated controls, also dilutions spread on agar
6/12/07	Microscopy (SYTO11/SYTOX Orange live/dead staining)	EAB06-017 (Arkashin) and EAB06-019 (Zavarzin)	AZ Arsenate and AZ Sulfate reducer media, AZ Sulfur Agar	growth not abundant, but some green, cell-shaped objects in all slides
6/29/07	Culture observations, microscopy (SYTO11/SYTOX Orange live/dead staining)	EAB06-017 (Arkashin) and EAB06-019 (Zavarzin)	AZ Arsenate and AZ Sulfate reducer media, AZ Sulfur Agar	some improvement
7/9/07	Measuring time 1 carbon dioxide	EAB06-017 (Arkashin) and EAB06-019 (Zavarzin)	AZ Arsenate and AZ Sulfate reducer media, AZ Sulfur Agar	unexplained variance
7/10/07	Make glycerol aliquots	n/a	Glycerol aliquots	~50 aliquots
7/13/07	Microscopy (SYTO11/SYTOX Orange live/dead staining)	EAB06-017 (Arkashin) and EAB06-019 (Zavarzin)	AZ Arsenate and AZ Sulfate reducer media, AZ Sulfur Agar	many green and orange specks
7/??/07	50% glycerol stocking of cultures	EAB06-017 (Arkashin) and EAB06-019 (Zavarzin)	AZ Arsenate and AZ Sulfate reducer media, AZ Sulfur Agar, Glycerol aliquots	for -80°C storage

APPENDIX C

MEDIA AND METHODS USED IN ENRICHMENT AND ISOLATION EFFORTS

EAB Recipe Log
10/2003-6/2007

Recipe name: **Carbon Sources 1**
1M pyruvate ($CH_3COCOOH$)
Ingredients: pyruvic acid, diH_2O
Preparation: dissolve 4.4 g of FW 110.0 pyruvic acid in 20 mL diH_2O with no heat, stirring and gassing (N_2). Bring voume to 40 mL with diH_2O, bubble gas with N_2 for ~10-15 mins. Bottle anaerobically, transfer to sterile, anaerobic bottle via filter sterilization through syringe

1M Acetate (CH_3COOH)
Ingredients: FW 98.14 acetic acid, diH_2O
Preparation: Dissolve 3.93 g acetic acid in 20 mL diH_2O with heat, stirring, and gassing (N_2), bring volume to 40 mL with diH_2O, heat to boiling with gassing cool with bubbling N_2. Transfer to anaerobic bottle, autoclave.

1M Lactate ($CH_3CHOHCOOH$)
Ingredients: NaLactate, diH_2O
Preparation: add 7.47 mL 60% Nalactate to 32.53 mL diH_2O bring to a boil while gassing, cool while bubbling with N_2 transfer to anaerobic bottle, autoclave.

0.5M Benzoate (C_6H_5COOH)
Ingredients: benzoic acid, diH_2O, NaOH
Preparation: add 4.88 g benzoic acid to ~40 mL diH_2O with heating, gassing and stirring, neautralize with NaOH to facilitate dissolving bring to 80 mL, bring to boil under N_2 gassing cool with bubbling with N_2 transfer to anaerobic bottles, autoclave.

Recipe name: **Vitamin solution 1**

Ingredients:		
	2.0 mg biotin	2.0 mg folic acid
	10.0 mg Pyridoxing-HCl	5.0 mg thiamine-HCl·$2H_2O$
	5.0 mg riboflavin	5.0 mg nicotinic acid
	5.0 mg D-Ca-pantohenate	0.1 mg vitamin B12
	5.0 mg p-aminobenzoic acid	5.0 mg lipoic acid

Preparation: Dissolve in 1000 mL (1L) diH_2O. Filter-sterilize and store at 2-8°C

Recipe name: **Sulfate-reducer 1**
Ingredients per 1 L volume:

1.0 g NaCl	1.42g $NaSO_4$
0.5 g NH_4Cl	0.5 g KH_2PO_4
0.5 g KCl	0.08 g $MgCl_2·6H_2O$
0.08 g $CaCl_2·2H_2O$	0.025 g Fe citrate

0.1 g yeast extract	0.05 g cystine
1.0 mL trace element solution	1.0 mL vitamin solution
1.0 mL selenite-tungstate solution	resazurin
0.98 g acetate	1.87 mL 60% Nalactate
1.22g benzoic acid	0.096 g propionic acid

0.2 mL of 1 M filter sterilized (~5mM) pyruvate to each after autoclave

Preparation: To make medium, all ingredients but cystine and pyruvate are combined in 1 L flask and mixed to dissolved with stirring and heat. Medium is brought to a boil under N_2 gassing and aluminum foil cover. As resazurin goes pink, bubble liquid with N_2 and put on ice. Add cystine when cool. Check pH and titrate to desired [H+]. Distribute to serum bottles using Hungate technique. Autoclave at 121°C for 45 minutes.

Recipe name: **C-free**

Ingredients per 1L volume

1.0 g NaCl	1.42 g $NaSO_4$
0.5 g NH_4Cl	0.5 g KH_2PO_4
0.5 g KCl	0.08 g $MgCl \cdot 6H_2O$
0.08 g $CaCl_2 \cdot 2H_2O$	0.025 g Fe citrate
0.1 g yeast extract	0.05 g cystine
1.0 mL trace element solution	0.2 mL selenite-tungstate solution
1.0 vitamin solution	Few drops of resazurin

Preparation: diH_2O to 1 L, boil and bubble with N_2, cool under gass and add cystine, distribute 10 mL to Hungate tubes anaerobically. Autoclave at 121°C for 45 min.

Recipe name: **Glycerol aliquots**

Ingredients: Glycerol

Preparation: ~125 mL of glycerol in a flask with a stir bar. Heat with bubbling of N_2 to 100° C (from thermometer). Then cool the glycerol to room temperature under N_2 headspace and aliquot 2-5 mL stocks to smaller serum bottles.

Recipe name: **Sulfate reducer 1 agar**

Ingredients per 1 L volume (salts and Fe citrate from stock solutions):

17 mM NaCl	10 mM $NaSO_4$
9.3 mM NH_4Cl	4.0 mM KH_2PO_4
6.7 mM KCl	0.4 mM $MgCl_2 \cdot 6H_2O$
0.5 mM $CaCl_2 \cdot 2H_2O$	0.1 mM Fe citrate
0.02 g yeast extract	0.01 g cystine
0.2 mL trace element solution	0.2 mL vitamin solution
0.2 mL selenite-tungstate solution	0.02 g proprionic acid
10 mM acetate	10 mM lactate
10 mM benzoic acid	5 mM pyruvate (add after autoclave)

2% wt/vol agar

Preparation: To make medium, all ingredients but cystine and agar are combined. Added distilled water to volume (200 mL). Brought to boil with bubbling N_2 allowed to cool slightly with N_2 in headspace. Added agar and cystiene. Checked pH. Distributed 5 mL to tubes under gas autoclaved at 121° for 45 min along with unautoclaved stock solutions.

Recipe name: **C-free agar**

Ingredients per 1 L volume (salts and Fe citrate from stock solutions):

18 mM NaCl	10 mM $NaSO_4$
9.3 mM NH_4Cl	4.0 mM KH_2PO_4

6.7 mM KCl	0.4 mM $MgCl_2 \cdot 6H_2O$
0.5 mM $CaCl_2 \cdot 2H_2O$	0.1 mM Fe citrate
0.02 g yeast extract	0.01 g cystine
0.2 mL trace element solution	0.2 mL vitamin solution
0.2 mL selenite-tungstate solution	2% wt/vol agar

Preparation: To make medium, all ingredients but cystine and agar are combined. Added distilled water to volume (200 mL). Brought to boil with bubbling N_2 allowed to cool slightly with N_2 in headspace. Added agar and cystiene. Checked pH. Distributed 5 mL to tubes under gas autoclaved at 121° for 45 min along with unautoclaved stock solutions.

Recipe name: **MPN media** (for use with one of two terminal electron acceptors)*
Ingredients per 1200 mL volume:

1.2 gNaCl	0.6 g NH4Cl
0.6 g KH_2PO_4	0.6 g KCl
0.024 g $MgCl_2 \cdot 6H_2O$	0.012 g $CaCl_2 \cdot 2H_2O$
0.03 g Fe citrate	0.12 g yeast extract
0.006 g cystine	1.2 mL trace element solution
1.2 mL vitamin solution	1.2 mL selenite-tungstate solution
0.1152 g proprionic acid	10 mM k acetate
10 mM Na lactate	10 mM benzoatic acid
5 mM pyruvate	

*10 mM sulfate and 1 mM ferrous sulfate for sulfate-reducers OR 10 mM arsenate for arsenate-reducers

Preparation: Add diH_2O to 1200 mL, boil and bubble with N_2, cool-add cystiene, distribute to bottles under gas, autoclave, add pyruvate.

Recipe name: **MPN media agar** (for use with one of two terminal electron acceptors)*
Ingredients: same as MPN media with 2% w/v agar
Preparation: combine ingredients except cystiene and agar, boil and bubble with N_2, cool-add cystiene and agar, distribute to bottles under gas, autoclave, add pyruvate.

Recipe name: **Minimal medium**
Ingredients: same as MPN media with no C sources [except yeast extract]
Preparation: same as MPN media

Recipe name: **Modified Wolfe's mineral solution**

Ingredients:	3.0 g $MgSO_4 \cdot 7H_2O$	1.5 g Nitrolotriacetic acid
	1.0 g NaCl	0.5 g $MnSO_4 \cdot H_2O$
	0.1 g $CaCl_2$	0.1 g $CoCl_2 \cdot H_2O$
	0.1 g $FeSO_4 \cdot 7H_2O$	0.1 g$ZnSO_4 \cdot 7H_2O$
	0.01 g $AlK(SO_4)_2 \cdot 12H_2O$	0.01 g $CuSO_4 \cdot 5H_2O$
	0.01 g H_3BO_3	0.01 $Na_2MoO_4 \cdot 2H_2O$
	0.01 g Na_2SeO_3	0.01 g $NaWO_4 \cdot 2H_2O$
	0.01 g $NiCl_2 \cdot 6H_2O$	

Preparation: Dispensed 1 L ddH_2O from Barnstead Nanopure Diamond system into 1000 mL flashed, marked 1 L. Removed 500 mL and set aside. Added Nitrilotriacetic acid and stirred, pH's to 6.55 with ~1.5 mL 4 M KOH, 0.25 mL 1 M KOH, 0.5 mL HCl. Added remaining components one at a time, rinsing weigh boats into flash with di or ddH_2O. Brought to ~850 mL, adjusted to pH 6.8 with ~3 mL 1 M KOH. Brought to 1L mark with remaining ddH_2O.

Recipe name: **Wolfe's vitamin solution**

Ingredients: Per L vol:

0.01 g pyridoxine·HCl	0.005 g p-aminobenzoic acid
0.005 g Lipoic acid	0.005 g Nicotinic acid
0.005 g Riboflavin	0.005 gThiamine·HCl
0.005 gCalcium DL-pantothenate	0.002 g Biotin
0.002 g Folic acid	0.0001 g Vitamen B12

Preparation: Marked 1 L on flask with water measured in graduated cylinder, removed ~1/2 the water. Weighed and added ingredients in order. Mixed well, filled to 1 L, mixed well. Transferred to 1L wheaton bottle to take to SREL. Filter sterilized using two 500 mL coring filter systems with 0.2 um nylon filters. Dispensed to sterile 125 mL serum bottles in laminare flow hood, stoppered, sealed. Wrap with foil or store in closed cardboard box. Stored at 4°C.

Recipe name: **YP87 Medium with acetate**

Ingredients: per 1 L volume:

4.0 g $NaSO_4$	[1.3 g] $NaHCO_3$
0.5 g KCl	0.5 g yeast extract
0.4 g $MgCl_2·6H_2O$	0.25 g NH_4Cl
0.2 g L-Ascorbic Acid	0.2 g NaH_2PO_4
0.2 g Sodium thioglycolate	0.15 g $CaCl_2·2H_2O$
1 mg Reazurin	10 mL Modified Wolfe's minerals
10 mL Wolfe's Vitamins	1.5 mL sodium lactate (60%)
0.113 g Amonium Acetate (anhydrous)	

Preparation: Combine all ingredients except NaHCO3, ascorbic acid and sodium thioglycolate, boil while bubbling with N2, cool under N2, add ascorbic acid and sodium thioglycolate, bottle under N2 and autoclave, cool, add $NaHCO_3$ from stock solution to achieve appropriate concentration (~15 mM)

Recipe name: **M1 Sulfate**

Ingredients per 500 mL volume:

- 0.95 g $(NH_4)_2SO_4$
- 7.5 mL phosphate buffer (30.0g KH_2PO_4, 66.1 g K_2HPO_4, ddH_2O to 1 L, pH to 7)
- 5 mL Modified Wolfe's minerals
- 5 mL Wolfe's vitamins
- 0.25 g yeast extract
- 50 mL basal salts (2.0g $MgSO_4·7H_2O$, 0.57 g $CaCl_2·2H_2O$, 0.2 g EDTA, disodium salt, 0.012 g FeSO4·7H_2O, ddH_2O to 1 L)
- 0.05 mL 0.115 M $NaSeO_4$
- 0.05 mL metal supplement (1.41 g $CoSO_4·7H_2O$, 1.98 g $NiCl_2·6H_2O$, 0.58 g NaCl 100 mL ddH_2O)
- 3 mL 60% sodium lactate
- resazurin
- 0.1 g sodium thioglycolate
- 0.1 g L-ascrobic acid
- 2 mM sodium bicarbonate

Preparation: combine all ingredients except sodium thioglycolate, L-ascorbic acid, and sodium bicarbonate, adjust to pH 7, add ddH2O to 500 mL, boil and bubble with N2, cool under N2, add sodium thioglycolate and L-ascrobic acid, bottle under N2, autoclave, cool, add sodium bicarbonate from anaerobic stock solution

Recipe name: **M1 Sulfur**

Ingredients: Same as M1 Sulfate medium, EXCEPT

- 0.00 g $(NH_4)_2SO_4$

0.68 g sodium acetate
1 g elemental sulfur per 10 mL medium in each culture bottle/tube
Preparation: Same as M1 Sulfate medium

Recipe name: **M1 Arsenate**
Ingredients: Same as M1 Sulfate medium, EXCEPT
0.00 g $(NH_4)_2SO_4$
15 mM arsenate
0.68 g sodium acetate
no resazurin
Preparation: Same as M1 Sulfate medium

Recipe name: **M1 NoTEA**
Ingredients: Same as M1 Sulfate medium, EXCEPT
0.00 g $(NH_4)_2SO_4$
0.68 g sodium acetate
no resazurin
Preparation: Same as M1 Sulfate medium

Recipe name: **M1 Arsenate or Sulfate Agar**
Ingredients: Same as M1 media, EXCEPT
1-5 % wt/v agar
Preparation: Same as M1 media, add agar before boiling

Recipe name: **M1 Sulfur Agar**
Ingredients: Same as M1 Sulfur, EXCEPT
1-5 % wt/v agar
no resazurin
8.4 mL polysulfide
Preparation: combine ingredients as for M1 sulfur, add agar before boiling, do not need to bubble under N_2, do not dispense before autoclaving, add polysulfide with bicarbonate after autoclaving, cool slightly, pour into Petri dishes, cover and cool overnight, store in anaerobic chamber ~3 days before use

Recipe name: **AZ Mineral Medium**
Ingredients per 1 L:
10 mL 100X Mineral Solution (80 g NaCl, 100 g NH_4Cl, 10 g KCl, 10 g KH_2PO_4, 20 g $MgSO_4 \cdot 7H_2O$, 4 g $CaCl_2 \cdot 2H_2O$, ddH_2O to 1 L, store in sterile containers at room temperature)
2.5 mL Wolfe's Vitamin Solution
2.5 mL Modified Wolfe's Mineral Solution
6.7 g PIPES buffer
1 mL 1 M sodium acetate
0.8 mL 1 M Na_2S
0.1 g sodium thioglycolate
0.1 g ascorbic acid
Preparation: combine first five ingredients in ~800 mL ddH_2O, stir to dissolve, pH to 7, add water to ~999.2 mL, boil and bubble with N_2, cool under N_2, add remaining three ingredients, dispense to bottles under N_2, autoclave, store at 4°C

Recipe name: **AZ Stock Solutions**
Arsenate

Ingredients: $Na_2HAsO_4 \cdot 7H_2O$
Preparation: combine ddH_2O and $Na_2HAsO_4 \cdot 7H_2O$ to make 0.5 M stock solution, bring to boil with bubbling N_2, cool under N_2, bottle under N_2, autoclave

Sulfate
Ingredients: Na_2SO_4
Preparation: combine ddH_2O and Na_2SO_4 to make 1 M stock solution, bring to boil with bubbling N_2, cool under N_2, bottle under N_2, autoclave

Acetate
Ingredients: $NaOOCCH_3 \cdot 3H_2O$
Preparation: combine ddH_2O and $NaOOCCH_3 \cdot 3H_2O$ to make 1 M stock solution, bring to boil with bubbling N_2, cool under N_2, bottle under N_2, autoclave

Sulfide
Ingredients: $Na_2S \cdot 9H_2O$
Preparation: combine ddH_2O and $Na_2S \cdot 9H_2O$ to make 1 M stock solution, bring to boil with bubbling N_2, cool under N_2, bottle under N_2, autoclave

Bicarbonate
Ingredients: $NaHCO_3$
Preparation: combine ddH_2O and $NaHCO_3$ to make 1 M stock solution, bring to boil with bubbling N_2, cool under N_2, bottle under N_2, autoclave

Recipe name: **AZ Sulfur Agar**
Ingredients per 0.5 L:

- 5 mL 100X Mineral Solution (80 g NaCl, 100 g NH_4Cl, 10 g KCl, 10 g KH_2PO_4, 20 g $MgSO_4 \cdot 7H_2O$, 4 g $CaCl_2 \cdot 2H_2O$, ddH_2O to 1 L, store in sterile containers at room temperature)
- 1.25 mL Wolfe's Vitamin Solution
- 1.25 mL Modified Wolfe's Mineral Solution
- 3.35 g PIPES buffer
- 0.5 mL 1 M sodium acetate
- 7.5 g agar
- 8 mL polysulfide

Preparation: combine first five ingredients in ~400 mL ddH_2O, stir to dissolve, pH to 7, add agar, add and water to 500 mL, boil, autoclave, add polysulfide, pour into Petri plates