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Controls on microbial community structure in thermal environments : exploring bacterial diversity and the relative influence of geochemistry and geography

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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
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The University of New Mexico
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For Doug

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ABSTRACT

Community wide molecular surveys have revealed incredible hidden phylogenetic and metabolic diversity in microbial habitats. We have conducted the first microbial survey of Yellowstone National Park thermal environments, sampling 103 communities from across the park and across the range of conditions found. Yellowstone is particularly suited for this type of research because of the large number and wide variety of thermal springs, which are naturally occurring chemostats enabling examination of the factors that control microbes and drive community structure. In addition to samples for molecular microbial analysis, we collected water for extensive geochemical analysis in order to begin to deduce the microbes' role *in situ*. With this data we investigated patterns and correlations among the microbial communities, environmental geochemistry, and theoretical energy yield from 179 reactions that could be catalyzed by microbes. Prior to this work it was believed that temperature was the driver of microbial diversity in thermal communities, we have shown that pH is the most important factor controlling where communities are found.

Through this extensive sequencing effort we have identified five major community types that can be described by the dominant organisms: Thermocrinis/Thermus, Phototrophs, Sulfurihydrogenibium, Hydrogenobaculum, and Proteobacteria/Bacteriodetes. The last group has never before been noted to be an important community type in thermal areas. The Proteobacteria/Bacteriodetes group is also interesting because it seems to thrive in the harshest conditions measured, low pH and high concentrations of metals. Additionally, sequences from 15 putative candidate phyla were recovered from multiple springs. The ecosystems described in this study are ideal for further application of ecological theory, especially community assembly patterns, biogeographic theory, and macroecological experiments that take advantage of the high diversity of habitats and short generation time of thermal communities. This work establishes a baseline of the communities inhabiting the range of thermal features in Yellowstone which will provide a foundation for future microbial research.

The taxa-area relationship is regarded as one of the few laws in ecology. Although it has been investigated for decades in plants, animals, and insects; the taxa-area relationship has only begun to be examined in microbes. We evaluated the taxa-area and taxa-energy relationships in bacterial diversity of terrestrial hot spring "islands" representing the range of environmental conditions found in Yellowstone National Park. There was no significant relationship between species richness and either island size or energy available. Clone libraries of microbial communities under sample the diversity of those communities; therefore we also tested these relationships on estimated diversity. This study is the first to examine a large number of natural isolated microbial

communities, but it is still possible that more extensive sampling is needed to detect the relationship between richness and island size.

The work described here is unique in the number of microbial habitats studied, the intensity of the molecular sequencing effort, and the concurrent geochemical investigations. It is also the broadest application of thermodynamic energetic modeling done to date, which has enabled us to examine microbial communities across differing metabolic regimes as well as across geographic space. The combination of molecular and geochemical analysis of a wide variety microbial communities with energetic modeling of potential metabolisms forms a basis for future ecological studies of these environments.

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Chapter 1

Microbial ecology and the Bacteria that inhabit Yellowstone hot springs

Background

Microorganisms account for most of the evolutionary diversity on Earth. However, until recently knowledge of the extent of this diversity was severely limited because microbiologists could only study the organisms that are capable of growing in isolation in the laboratory. Through the now widespread use of molecular techniques, it is apparent that less than 1% of microbes in the environment can be cultivated using current techniques (Pace, 1997, Schoenborn et al., 2004). Community wide molecular surveys have revealed incredible hidden phylogenetic and metabolic diversity in microbial habitats (Barns et al., 1994, Casamayor et al., 2002, Ferris and Ward, 1997, Ghosh et al., 2003, Giovannoni, 2004, Hugenholtz et al., 1998a, Sogin et al., 2006). Twenty years ago, just as researchers were beginning to characterize microbial communities using cultivation independent approaches, there were 11 recognized phyla of Bacteria (Woese, 1987). One of these original 11 phyla, the Gram positive bacteria, has now been split into 2 phyla, the Firmicutes and Actinobacteria (Boone et al., 2001). The first decade of the molecular revolution expanded our knowledge of the Bacteria and increased the recognized phyla to 23 with an additional 13 candidate phyla (members of these phyla are only known through their 16S rDNA sequence) (Hugenholtz et al., 1998a). The most recent paper summarizing known phyla and candidate divisions, which is already 5 years

old, increases the number of phyla represented by isolates in collection to 27 and published candidate phyla to 26 (Rappe and Giovannoni, 2003).

Just as the phylogenetic diversity of extreme environments is largely unknown, so is the metabolic diversity. Recent discoveries have included major new metabolic pathways. For example two novel photosynthetic organisms, an anaerobic phototroph from Mono lake that uses arsenite as the electron donor (Kulp et al., 2008) and a novel aerobic phototroph from Octopus spring (Bryant et al., 2007) have been described in the past year. These discoveries were made using two different approaches. The arsenite phototroph was discovered and cultivated using traditional microbiological tools (*i.e.* scientists noticed an interesting community in the environment and from that observation worked to isolate the organisms responsible). While the novel aerobic phototroph was first discovered through a metagenomic analysis of one of the best studied hot springs in the world (Ward et al., 1998), then cultivated and characterized. A third approach that could be fruitful in discovering novel metabolisms is to search not for the organisms directly but to use thermodynamic calculations to determine which metabolisms are likely energy yielding in a particular environment (Amend and Shock 2001, Spear 2005, Meyer-Dombard 2005). This final approach has the advantage of being very broad. Cultivation based discovery is limited to the organisms that are being targeted while metagenomic sequencing is still too expensive to be commonly used. Results from thermodynamic modeling of the metabolisms in the environment can be used to guide culturing efforts.

History of microbiology in Yellowstone National Park

The organisms that inhabit the thermal features of Yellowstone National Park (YNP) have been studied throughout the history of the park. One of the earliest research permits was granted to W. A. Setchell to study the "algous growth" in hotsprings (Wondrak Biel, 2004). Another early microbiologist in YNP was Joseph Copeland who examined bacteria from a large number of springs in collaboration with the park wide geothermal work of Allen and Day (Allen and Day 1935; Copeland 1936). While the applicability of either Setchell or Copeland's work to modern microbiology is difficult to discern, given the radical transformation of the known bacterial world through molecular techniques, their studies were an attempt to conduct a microbial survey across Yellowstone and are the ideological ancestors of work described in this dissertation.

The next wave of intensive work on YNP thermophiles began in the early 1960's. Thomas Brock and collaborators isolated organisms from a number of springs across the park and studied the ecology of those organisms in situ (summarized in (Brock 1978)). This work is significant not only in the application of ecological theory to the study of a variety of springs and organisms, but also because two of the most significant microbiological findings were, at least in part, based on that work. The first is isolation of *Sulfolobus* which was one of the organisms that Carl Woese used in demonstrating the existence of the Archaea (Woese et al. 1978), fundamentally changing our knowledge of the organization of life on Earth. The second is the isolation of *Thermus aquaticus* who's thermostable polymerase was integral to the development of polymerase chain reaction which lead to the molecular revolution in microbiology, allowing microbiologists to access the vast microbial diversity that is not amenable to cultivation. Richard

Castenholtz and co-workers also began studying the phototrophic algae and bacteria in hot springs in the early 1960's; studies which continue to the present. Castenholtz's group discovered anaerobic photosynthesis, rewriting our understanding of the evolution of photosynthesis (Pierson and Castenholtz 1974). It is now believed that the earliest forms of photosynthesis on Earth were anaerobic and only later evolved to produce oxygen as a byproduct (Boone et al. 2001).

Yellowstone is currently in a period of intensive research, started in the mid 1990's, that has been fueled to a large degree by the advances in molecular techniques. Examples of the insight gained from this research include expansion of known bacterial lineages (Reysenbach et al. 1994; Hugenholtz et al. 1998; Hall et al. 2008), evidence of biogeographic differentiation of microbial species (Papke et al. 2003; Whitaker et al. 2003), and, by combining molecular analysis of communities with geochemical analysis and energetic modeling, the first estimates of the relative impact of various metabolisms on thermal communities (Meyer-Dombard et al. 2005; Shock et al. 2005; Spear et al. 2005). However, only a few studies have had the goal of inventorying the diversity of microorganisms populating thermal areas. Those studies that have sought to assess the diversity of life in the thermal features have focused on only a few springs, usually in the front country of the park (Meyer-Dombard et al. 2005, Barns et al., 1994, Hugenholtz et al., 1998b, Ward et al., 1998) or have focused only on specific lineages (Boomer et al., 2002, Fishbain et al., 2003, Papke et al., 2003). The work reported here was designed to fill that gap in the knowledge of thermophiles in YNP by sampling microbial communities from across the park and across the range of conditions found. In addition to samples for molecular microbial analysis, we collected water for extensive

geochemical analysis in order to begin to deduce the microbes' role *in situ*. With this data we investigated patterns and correlations among the microbial communities, environmental geochemistry, and theoretical energy yield from 179 reactions that could be catalyzed by microbes. Yellowstone is particularly suited for this type of research because of the large number and wide variety of thermal springs that are naturally occurring chemostats enabling research into the factors that control the organisms and drive community structure.

Despite an ever-expanding database of newly discovered diversity, the application of ecological theory to microbial ecology is largely lacking (Prosser et al., 2007). Terrestrial thermal springs harbor ideal microbial communities for testing ecological theory because the diversity is low relative to soils or more temperate habitats and, owing to the inability of thermophiles to thrive at lower temperatures, a single thermal spring represents an isolated habitat. The studies that best demonstrate that microbes can be biogeographically isolated have been done in thermal environments (Takacs-Vesbach et al., 2008, Papke et al., 2003, Whitaker et al., 2003). Given that little is known about the potential of thermophiles to disperse, examination of the ecological theories such as the taxa area relationship among thermal communities may provide clues about the dispersal and extinction rates of these organisms.

Description of Study Sites

The thermal areas in YNP are the most varied and largest intact thermal areas in the world (Rodman and Maas, 2002). There are over 12,000 thermal features in YNP. We collected samples from 103 representative sites across the park, sampling from every major thermal area. The close spatial relationship of thermal areas and features combined

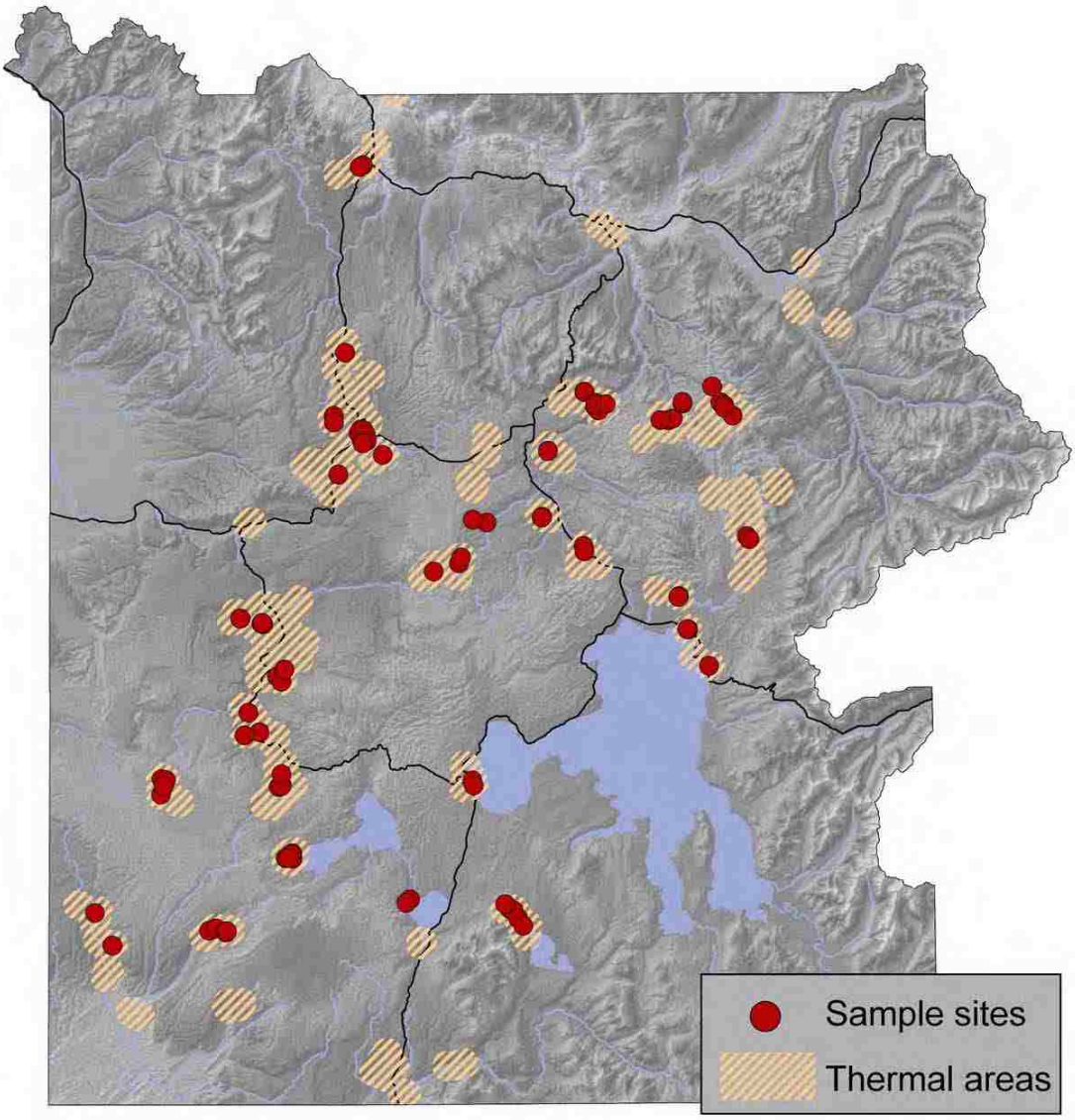

Figure 1.1 Sampling locations of the sites examined for this study.

with variable geochemistry make YNP ideally suited as a natural experiment in microbial biogeography. Each thermal feature is essentially it's own ecosystem with occasional inputs from outside the system. The samples collected span the range of temperature, pH, and geochemical conditions found in YNP (34.8-94.7°C and 1.68-9.19)

My dissertation research has focused on the study of thermophilic bacterial communities, with the goal of identifying the relative importance of geochemistry and geography in controlling community structure. Using molecular techniques, I have explored the diversity and distribution patterns of the Bacteria inhabiting these springs. In order to better understand the conditions in the hot springs, I examined both the bulk water geochemistry and thermodynamically modeled the energy available from potentially microbial mediated redox reactions. These data allowed me to address the following questions: 1) What is the structure of thermal microbial communities?, 2) What are the most significant abiotic factors controlling thermophile diversity?, 3) Does energetic modeling of reactions that are potentially metabolic help differentiate the communities?, and 4) Can established ecological theories such as taxa-area and taxa-energy relationships be used to explain the community structure of Bacteria in thermal environments?

This study is unique in the breadth of the samples collected, therefore it was important to systematically determine the best techniques for sample preservation and DNA extraction that worked for all sample types, Chapter 2. The bulk of the data generated by this project is reported in Chapter 3, with an emphasis on using this large dataset to tease apart the abiotic parameters and potential metabolic processes that impact the thermal communities. In Chapter 4, I test the taxa-area and taxa-energy relationships

in these communities. I summarize my major findings and suggest future research in Chapter 5.

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Chapter 2

A comparison of methods for total community DNA preservation and extraction from various thermal environments

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Abstract

The widespread use of molecular techniques in studying microbial communities has greatly enhanced our understanding of microbial diversity and function in the natural environment and contributed to an explosion of novel commercially viable enzymes. One of the most promising environments for detecting novel processes, enzymes, and microbial diversity is hot springs. We examined potential biases introduced by DNA preservation and extraction methods by comparing the quality, quantity, and diversity of environmental DNA samples preserved and extracted by commonly used methods. We included samples from sites representing the spectrum of environmental conditions that are found in Yellowstone National Park thermal features. Samples preserved in a non-toxic sucrose lysis buffer (SLB), along with a variation of a standard DNA extraction method using CTAB resulted in higher quality and quantity DNA than the other preservation and extraction methods tested here. Richness determined using DGGE

revealed that there was some variation within replicates of a sample, but no statistical difference among the methods. However, the sucrose lysis buffer preserved samples extracted by the CTAB method were 15-43% more diverse than the other treatments.
Keywords: DGGE, DNA extraction, environmental microbiology, thermophile

Introduction

The impact of molecular studies on our knowledge of microbial diversity cannot be overstated. As a consequence, the entire field of environmental microbiology, from basic ecological research into the organization of microbial communities to bioprospecting for commercially relevant enzymes has changed. Even with recent advances in culturing efforts (Schoenborn et al., 2004, Hobel et al., 2004), the majority of microbes in the environment still cannot be cultivated in the laboratory (Pace, 1997). However, inability to maintain an organism in culture is no longer a major impediment to accessing its genetic diversity. Metagenomic studies similar to those that have been useful in exploring the diversity of uncultivated organisms have also been used to mine for enzymatic diversity (Kowalchuk et al., 2007). The biotechnology applications that are currently targeting microbial metagenomic studies range from the search for new antibiotics to environmentally sound biocatalysts such as amylases (Lorenz and Eck, 2005, Pontes et al., 2007).

Thermal environments have been a particularly rich source of unique organisms (Hugenholtz et al., 1998, Barns et al., 1994, Ghosh et al., 2003, Harris et al., 2004, Takacs-Vesbach et al., 2008), processes (Bryant et al., 2007), novel enzymes (Hobel et al., 2004, Schoenfeld et al., 2008), and on-going research into the origin and diversity of microbes. Fundamental to any of these studies is maximizing the detectable diversity by optimizing the quality and quantity of DNA examined and minimizing the biases of the methods. There are layers of potential bias in molecular studies because of the sequential nature of the process, including those inherent in PCR (von Wintzingerode et al., 1997) which have been well studied and will not be addressed in this paper. The first potential

source of bias in the molecular study of environmental samples is determined by the method used to preserve the biomass. Cultivation based studies have shown that the groups of organisms that can be cultured from samples change drastically if the sample is not adequately preserved (Haldeman et al., 1994). However, the impact of sample preservation on molecular diversity surveys is rarely examined in the literature (except see (Harry et al., 2000)).

Another possible source of bias includes DNA extraction methods and post-extraction purification. The effect of DNA extraction method on detectable diversity has been examined in soil (Frostegard et al., 1999, Gabor et al., 2003), marine sediments (Luna et al., 2006), compost (LaMontagne et al., 2002, Yang et al., 2007), and volcanic environments (Herrera and Cockell, 2007). The results of these studies are mixed; certain methods are more effective for a particular type of sample than others. Additionally, no reports have examined the impact that the range of environmental pH extremes may have on extraction efficiency, which is of particular importance for thermal samples since they can come from springs on either end of the pH spectrum. While there are plenty of studies that examine effectiveness of extraction method on a particular type of sample, we aim to determine the method that is best for a wide variety of sample types.

Here we evaluated sample preservation and DNA extraction methods, to identify a procedure that results in high molecular weight DNA that is relatively free from contaminants and maximizes detectable diversity. We compared three preservation and four nucleic acid extraction methods. The ideal preservation and extraction method would work well with a variety of samples that included high and low biomass, a wide range of pH, as well as different sample types including microbial mats, filamentous

biofilms, and sediments. Additionally, this method should be quick to accommodate high throughput of large sample numbers and facilitate sample collection from the remote backcountry where liquid nitrogen and dry ice are not practical. We included samples from the whole spectrum of environmental conditions that are found in Yellowstone National Park, USA (YNP) thermal features and our results are applicable to future studies in YNP, other extreme environments, and microbial surveys in general.

Methods and Materials

Site description and sampling

Samples were collected from thermal features throughout YNP during the summer of 2002. A subset of fifteen samples was selected for this study that encompassed the full range of pH, temperature, and biomass types found in the park (YNP sample n=15, see Table 2.1). The pH and temperature of the samples were split into three ranges, low (pH 0 to 4, temperature 40 to 60 degrees Celsius), mid (pH 4.01 to 8, temperature 60.1 to 80), and high (pH above 8.01, temperature above 80.1). The samples were also categorized by the type of biomass collected: microbial mat, filaments, or sediment. Microbial mat samples are expected to be the highest biomass, for example, mats may contain up to 6.1 x 10^8cells cm^{-3} and are also high in pigments and extracellular proteins and polysaccharides (Brock, 1978). Filament samples represent an intermediate amount of biomass (maximum cell density in culture 4.6 x 10^7 cells ml^{-1} (Nakagawa et al., 2005)) and are generally non-pigmented. The sediment samples are expected to be the lowest biomass (2.1-3.6 x 10^6cells cm^{-3} (Meyer-Dombard et al., 2005)) and the highest clay content and heavy metal concentration (Allen and Day, 1935). Additionally, we collected samples from two neutral thermal springs in the Jemez Mountains, New

Table 2.1 Sample site, description and environmental parameters.

Sample ID	Sample Area[1]	pH	T (C°)	Sample description	Northing[2]	Easting[2]
007-L	YNP Lower GB	7.54	79.9	black powder sediment	4932385	517103
010-L	YNP Lower GB	3.55	90.6	gray clay-like sediment	4933099	515316
022-L	YNP Lower GB	6.89	85.9	black powder sediment	4933820	513269
045-L	YNP Lower GB	2.68	42.4	brown foam, water, and sediment	4934500	513403
048-L	YNP Lower GB	3.39	48.8	grey mud sediment	4934417	513990
058-L	YNP Lower GB	2.96	61.4	yellow and tan powder sediment	4953191	522871
066-MV	YNP Mud Volcano	6.41	67.4	orange mat and black filaments	4939784	544533
072-CH	YNP Crater Hills	5.47	55.6	yellow powder sediment	4952741	523490
088-L	YNP Lower GB	8.46	52.8	orange and green mat	4935485	515973
126-MM	YNP Mary Mountain	6.58	79.8	grey powder sediment	4940597	532861
131-LS	YNP Lone Star GB	4.24	43.9	yellow filaments	4916381	514106
139-LS	YNP Lone Star GB	2.49	54.8	green mat and gray sediment	4919322	515169
171-S	YNP Shoshone GB	8.63	68.7	orange and green mat	4911109	515791
184-S	YNP Shoshone GB	8.92	77.3	tan filament	4911522	515932
190-S	YNP Shoshone GB	9.08	44.5	layered orange mat	4911267	515925
BH	Jemez Bath House	7.15	76.0	yellow filaments	3959977	347240
GS	Jemez Giggling Star Resort	6.45	53.4	green mat	3959468	347235

[1]Geyser Basin is abbreviated GB. [2]Northing and Easting are given in UTM, grid 12N for YNP, 13N for Jemez Springs, NM, datum NAD83

Mexico. Samples (1-2.5 ml) were collected at each site with either sterile forceps or a syringe.

Sample preservation

Two replicate YNP samples were collected from each sampling site: one was preserved in an equal volume of sucrose lysis buffer (SLB) (20 mM EDTA, 200 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 9.0) (Giovannoni et al., 1990) and the other replicate was preserved in an equal volume of GIT (5 M guanidine isothiocyanate, 50 mM Tris pH 7.4, 25 mM EDTA pH 8, 0.8% 2-mercaptoethanol)(Cary et al., 1993). The YNP samples were held at ambient air temperature (10 to 26 ºC) for up to five days before they were stored at –80 degrees Celsius.

The two Jemez Springs, New Mexico samples were collected from neutral thermal springs located at the Jemez Springs Bath House (BH) and Giggling Star Resort (GS). Three replicate samples were collected. Two of the replicates were preserved in SLB, one of these replicates was frozen immediately in liquid nitrogen and the other was held at 20°C for 7 days before being frozen at –80°C. The third replicate sample was collected and mixed with molten 2% agarose while in the field for extraction the noodle method (see below).

Extraction methods

We used four extraction methods: lysis by pulse boil, a CTAB (hexadecyltrimethylammonium) extraction, high molecular weight DNA agarose noodle (Stein et al., 1996), and the Mo Bio Soil DNA Purification kit, which combined with the preservation methods results in six treatments. With the pulse boil method, nucleic acids were extracted from 200 μl of the YNP and Jemez samples that were preserved in

SLB (referred to here as "Boil") (Reysenbach et al., 2000). Briefly: the samples were boiled at 96°C then cooled to 4°C three times in a thermocycler, sodium dodecyl sulphate (SDS) (to a final concentration of 2%) and proteinase K (final concentration 250 μg ml^{-1}) were added and the sample was incubated at 42°C for 2.5 h, then incubated at 60°C for 30 min, extracted once with phenol/chloroform then twice with chloroform, finally the DNA was precipitated and washed with ethanol.

We extracted nucleic acids from 200 μl of the YNP and Jemez SLB preserved samples using a variation of the CTAB method (referred to as CTAB S) (Zhou et al., 1996). Briefly: 2 volumes of 1% CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 μg ml^{-1}) were added to the SLB preserved samples; incubated for one hour at 60°C, SDS (final concentration 2%) was added and incubated one hour at 60°C, extracted once with phenol/chloroform then twice with chloroform, finally the DNA was precipitated with ethanol. The YNP samples that were preserved in GIT (referred to as CTAB G) were also extracted using the CTAB method with the modification of washing the sample three times with filter sterilized water before the addition of CTAB to remove the GIT from the sample. GIT is a protein denaturant and inhibits proteinase K activity if not removed.

SLB (Mo Bio S) and GIT (Mo Bio G) preserved YNP samples were extracted using the Mo Bio Soil DNA purification kit following manufacture's suggested protocol (Mo Bio Laboratories, Inc., Solana Beach, CA).

Nucleic acids were extracted from YNP SLB preserved samples and all Jemez samples using the high molecular weight noodle extraction method (Noodle) (Stein et al., 1996). Briefly: the sample was mixed with molten 2% agarose and cooled in 1 mL

syringes forming agarose noodles, the noodles were incubated for 3 h at 37°C in a lysis buffer (10 mM Tris, 50 mM NaCl, 50 mM EDTA, 0.2% SDS, 1% Sarkosyl, 1mg/ml lysozyme) then incubated at 37°C in ESP buffer (1% Sarkosyl, 0.1 M EDTA, 1 mg/ml proteinase K), the ESP buffer was changed once a day for a total of four days, the noodles were then stored at 4°C in TE storage buffer (10 mM Tris and 50 mM EDTA). The purified nucleic acids were extracted from the agarose noodles by incubating at 60°C to melt the agarose then adding agarase and incubating overnight at 37°C. The nucleic acids were purified from the agarose/agarase slurry by extracting once with phenol/chloroform then twice with chloroform. The nucleic acids were precipitated with ethanol.

Measuring extraction success

Environmental DNAs were electrophoresed on an ethidium bromide stained 1.2% agarose gel. The size and quality (evidence of shearing etc.) of the DNA was evaluated using a size standard DNA ladder (EZ Load 1Kb, BioRad Laboratories).

The purity of the extracted DNA was quantified by calculating the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}). Nucleic acids extracted from environmental samples are often contaminated with humic organic carbons, metals, and other compounds which cause the DNA concentration calculated from 260nm absorbance to be unreliable. To circumvent this problem, we also quantified the DNA using PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Inc., Eugene, OR, USA). Extracted DNA (5 μl) was mixed with a 1:200 solution of PicoGreen in 1X tris-acetate buffer (TAE) and absorbance was read in a fluorometer.

The third parameter that was qualified was the ability to amplify 16S rRNA gene from the environmental genome of each extraction replicate. Dilutions of the genomic DNA were used as template. The reaction included (1X Promega buffer with 1.5 mM $MgCl_2$, bovine serum albumin (0.04 % final), 2.5 U Taq DNA polymerase (Promega U.S.) 2.5% Igepal CA-630 (Sigma-Aldrich), 10 μM each dATP, dGTP, dCTP, dTTP (BioLine USA, Inc.), 20 μM Bacterial specific primers (Lane, 1991) 338FGC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGC AGCAG) and 519R (ATTACCGCGGCTGCTGG)). The PCR reaction (50 μl) was incubated in a thermocycler (ABI GeneAmp 2700) for 5 min at 94.0°C then for 30 cycles of 30 s at 94.0°C, 30 s at 50.0°C and 30 seconds at 72.0°C. The reaction was incubated at 72.0°C for 7 min for final extension.

Richness

Differences in relative species richness among the preservation and extraction methods were determined using denaturing gradient gel electrophoresis (DGGE). Ten μl of PCR product (approximately 500 ng) was run on a 6% (wt/vol) acrylamide gel with 1X TAE (40 mM Tris, 20 mM acetate, and 1mM EDTA) with a denaturing gradient of 20% (8% (vol/vol) formamide and 8.4% (wt/vol) urea) to 60% (24% formamide and 25.2% urea). The gels were run in a BioRad DCode Universal Mutation Detection System (BioRad Laboratories, Hercules, CA) at 180V for 3.5 h. The gels were stained in 1X TAE containing SybrGreen (100 μl L^{-1}) for 30 min then destained in 1X TAE for 15 min. The gels were photographed under UV light and DGGE bands were identified and analyzed using Kodak 1D software. For our analyses, only bands with a minimum intensity of 72% were recognized (program default). Bands were distinguished based on

migration distance within each gel, as determined by the software using the DGGE product from *Escherichia coli* as a standard (Fromin et al., 2002). Although re-amplification and sequencing of individual DGGE bands can be particularly important in resolving heteroduplex fragments, we did not sequence the bands we detected in this study because we were interested in detecting the greatest number of bands possible. The effectiveness of methods within each sample was calculated by dividing the number of bands detected within a treatment by the total number of unique bands found within a sample. This calculation of relative species richness was computed for samples where at least 2 of the treatments resulted in successful amplification (YNP samples n=12).

Statistical analysis

Statistical analyses were performed using Minitab software version 13 and SPSS 11 (for Mac OS X). The purity of the DNA among treatments was compared using the absolute value that resulted from subtracting the A_{260}/A_{280} from 1.8, the $A260/A_{280}$ of pure DNA (Sambrook et al., 1989). Univariate ANOVA was used to detect statistical differences in the quantity and purity of the DNA and relative species richness (dependent variables) among the treatments and by pH, temperature, and biomass type (fixed factors). For analysis of the DGGE bands, only samples where at least 2 of the treatments amplified were included, otherwise a statistical comparison would not be possible. The Bonferroni test (which is more powerful for small sample sizes than Tukey's test) was performed to identify treatments that were significantly different. Rarefaction curves were calculated in EstimateS version 8.0.0.

RESULTS

Sample preservation

We extracted more DNA from the samples preserved in SLB than from the GIT replicates (paired t-test p=0.01). The extracted DNAs were quantified using the PicoGreen reagent. Using our protocol, the PicoGreen assay has a dsDNA detection limit of 500 pg/ml. We also compared the amount of DNA recovered from the Jemez samples that were frozen or mixed with agarose in the field with those that were held at ambient temperature; there was no statistical difference between the two replicates (paired t-test α=0.05). Even when samples cannot be frozen immediately, the DNA is adequately preserved in SLB.

There was no significant difference among the preservation methods in the richness we detected (paired t-test α=0.05). However, the 16S rRNA gene from the samples that were preserved in SLB were amplified by PCR more successfully; 80.5% of SLB preserved samples PCR amplified compared to just 50% of the GIT preserved samples.

DNA extraction

The CTAB extraction on SLB preserved samples resulted in the greatest yield of DNA. The results of the quantification are displayed in Fig. 2.1a. ANOVA showed a statistically significant difference in DNA yield among the extraction methods ($p<0.001$). Post-hoc multiple comparison analysis indicates that the CTAB S extracted samples resulted in the highest quantity of DNA.

DNA purity, as measured by A_{260}/A_{280} ratio, was greatest in samples extracted using the Mo Bio extraction kits, however the kit results in less DNA and smaller fragments (presumably from shearing and the filter column used in the kit (Braid et al., 1999)) (Fig. 2.1b). There was no significant difference in the purity of the DNA extracted with the Boil, CTAB S, or CTAB G methods (ANOVA α=0.05). The Noodle method did not result in sufficient DNA quantity to obtain reliable absorbance data, therefore it was not included in the DNA purity comparison. Most of the amplification success we observed was in sample extracted with either the CTAB method (89% amplified) or the Mo Bio kit (95% amplified) (ANOVA and Bonferroni test $p<0.001$).

The sequence diversity detected by DGGE, number of bands, varied within the replicates of the samples (Fig. 2.2). The only statistically significant difference we detected was between the CTAB S and the noodle method ($p = 0.014$). However, CTAB S resulted in 15% to 43% more bands than the other treatments (Fig, 2.3). There were no differences at the fixed effects level (pH, temperature, or biomass type), nor were any interactions (e.g., pH X method) among the factors detected. This suggests that CTAB S is appropriate for a wide range of sample types.

DISCUSSION

Sample preservation

SLB preserved our extreme environment samples better than the GIT solution. This was likely due to more effective lysing, as the SLB initiates the lysis process while the sample is being stored. Additionally, the alkaline SLB raised the pH within the sample vials, potentially slowing the degradation of the DNA in low pH samples. Raising the pH of acidic samples is likely to have two positive impacts on preserving the

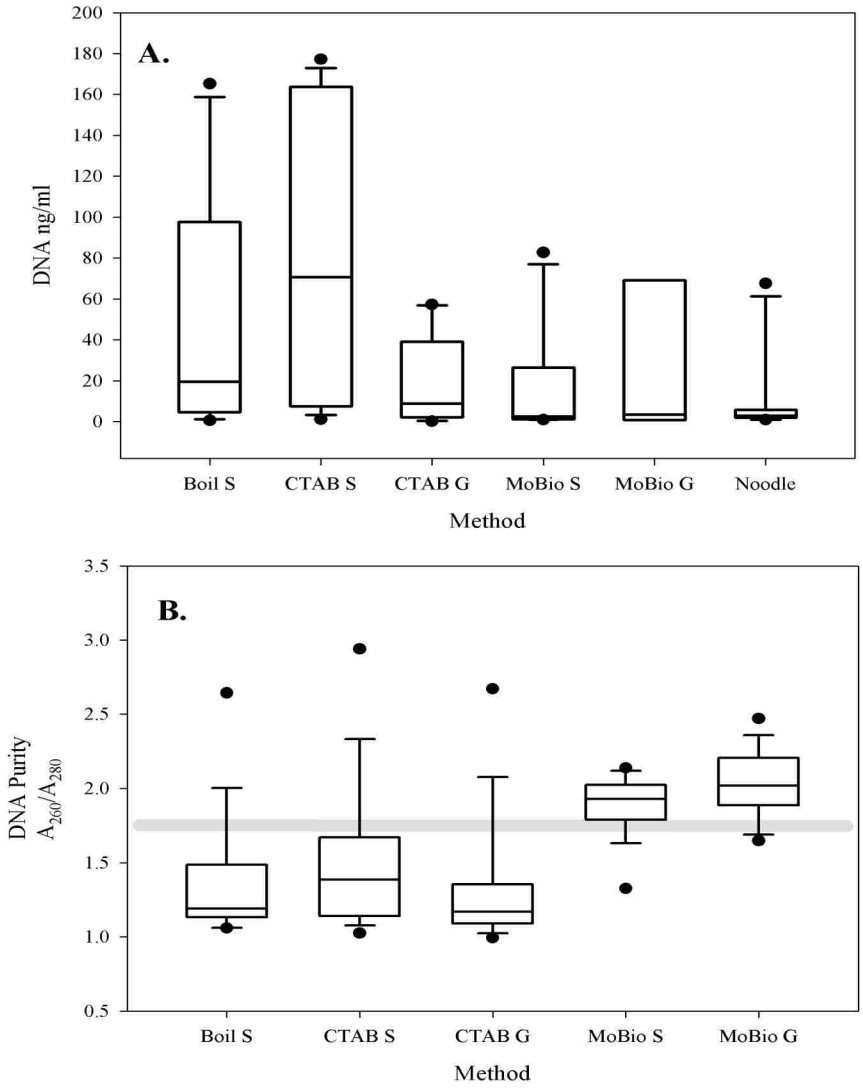

Fig. 2.1 a) Mean DNA concentration as measured using PicoGreen assay for each preservation and extraction method with 95% confidence intervals, outliers are filled circles. b) Mean DNA purity with 95% confidence interval and outliers. Pure DNA has a A_{260}/A_{280} ratio of 1.8, shown as the gray bar. The concentrations of the DNAs from the Noodle extractions were not high enough to be detected by the florometer.

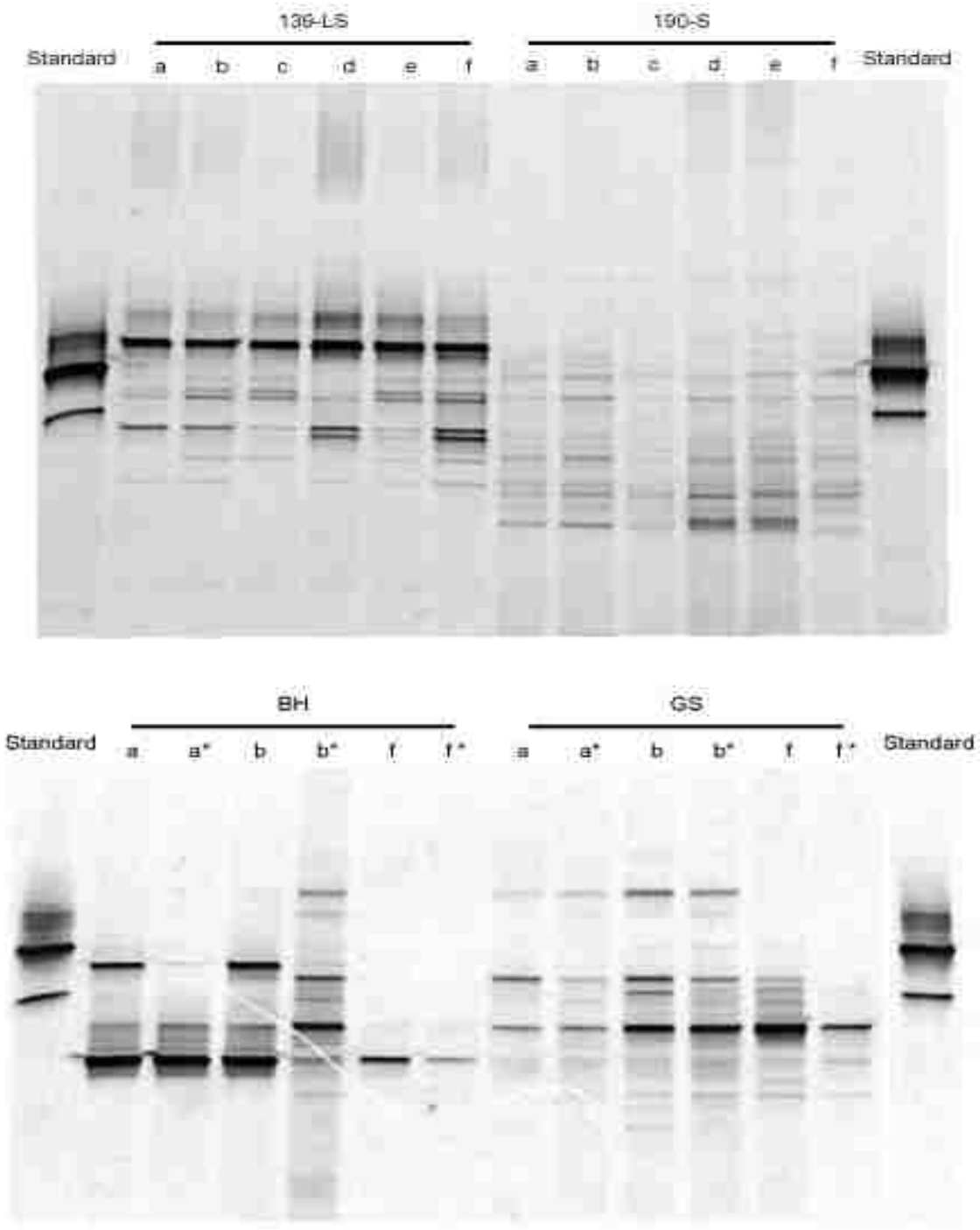

Fig. 2.2 Example DGGE of PCR products on 6% acrylamide gel with 20%-60% urea/formamide denaturing gradient, stained with SybrGreen [Treatments: a) Boil; b) CTAB S; c) CTAB G; d) Mo Bio S; e) Mo Bio G; f) Noodle].

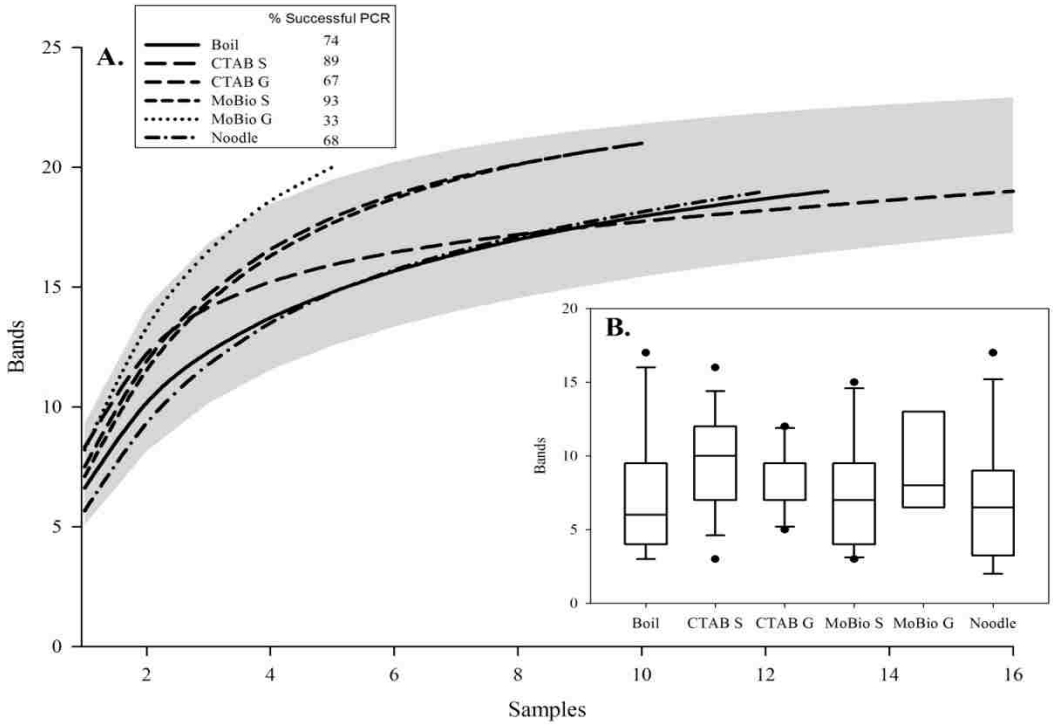

Fig. 2.3 a) Rarefaction curves of DGGE bands detected with each extraction method. The 95% confidence interval for the diversity of all samples and extraction methods combined is in gray to demonstrate that there was no significant difference in diversity detected by each method. b) Mean diversity detected as number of DGGE bands with 95% confidence intervals, outliers are filled circles.

DNA in those samples. First, DNA degrades in acidic solutions due to depurination (Garrett and Grisham, 1995), raising the pH of the solution reduces the rate of this reaction. Second, DNA binding to clay minerals increases as the pH decreases (Khanna and Stotzky, 1992). Even when samples could not be frozen immediately, the DNA is adequately preserved in SLB. This result is especially important because of restrictions on carrying and shipping dry ice and for samples that are collected from remote areas where it is logistically very difficult to freeze samples immediately and maintain them frozen. The non-toxic nature of SLB may be an additional attraction for field microbiologists, and for those shipping samples either commercially or hand carrying on airplanes. Although this study did not assess the relative effectiveness of the different methods in extracting RNA, we have successfully produced cDNA from samples preserved in SLB using reverse transcriptase PCR (unpublished data).

Previous studies have suggested that the Noodle method, for very high molecular weight DNA (Stein et al., 1996), and storage in absolute ethanol, for samples where immediate freezing is not possible (Harry et al., 2000), adequately preserve DNA. However, based on the results from this study, the noodle preservation method does not work well with the samples we collect which often have low biomass in a dense extracellular matrix. In many samples, the concentration of DNA recovered using the Noodle method was an order of magnitude less than recovered using the CTAB protocol. Similarly, we did not test ethanol preservation because the ethanol must be nearly absolute to prevent DNA degradation (Frantzen et al., 1998, Mandrioli et al., 2006) which would not be possible for samples that contain significant amounts of water. Therefore,

we do not think that either the Noodle method or absolute ethanol preservation are appropriate for thermal environmental samples.

DNA extraction efficiency and purity

When considering preservation alone, among the techniques we tested, SLB with or without freezing preserved environmental DNA best. However, we also were interested in the effect of extraction method on the amount and quality of extracted DNA and detectable diversity. Extracting pure DNA from environmental samples is nearly as important as extraction efficiency and is one of the most difficult technical problems in using molecular techniques on environmental samples. Most DNA extraction procedures co-extract humic organic carbon, pigments, heavy metals, and other contaminants. These contaminants play havoc with PCR reactions and can degrade the DNA during storage. We found that CTAB extraction combined with SLB preserved samples resulted in the most DNA. The greater efficiency of the CTAB extraction method has previously been found in several environments, marine sediments (Luna et al., 2006), basalt rock (Herrera and Cockell, 2007), and by one of us in caterpillar intestinal tracts and filtered water (unpublished data) indicating that this method is effective on a wide range of sample types. An extraction comparison on compost microbial communities found no difference in the amount of DNA recovered by the CTAB method, but it resulted in the highest percentage of cells lysed (Yang et al., 2007). It is likely that the combination of proteinase K and hot SDS lyses more cells, including cell types more resistant to lysis (e.g., gram-positive bacteria)(More et al., 1994). We found two exceptions where larger quantities of DNA were not recovered by the CTAB method. The PicoGreen analysis on two samples, 066-MV and 139-LS, indicates that more DNA was extracted using the Mo

Bio kit than was extracted with the Boil and CTAB methods. The Boil and CTAB extracted DNA from these two samples was dark brown, which was most likely caused by pigments that were co-extracted with the DNA. The Mo Bio kit, which produced clear DNA extracts, was effective at removing these organics because it is the only method that incorporated a post-extraction purification (Braid et al., 1999). Work on basalt rock samples found that only Mo Bio extracted DNA could be amplified (Herrera and Cockell, 2007), which is contrary to our results. We suspect that the co-extracted pigments interfered with PicoGreen fluorescence, but both of these samples were successfully amplified with all of the extraction methods. The co-extracted pigments from the Boil and CTAB methods do not appear to inhibit the PCR reaction. It should be noted that some of the samples with no detectable DNA, as determined using the PicoGreen fluorometer quantification, still had enough DNA to amplify with PCR (theoretical detection limit for PCR amplification < 1 pg/ml(Steffan and Atlas, 1998)).

Richness

Although the CTAB S treatments consistently had more bands than any of the other methods (15-43%), the only statistical difference detected was between the CTAB S and the noodle extraction method. We also tested for fixed effects such as pH, temperature, and biomass type, but did not detect any statistical differences. However, there were three samples that we were unable to amplify using two or more of the extraction treatments, those samples could not be included in the richness analyses. It is interesting to note that all three of these samples were sediments from low pH systems (<pH 3.5), indicating that there may be a sample type or geochemistry effect on extraction efficiency or amplification success. This result could have been due to

inefficient extraction of DNA, co-extraction of PCR inhibitors, or too little target DNA in the original sample (i.e. if the community was dominated by Archaea to the exclusion of Bacteria). For those types of extremely recalcitrant samples, it may be necessary to crush the sample using a sterile mortar before the enzymatic lysis step (Herrera and Cockell, 2007). However, CTAB worked on a majority of the samples and we suggest it should be used as a good general extraction method. If unsuccessful, alternate approaches should be used that may more effectively lyse the biomass or better remove humics.

There are many methods for assessing microbial community richness, generally based on the 16S rRNA gene, such as clone libraries, DGGE, and T-RFLP (terminal restriction fragment length polymorphisms). These analyses have greatly increased our understanding of microbial ecology and revealed novel enzymes. DGGE is an attractive method for microbial ecology studies because of the relatively rapid ease with which a community profile can be generated. DGGE can resolve minor base differences among samples; therefore, distinct bands may represent sequences that differ by less than 1% (Moeseneder et al., 1999). Depending on the nature of the study being performed, 1% may or may not be significant to the research question. Conversely, we are aware that individual bands could be heteroduplexes (Speksnijder et al., 2001, Ferris and Ward, 1997), comprised of several different sequences, however, because our methods were standardized across samples, this effect does not figure in our conclusions. We repeated the PCR amplification and DGGE for selected samples to ensure reproducibility in the number of bands detected (data not shown, but available on request).

It is standard practice that investigators informally evaluate methods to determine which are appropriate for their study. However, few of these studies have been published

(Gabor et al., 2003) and often, statistical analyses are not used to determine the most appropriate methods (Fromin et al., 2002, Herrera and Cockell, 2007). Additionally, other published studies did not evaluate the effect of extraction method on the diversity detected, only the quantity of DNA (Miller et al., 1999, Leuko et al., 2008). This study presents a framework for the systematic comparison and evaluation of methods by statistical analysis. The current interest in the organisms that inhabit extreme environments makes this study, which used samples from a wide variety of thermal environments, of importance to thermal biology researchers, and applicable to other microbial studies in general.

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Chapter 3

Exploration of Thermal Microbial Community Ecology through analysis of Bacterial Diversity, Geochemistry, and Metabolic Energetic Modeling

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Abstract

Molecular surveys have revealed hidden phylogenetic and metabolic diversity in many microbial habitats, indicating that much still remains to be discovered. We have conducted a microbial survey of Yellowstone thermal environments, sampling 103 communities representing the full range of areas and conditions found in the park. In addition we also collected water for geochemical analysis in order to begin to deduce the microbes' role *in situ*. Prior to this work it was believed that temperature was the driver of microbial diversity in thermal communities; we have shown that pH is the most important factor controlling thermophile distribution. Additionally, sequences from 15 candidate phyla were discovered from multiple springs. With this data we investigated patterns and correlations among the microbial communities, environmental geochemistry, and theoretical energy yield from 179 reactions that could be catalyzed by microbes. Five major groupings of community types were found through MRPP analysis that can be described by the dominant organisms; *Thermocrinis/Thermus*, phototrophs,

Sulfurihydrogenibium, *Hydrogenobaculum*, and Proteobacteria/Bacteriodetes. The last group has not previously been noted to be an important community type in thermal areas. The Proteobacteria/Bacteriodetes group is also interesting because it seems to thrive in the harshest conditions measured, low pH and high concentrations of metals. The ecosystems described in this study are ideal for further application of ecological theory, especially community assembly patterns, biogeographic theory, and macroecological experiments that take advantage of the high diversity of habitats and short generation time of thermal communities.

Introduction

Microorganisms account for most of the evolutionary diversity on Earth. However, until recently, knowledge of the extent of this diversity was limited to the organisms that are capable of growing in isolation in the laboratory. Through the now widespread use of molecular techniques, it is apparent that less than 1% of microbes in the environment can be cultivated using current techniques (Pace 1997; Schoenborn et al. 2004). Community wide molecular surveys have revealed hidden phylogenetic and metabolic diversity in microbial habitats (Barns et al. 1994; Ferris and Ward 1997; Hugenholtz et al. 1998a; Casamayor et al. 2002; Ghosh et al. 2003; Giovannoni 2004; Sogin et al. 2006). The known diversity of microbes has significantly expanded in the past 20 years. In 1987, there were 11 described phyla of Bacteria (Woese 1987) while today 52 phyla have been described and 26 of those are only known through gene surveys (Rappe and Giovannoni 2003). The diversity of the microbial world is one of the great unknowns left to be explored on Earth.

Just as the phylogenetic diversity of extreme environments is largely unknown, so is the metabolic diversity. Recent discoveries include two novel organisms, an anaerobic phototroph from Mono Lake that uses arsenite as the electron donor (Kulp et al. 2008) and an aerobic phototroph from Octopus spring (Bryant et al. 2007). The arsenite phototroph was discovered and cultivated using traditional microbiological tools. Scientists noticed an interesting community in the environment and from that observation worked to isolate the organisms responsible. The novel aerobic phototroph was first discovered through metagenomic analysis from one of the most extensively studied hot springs in the world (Ward et al. 1998), then cultivated and characterized. A third

approach that may be fruitful in discovering novel metabolisms is to search not for the organisms directly but to use thermodynamic calculations to determine which metabolic reactions yield useful energy in a particular environment (Amend and Shock 2001, Spear 2005, Meyer-Dombard 2005). This approach is applicable to many habitats. Cultivation based discovery is limited to the organisms that are being targeted while metagenomic sequencing is still too expensive to be commonly used. Results from thermodynamic modeling of the metabolisms in the environment can then be used to guide culturing efforts.

The organisms that inhabit the thermal features of Yellowstone National Park (YNP) have been studied throughout the history of the park (Wondrak Biel 2004). Previous surveys have focused on only a few springs, usually in the front country of the park (Barns et al. 1994; Hugenholtz et al. 1998b; Ward et al. 1998) or have focused only on specific lineages (Boomer et al. 2002; Fishbain et al. 2003; Papke et al. 2003). The work reported here was designed to form a baseline inventory of thermophiles in YNP by sampling microbial communities from across the park. In order to begin to deduce the microbes' role *in situ,* we collected water for extensive geochemical analysis. With these data we investigated patterns and correlations among the microbial communities and environmental geochemistry, by calculating theoretical energy yield from 179 reactions that could be catalyzed by microbes.

Methods

Site description and sampling

Samples were collected from thermal features throughout Yellowstone National Park, USA during the summers of 2003 and 2004. One hundred and three samples were

analyzed that encompassed the full range of pH, temperature, and biomass types found in the park. Two to five samples were collected from each major thermal area; sampling locations were chosen to reflect the range of temperature and pH found in the area. Temperature and pH were measured using a Thermo Orion 290A+ meter, electrical conductivity was measured with a WTW meter with temperature correction, and site locations were recorded using a Trimble GeoXM and digital photographs. Hydrogen sulfide was measured in the field using the methylene blue method (Hach method 8131) and a hand held colorimeter (Hach DR/850). The samples were also categorized by the type of biomass collected: photosynthetic mat, chemotrophic filaments, sediment, or water. Approximately 1-2.5 mL of sample was collected at each site with either sterile forceps or syringe. For water samples, biomass was collected by filtering 0.6-1 L water through a 0.02 μm filter (Millipore) then preserving the filter. Previously, we determined the most effective sample preservation and DNA extraction procedures to maximize the quality of DNA extracted and the diversity detected in the samples (Mitchell and Takacs-Vesbach 2008). Samples were collected from each spring and preserved in an equal volume of sucrose lysis buffer (SLB) (20 mM EDTA, 200 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 9.0) (Giovannoni et al. 1990). Samples were held at ambient temperature for up to eight days before they were stored at –80 ºC.

Water for geochemical analysis was filtered (0.2 μm), unless otherwise stated, and preserved as appropriate for the analysis to be performed (McCleskey et al. 2004). Briefly, water (125 mL) was collected into deionized water washed bottles for anion measurement. Water for cation analysis (30 mL) was collected in acid washed bottles and preserved by acidification with 0.3 mL 3N nitric acid. Water for Fe and As species

was collected into opaque acid washed bottles and preserved with 1 mL of 6N HCl. For SiO_2, one mL of water was immediately diluted with 9 mL deionized water to prevent precipitation. Water (30 mL) was preserved for ammonium analysis by the addition of 0.3 ml of 4.5N H_2SO_4. For sulfate, thiosulfate, and thiocyanate, 30 mL of water was preserved with 0.5 mL $ZnCl_2$, followed by 0.5 mL NaOH. The thiocyanate bottle also received 0.3 mL of KCN. The geochemical analyses were performed according to USGS standard procedures (McCleskey et al. 2004).

DNA Extraction

DNA was extracted using a modified CTAB extraction (Zhou et al. 1996). Briefly: 2 volumes of 1% CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 μg/mL) were added to the SLB preserved samples which were then incubated for one hour at 60°C. Sodium dodecyl sulphate (SDS) (final concentration 2%) was added to the samples and incubated for one hour at 60°C. The DNA was then extracted with chloroform and finally, precipitated with ethanol.

Dilutions of the environmental genomic DNA were used as template DNA for PCR amplification of the 16S rRNA gene. The reaction included 1X Promega buffer with 1.5 mM $MgCl_2$, bovine serum albumin (0.04 % final), 2.5 U Taq DNA polymerase (Promega U.S.) 2.5% Igepal CA-630 (Sigma-Aldrich), 10 μM each dATP, dGTP, dCTP, dTTP (BioLine USA, Inc.), 20 μM Bacterial specific primers 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACACTT) or 1391R (GACGGGCGGTGTGTRCA). The PCR reaction (50 μl) was incubated in a thermocycler (ABI GeneAmp 2700) for 5 min at 94.0°C then for 30 cycles of 30 s at

94.0°C, 30 s at 50.0°C and 30 s at 72.0°C. The reaction was incubated at 72.0°C for 7 minutes for final extension. The PCR products were ligated and cloned using the TOPO TA pCR2.1 kit (Invitrogen).

Sequence Analysis

Ninety-six clones were analyzed for each library by either restriction fragment length polymorphism (RFLP) (n=18) or by sequencing each clone from the 5' end (n=66). For libraries screened by RFLP, unique clones were fully sequenced as described below. Sequences with a PHRED score below 20 and less than 300 bases long were not included in subsequent analysis. Sequences were aligned using Greengenes (DeSantis et al. 2006), ensuring that only likely bacterial sequences were included in the analysis. A distance matrix of the aligned sequences was calculated in ARB (Ludwig et al. 2003). Dotur (Schloss et al. 2003) analysis on the distance matrix was used to determine 1, 2, 6, and 15% divergent operational taxonomic units (OTUs) and to calculate Chao1 estimated diversity index. A presence/absence by sample matrix was constructed for each of the OTU levels, which was used for the statistical analyses. Estimation of the shared richness between sample groups was calculated in SONS (Schloss and Handelsman 2006).

Sequence identification was based on BLAST against the nr database at NCBI and the Greengenes classify tool using the Simrank algorithm. Phylogenetic trees were built using the sequences generated by this study and a backbone of 400 high quality reference sequences spanning the range of bacterial diversity downloaded from Greengenes (July 2008). The sequences were all aligned using the NAST aligner in Greengenes. The whole dataset tree (5943 sequences from this study plus the 400

reference sequences) was constructed using the parsimony interactive add tool in ARB. For the phylogenetic tree presented here only the reference sequences were complete gene sequences, however simulations have shown that accuracy in trees based on 1000 characters remains high as long as the missing data is less than 80% of the total characters (Weins 2003).

Energetic Modeling

Total energy available to the communities was modeled based on the activities of chemical species and compounds measured from the environment (Appendix Table A1) as described in (Amend and Shock 2001; Amend et al. 2003; Shock et al. 2005). The raw geochemical concentrations were speciated using the EQ3 program (Lawrence Livermore National Laboratories). The activities from this program were used to calculate the Gibbs free energy available in each spring for 179 reactions that may be mediated by microorganisms. This calculation estimates the amount of available energy per mole of electron transferred. We then multiplied this value by the modeled concentration of the limiting reactant for each reaction, resulting in determination of energy per kg of water that allows for the comparison of total energy available to each spring rather than comparison of individual reactions.

Statistical Analysis

Statistical analyses, other than liner regressions, were performed using PC-ORD v 5 (McCune et al. 2002). The samples were grouped using polythetic agglomerative hierarchical cluster analysis (Goodall 1973) based on the 4 OTU level matrices (1, 2, 6, and 15% divergent) with rare OTUs that were only found in one sample removed for this analysis. The number of clusters and clustering level were tested using multiple response

permutation procedure (MRPP) (Mielke 1984). The best fitting clustering scheme was used to classify the samples for further analyses. Indicator analysis (Dufrene and Legendre 1997) was performed on the OTU presence absence matrices, geochemical matrix, and energetic matrices to determine which OTUs, chemistries, or potential metabolic reactions defined the groups of samples. Nonmetric multidimensional scaling (Peterson and McCune 2001) was used to visualize the groups of samples based on OTU, geochemical, and energetic matrices. The importance of the two main drivers, pH and temperature, on the community structure was examined by Mantel test (Mantel 1967) of the phyla level NMS. Linear regression of the data and of NMS values were done in Excel (Microsoft Office 2007).

Results

The samples collected represent the range of conditions found in YNP hot springs (Figure 3.1). YNP spring water show a bimodal distribution of pH, with few springs having a pH between 4-5.5. The samples analyzed for this study show the same bimodal distribution. Earlier workers in thermal environments (e.g., Brock 1978) recognized four sample types: phototrophic mats, chemotrophic filaments, sediment and filtered water.

Sample type	Number of Samples	Temperature Range	pH Range
Phototrophic mat	20	51.7°-70.5°C	2.64-9.19
Chemotrophic filaments	24	50.9°-84.8°C	2.20-9.06
Sediment	21	36.7°-87.8°C	1.94-8.96
Water	14	34.8°-94.7°C	1.68-8.84

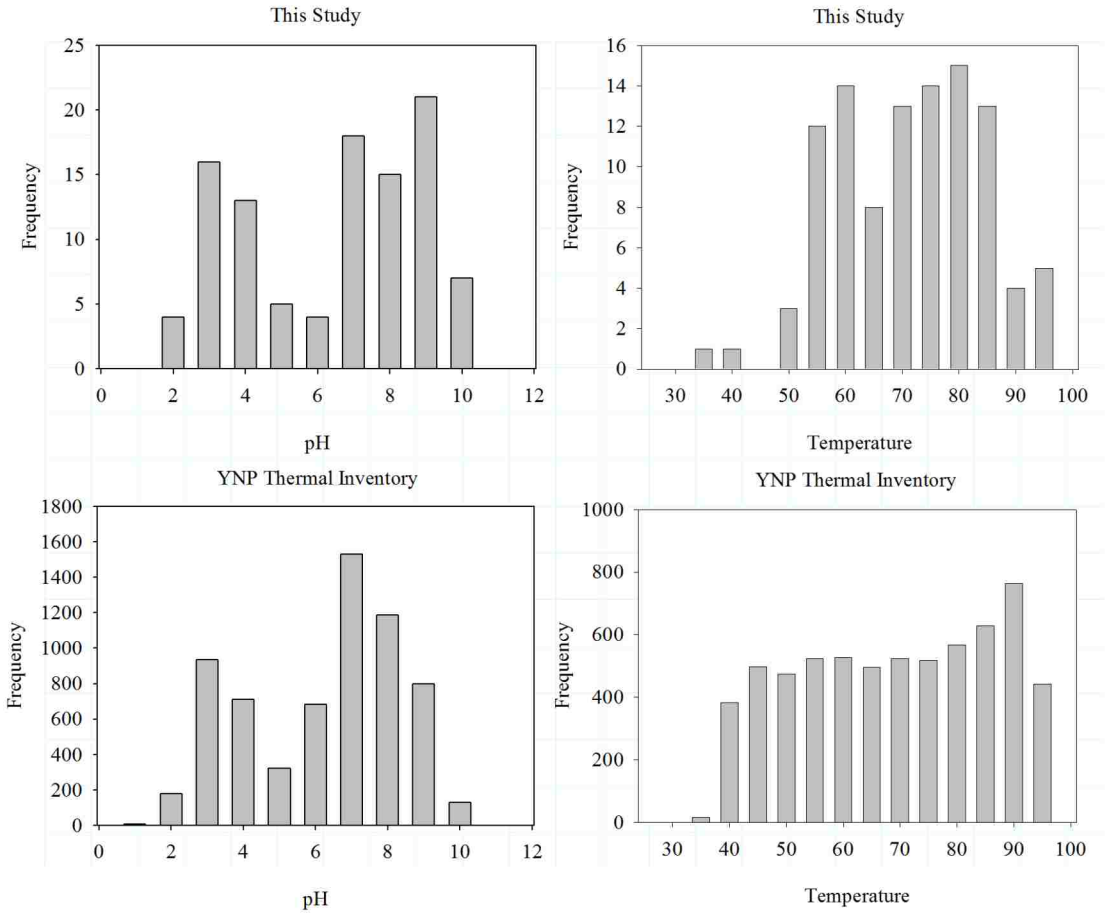


Figure 3.1. Comparison of the distribution of pH and temperature in samples collected for this study and in the YNP Thermal Inventory (unpublished, Ann Rodman).

We recovered bacterial 16S rRNA from 84 of the samples. The majority of the samples that we couldn't amplify bacterial 16S from were either sediment or water samples that likely have much lower biomass than the filament or mat samples. The energetic modeling was performed on all samples; however all other analyses were only performed on the 84 samples from which we were able to obtain bacterial sequences.

Extent of diversity

We amplified bacterial 16S rRNA from 84 of the 103 samples collected and RFLP screened or forward sequenced over 7000 clones. The sequences (N=5943) that met the minimum criteria (at least 300 bases long with phrap scores > 20) were included in the phylogenetic and community analyses. The diversity of the sequences detected is summarized both by numbers of clones analyzed and how those clones are distributed among the 1018 species level OTUs (2% divergence). Aquificales were the most commonly detected organisms with over 2200 clones. However, at the species level OTUs, there were more Proteobacteria, with 199 Proteobacteria OTU compared to 139 Aquificales OTU. Three percent of the clones (n=221) detected could not be identified (using either BLAST on the nr database NCBI or Simrank in Greengenes) even to the phyla level. Some of these unidentified sequences likely belong to previously described candidate divisions AD3 (Zhou et. al. 2003), OP1, OP5, OP8, OP9, OP12 (Hugenholtz et al. 1998b; Harris et al. 2004), or Toll (Hall et al. 2008). The other 30 OTUs, representing 174 clones, are more than 15% divergent from any described division although most of these sequences have at least a few closely related sequences present in GenBank from previous molecular surveys. These highly divergent sequences may represent up to 15 novel candidate phyla (Figure 3.2).

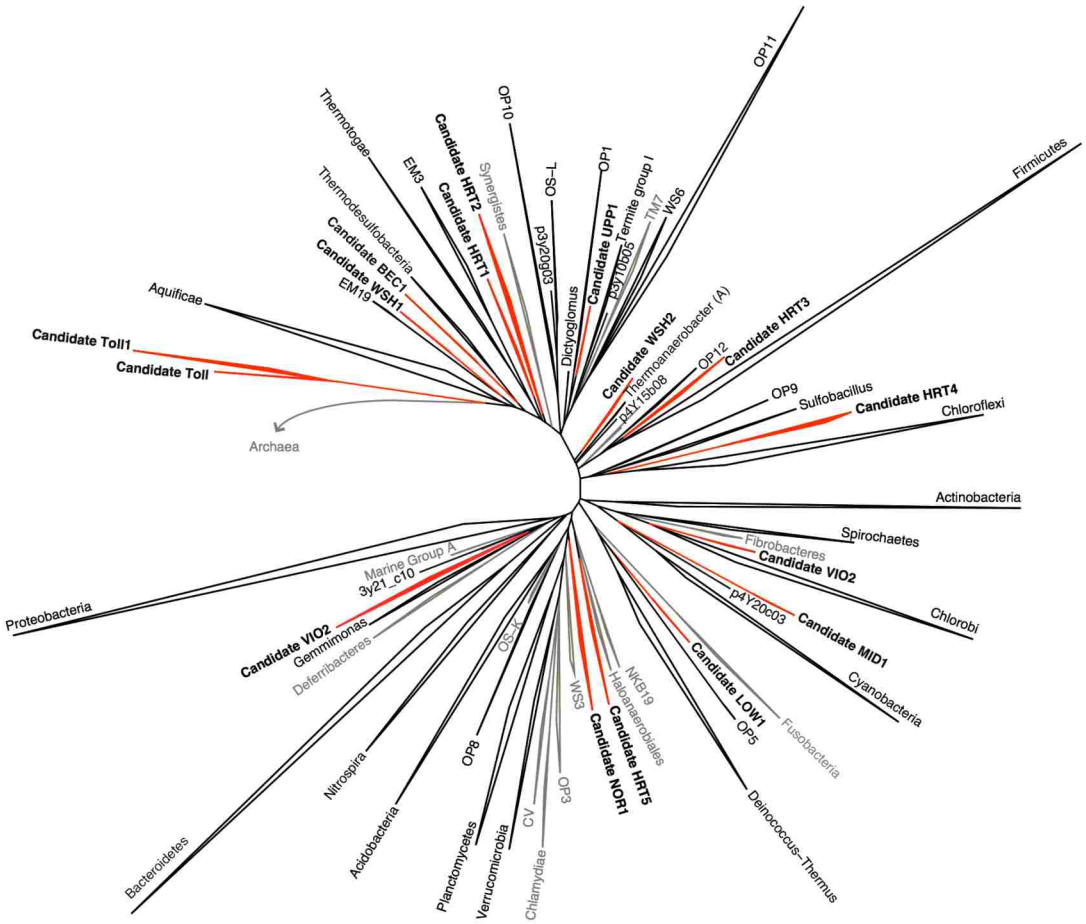


Figure 3.2. Phylogenetic tree of 5943 sequences produced by this study along with reference sequences. All sequences within a phylum were collapsed into a triangle, the width of the triangle is proportional to the number of sequences in that phyla, while the length is proportional to the evolutionary diversity contained in the phyla. All phyla that are black were detected in these samples, grey are phyla that were not found, and the putative candidate phyla discovered by this work are red and bolded.

Geochemistry

The samples studied represent the range of geochemical conditions found in YNP, not just in terms of the pH and temperature but also for major anions and cations, trace metals, nutrients, and stable isotopes (Truesdell and Fournier 1976; McCleskey et al. 2004; Meyer-Dombard et al. 2005; Nordstrom et al. 2005; Shanks et al. 2005), (Table A1). The waters are generally dominated by Na^+ and either Cl^- or SO_4^-, with some evidence for mixing trends (Figure 3.3). Geothermal water in YNP generally falls into two major types, acid sulfate or alkaline chloride. The 103 samples collected span most of the range of sulfate (7.7 – 3172 mg/l) and chloride (<1 – 642 mg/l) concentrations reported by other workers (McCleskey et al. 2004; Meyer-Dombard et al. 2005). The acid sulfate waters likely represent vapor dominated springs where meteoric or shallow ground water is heated by steam instead of heated by mixing with the deep hot aquifer (Fournier 2005). Alkaline chloride waters, hot-water dominated regions, are common in the western half of YNP and are generally in topographic lows. The source of the water in a sample can also be described using stable isotopes, specifically δD compared to either $\delta^{18}O$ or Cl^- (Figure 3.4 and 3.5 respectively). The isotopes also confirm that the samples represent both meteoric (i.e. vapor dominated) and at least some mixing with the deep hot aquifer (hot-water dominated).

Energetic Modeling

We evaluated the amount of energy available in each spring for 179 reactions under varying O_2 (0.1, 0.5, 3, and 6 mg/l) and H_2 (2, 10, 100, 325 nM) concentrations. When these reactions were evaluated by chemical affinity, kJ per mole of electron

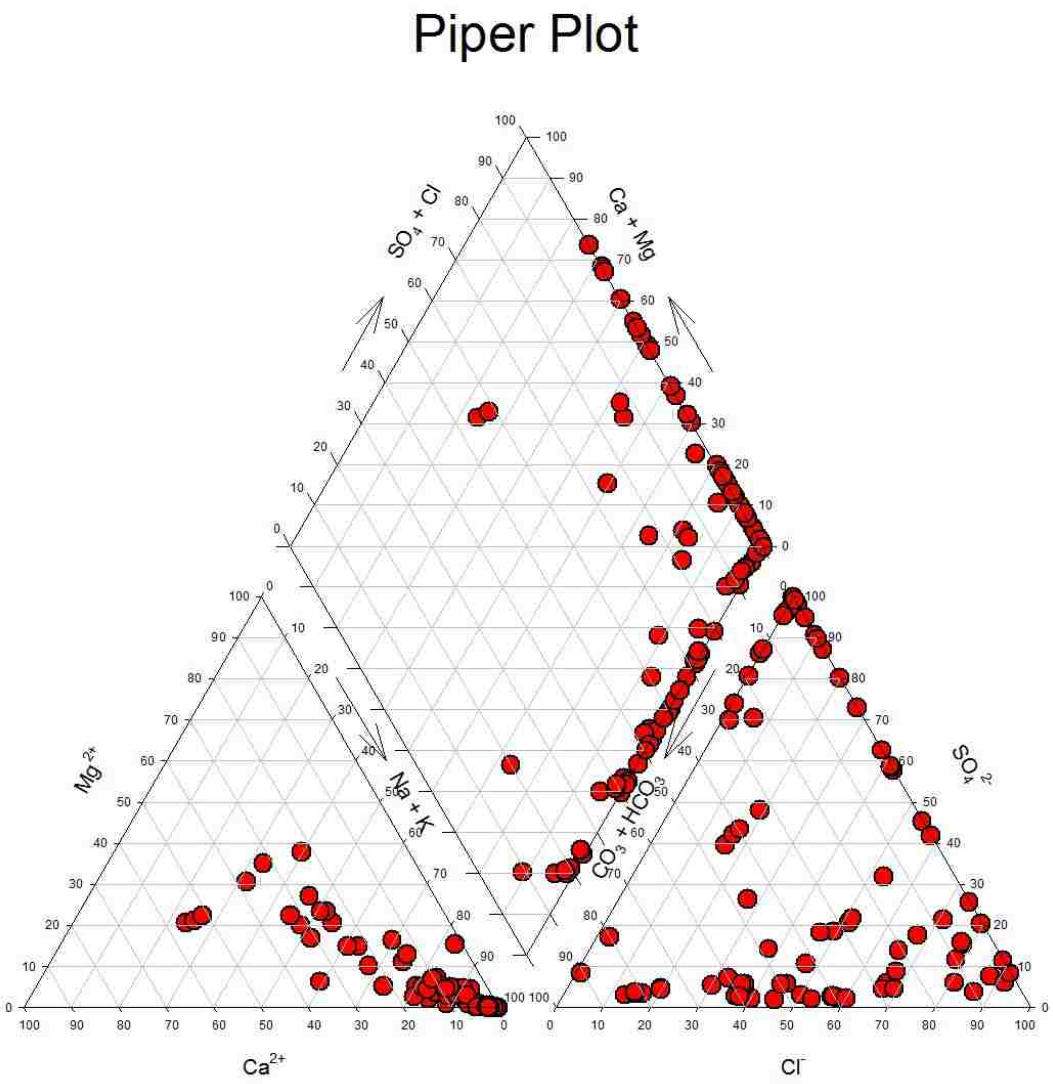


Figure 3.3 Piper diagram of the major anions (right ternary plot) and cations (left ternary plot). Both anions and cations in the lower triangles are projected up onto the diamond. The waters from these samples are usually dominated by a single anion cation pair (falling on the edges of the plots). A few of the samples are mixtures of waters which plot towards the center of the graphs.

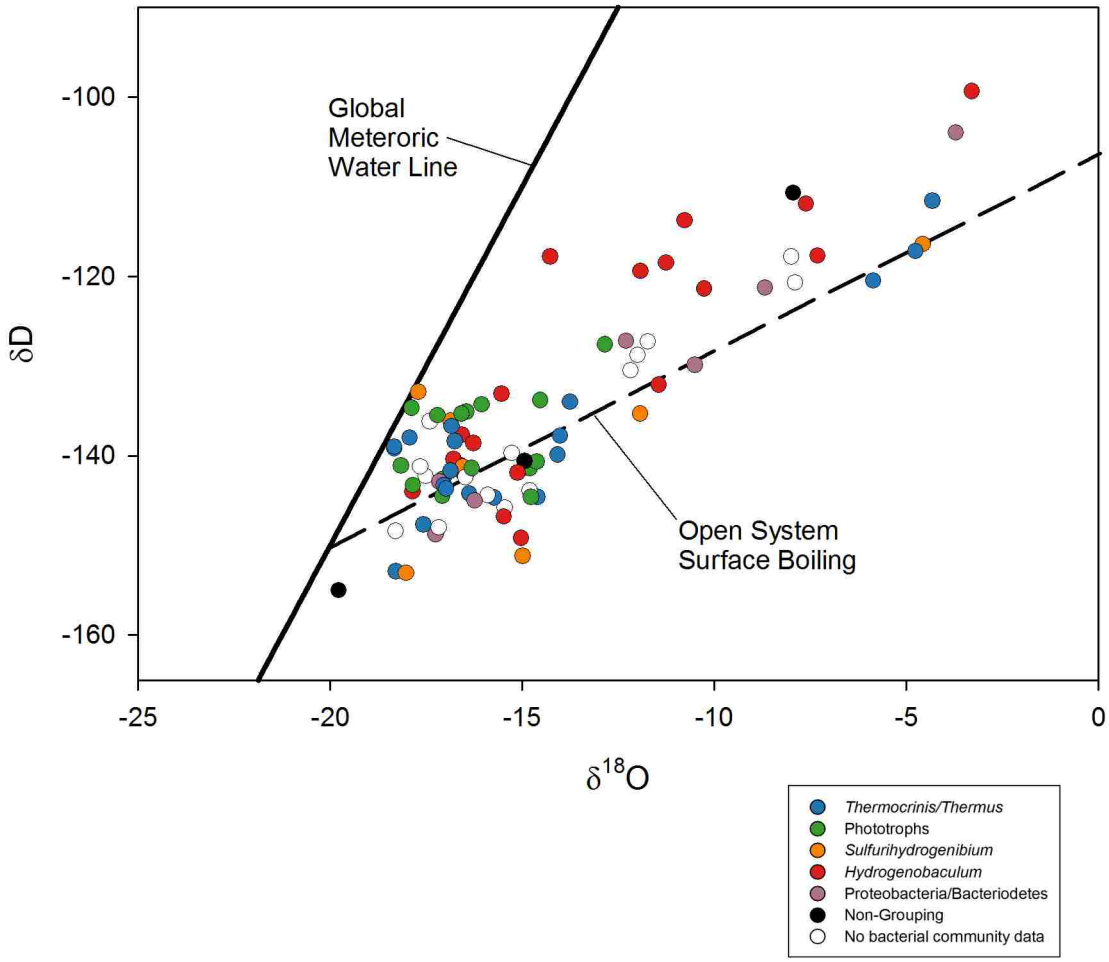


Figure 3.4. Hydrogen and oxygen isotope concentrations from the samples compared to Vienna Standard Mean Ocean Water (VSMOW), colored by the community group found in each site. The samples all fall below the global meteoric water line which could indicate mixing with the deep hot aquifer, subsurface boiling and steam separation, or open surface boiling (Shanks 2005).

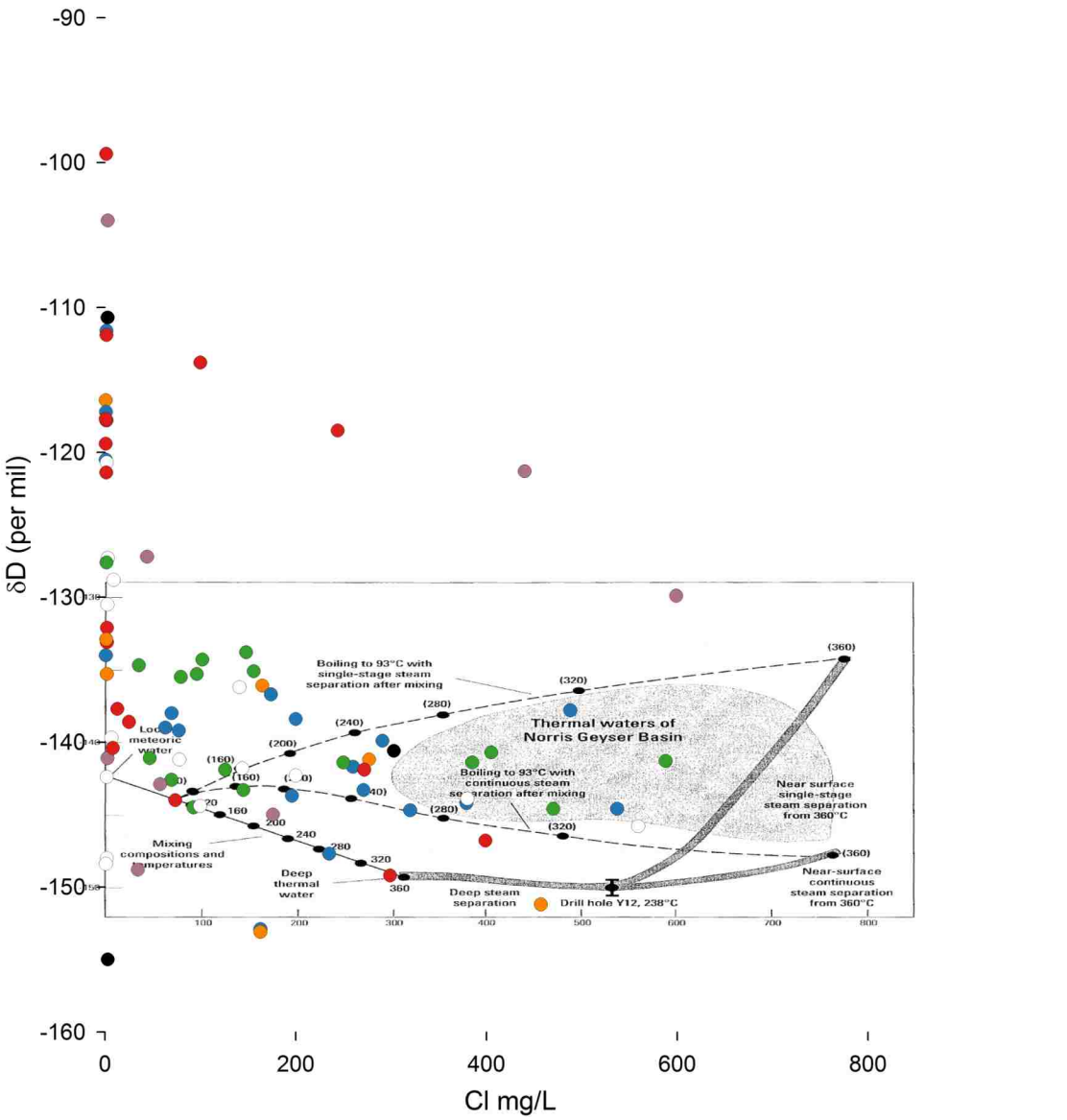
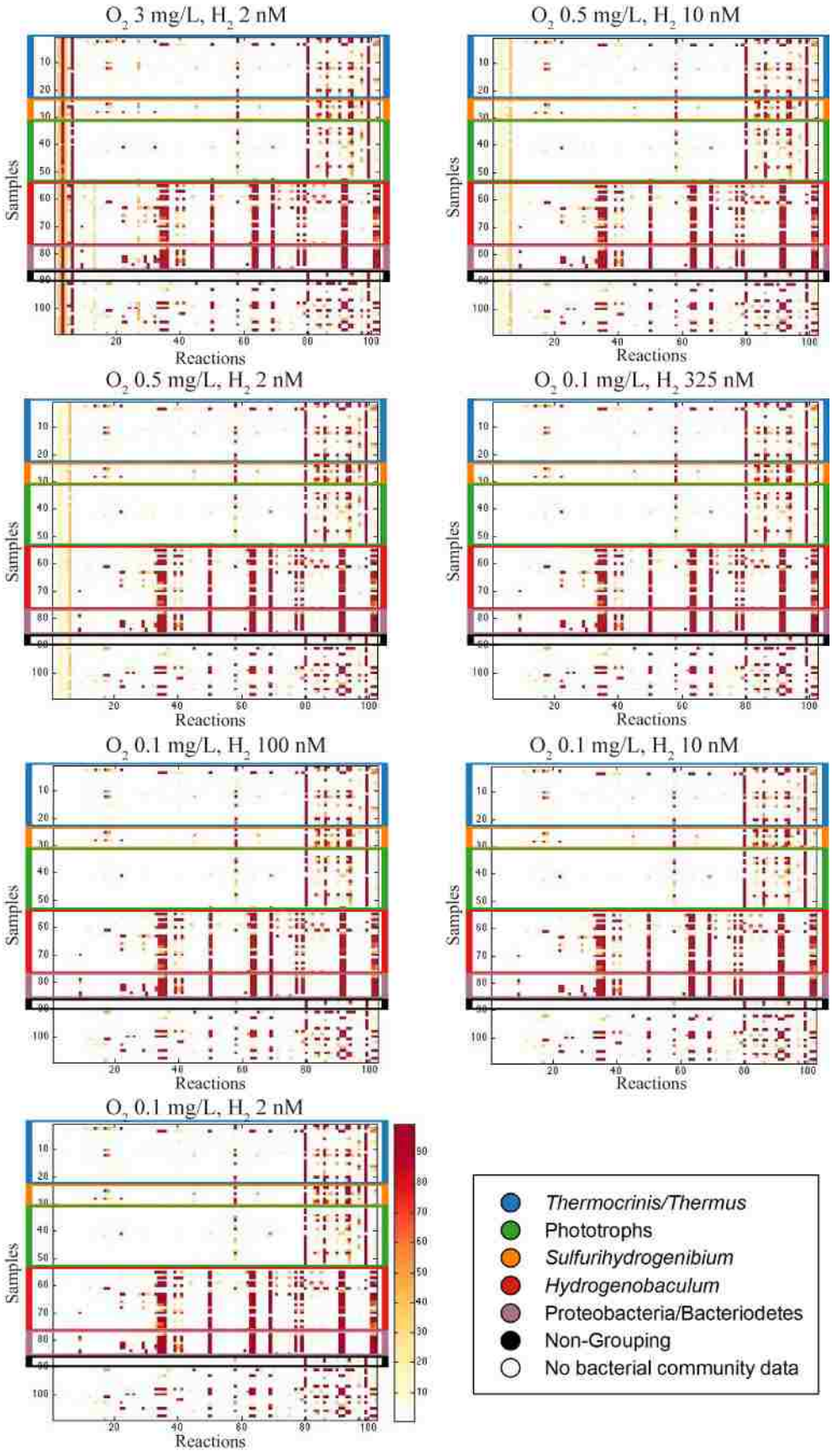


Figure 3.5. Water isotope and Cl^- concentration plotted on the theoretical mixing and boiling lines for the deep hot aquifer, colored by the community group found in each site. The samples on the left (very low Cl^-) are likely vapor dominated, while the others are meteoric water mixing with the deep hot aquifer to some degree. (Redrawn from (Rye and Truesdell 1993)

comparison of reactions by removing the impact of the size of the compound being reduced (e.g. directly comparing the energy from reduction of a small molecule such as H_2 by O_2, which involves the transfer of two electrons with the reduction of methane and iron II by sulfate where 56 electrons are transferred). However, this study is not concerned with comparing reactions but rather springs. To compare springs, it was necessary to express the energy available in units of water mass rather that moles of electrons. This was accomplished by multiplying the Gibbs free energy by moles of the limiting reactant, resulting in joules available per kilogram of water. The results of these analyses for the seven models (modeling a range of O_2 and H_2 concentrations) are displayed in Figure 3.6, with the samples (Y axis) and the reactions (X axis). Only reactions that resulted in greater than 1 J/kg water are included is this figure with those reactions bolded in Appendix Table A2.

Figure 3.6 (next page) Color-coded maps showing the amount of energy available for 102 reactions that produce at least 1 kJ/kg H_2O. More energy available is shown by darker color; each sample is a row and each reaction is a column. The samples that make up each community type are indicated by the color of the box around them. The 20 samples at the bottom of each map that are not color coded were the samples where we do not have bacterial sequences.


O2 3 mg/L, H2 2 nM
O2 0.5 mg/L, H2 10 nM
O2 0.5 mg/L, H2 2 nM
O2 0.1 mg/L, H2 325 nM
O2 0.1 mg/L, H2 100 nM
O2 0.1 mg/L, H2 10 nM
O2 0.1 mg/L, H2 2 nM
Samples
Reactions
Thermocrinis/Thermus
Phototrophs
Sulfurihydrogenibium
Hydrogenobaculum
Proteobacteria/Bacteriodetes
Non-Grouping
No bacterial community data

Clustering of samples by sequence, geochemistry, and energetics

Because of the large number and types of samples collected, it became possible to look for relationships between organisms within communities, and between community types and their geochemical setting. The initial step was to classify the 16S sequences into OTUs and determine which level or levels of classification best described the community. We tested four levels of sequence divergence, 1% (phylotype), 2% (species level), 6% (genus), and 15% (class or phylum) (Stackebrandt 2006), using polythetic hierarchical cluster analysis and MRPP to evaluate the numbers of clusters that best described the community and the level of clustering that best described the communities across the other phylogenetic levels (Fig. 3.7). Clusters based on presence absence matrices at 1% divergence and species level divergence were poor predictors of community groups compared to matrices at the higher phylogenetic levels. Class or phyla level (15%) divergence best described the communities across all phylogenetic levels. At this level, the samples collapsed into five community clusters with multiple members plus three samples that did not group with any other sample. Clusters based on the genus matrix resulted in similar groupings to the class/phyla matrices but with lower statistical significance through the randomization procedure. Therefore, the clusters presented are based on the 15% divergence matrix (Fig. 3.8).

The same type of classification was attempted for the geochemistry data and the energetic modeling results. The geochemistry data matrix produced no discernable pattern (Fig.3 9). Correlation between just pH and temperature and the community structure showed that both were significantly related ($p < 0.001$). However, pH showed a stronger relationship ($r = 0.502$) than temperature ($r = 0.162$). The matrices of the

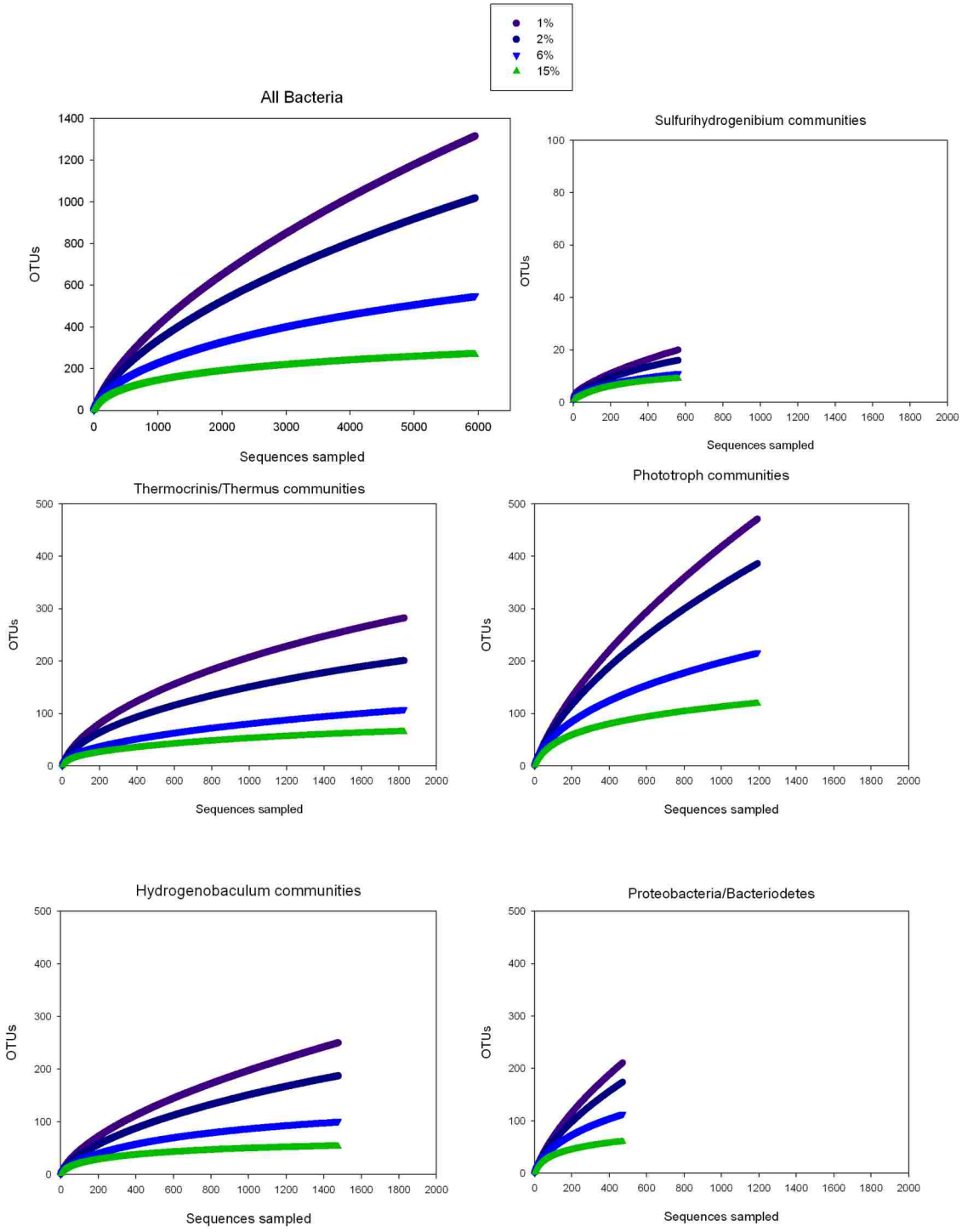


Figure 3.7 Rarefaction curves displaying sampling success for all the samples combined (top left) and for each of the five community types. Note that the scale is different for the Sulfurihydrogenibium group because of the very low diversity found in those samples.

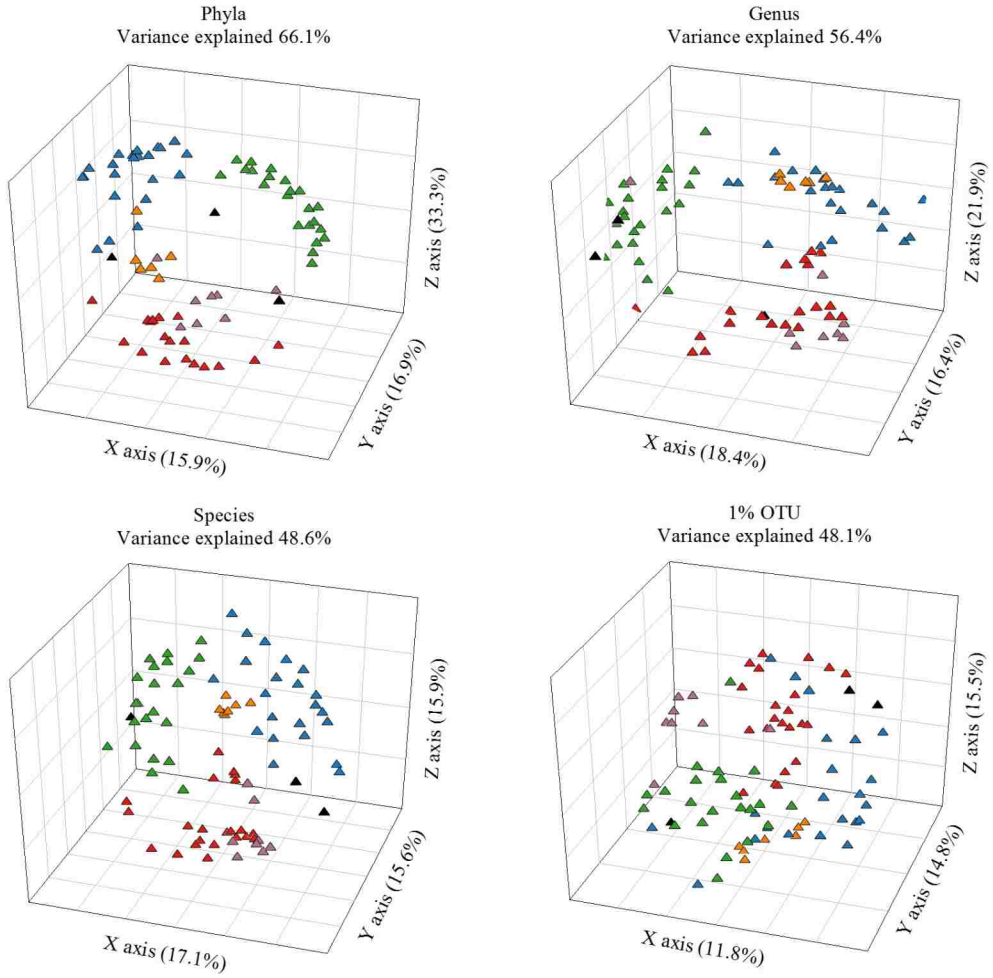


Figure 3.8. Plots of the results of the nonmetric multidimensional scaling at four OTU cutoff levels colored by the community group found in each sample. The community types cluster together across all the phylogenetic levels, however the groups are more distinct at the higher cutoff levels. The percent variance explained by each axis is the correlation between the original distance matrix and the three dimensional NMS matrix.

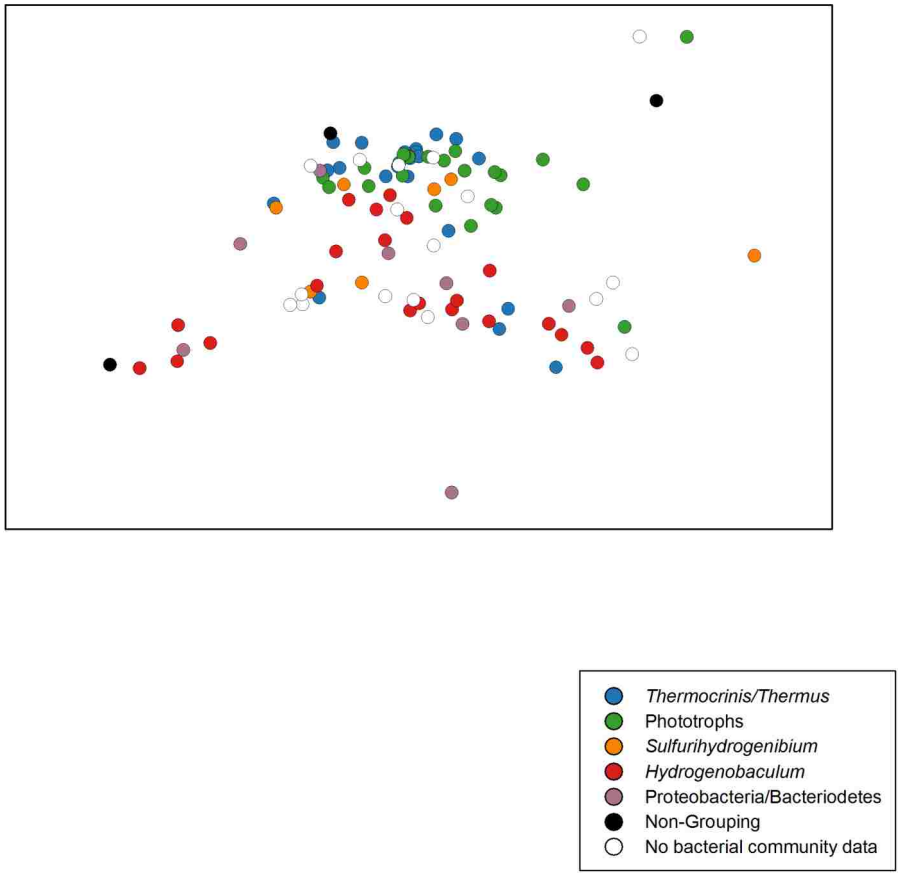


Figure 3.9. Nonmetric multidimensional scaling of geochemistry of the waters sampled, colored by the community group found in each site.

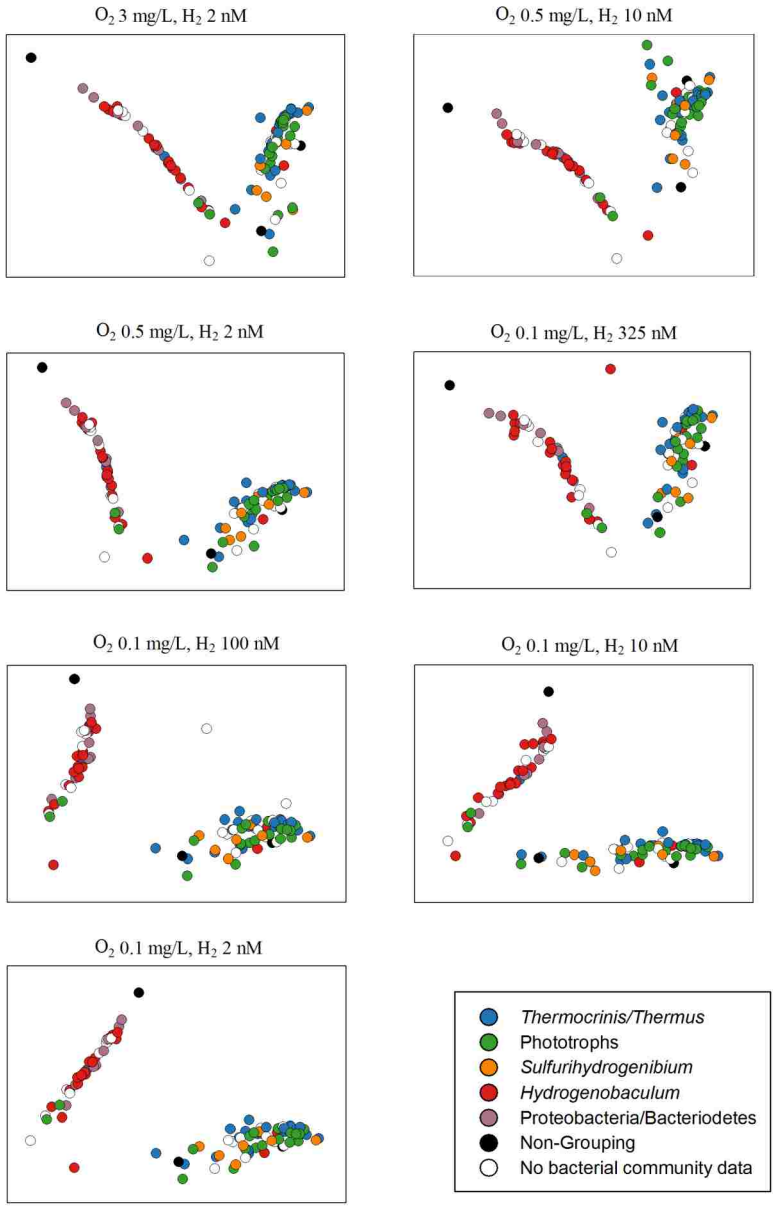


Figure 3.10. Nonmetric multidimensional scaling of energetic modeling of the springs sampled, colored by the community group found in each site.

energetic modeling showed a pattern that was consistent across all the models tested, with the samples split into a cluster of 64 samples distinct from a line of 39 samples (Fig 3.10).

Overview of five groups

We defined the five community groups by the most common genera or phyla found in that community by indicator analysis. Only those OTUs that are significantly indicated ($p<0.05$, indication percentage > 50%) are addressed here. Group 1 (n=22) is indicated by *Thermocrinis* (if a sample has *Thermocrinis*, it has an 89% chance of being assigned to group 1) followed by *Thermus* (52%). Groups 2 (n=7) and 3 (n=22) both only have one indicating genera, *Sulfurihydrogenibium* (89%) and *Hydrogenobaculum* (80%) respectively. Group 4 (n=22) consists mainly of the phototrophic communities, Chloroflexi (80%), Chlorobi (68%), Cyanobacteria (55%), and Acidobacteria (50%). The fifth group (n=8) is dominated by the Proteobacteria, including beta Proteobacteria (likely Comamonadaceae, 96%), alpha Proteobacteria (*Bradyrhizobiales*, 75% and *Sphingomonadales*, 71%), gamma Proteobacteria (*Moraxellaceae* 61%), and one member of the Bacteriodetes (*Flavobacteriales*, 88%). The relationships between these groups was explored using SONS (Schloss and Handelsman 2006), the estimated shared richness between the five groups is displayed in Table 3.1.

Discussion

This study is the first YNP wide survey of thermal springs for microbial diversity undertaken using molecular techniques. The earliest studies of Yellowstone thermophiles were based on microscopic observations and cultivation (Allen and Day 1935; Brock 1978). The most striking result of this work is that pH is shown to be much more

important in determining the resident microbial community than temperature (Figure 3.11), as others have suggested (Brock 1978; Ward et al. 1998; Skirnisdottir et al. 2000; Purcell et al. 2007). However, the majority of the research in thermophilic bacterial communities has been focused on neutral or basic springs (Reysenbach et al. 1994; Ward et al. 1998; Reysenbach et al. 2000; Skirnisdottir et al. 2000; Madigan 2003; Papke et al. 2003; Shock et al. 2005), as opposed to the focus on Archaea in acidic springs (Brock et al. 1972; Jackson et al. 2001; Whitaker et al. 2003). This historic bias in sampling locations may explain the assumptions that temperature is the driving environmental factor controlling microbial communities in thermal environments.

Much of the sequence diversity that we recovered spans the range of microbes that other researchers have found. Aquificales, a dominant phylum in the springs samples, as others have found across YNP and in other thermal areas (Jackson et al. 2001; Takacs et al. 2001; Blank et al. 2002; Kato et al. 2004; Meyer-Dombard et al. 2005; Nakagawa et al. 2005; Reysenbach et al. 2005; Spear et al. 2005; Purcell et al. 2007; Hall et al. 2008; Takacs-Vesbach et al. 2008), were found in 51 samples and were represented by 38% of the clones. Members of the Aquificales are also a defining taxon for 3 of the 5 groups that we found. This is likely due to the combined factors of the ubiquity of Aquificales in thermal areas coupled with the relatively low diversity of the sites they occupy (especially compared with phyla such as Proteobacteria).

The five types of bacterial communities that are found in our samples are not completely distinct from each other. We have estimated the amount of overlap between the groups at the species, genus, and class or phyla level using SONS, which calculates

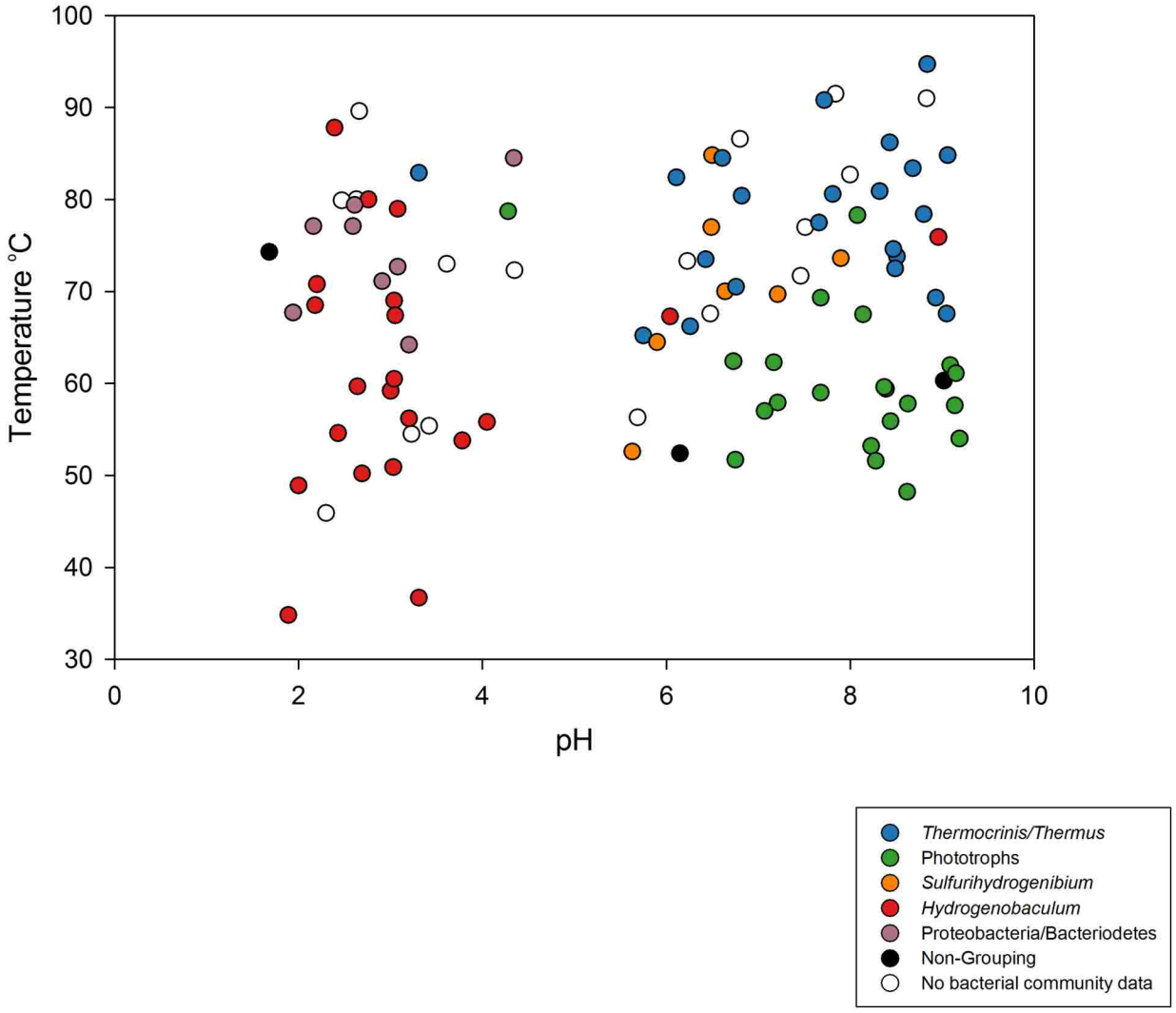


Figure 3.11. Temperature and pH scatter plot of the samples, colored by the community group found in each site.

Table 3.1.

	Estimated Richness (Confidence Interval)	Shared with all other samples pooled	Phototrophic	Sulfurihydrogenibium	Thermocrinis	Hydrogenobaculum	Bacteriodetes	3 non-grouping samples, pooled
Phototrophic								
2%	1063 (880-1321)	108	-	8	61	2	6	0
6%	393 (334-489)	271	-	8	164	2	5	0
15%	181 (153-241	123	-	10	57	12	43	2
Sulfurihydrogenibium								
2%	25 (18-61)	22	8	-	15	2	2	0
6%	13 (11-42)	9	8	-	5	2	1	0
15%	11 (9-24)	12	10	-	8	4	4	2
Thermocrinis								
2%	290 (253-354)	73	61	15	-	10	13	1
6%	165 (135-228)	151	164	5	-	11	19	1
15%	78 (70-103)	96	57	8	-	11	18	3
Hydrogenobaculum								
2%	342 (278-451)	45	2	2	10	-	28	0
6%	124 (110-155)	38	2	2	11	-	21	0
15%	58 (54-74)	29	12	4	11	-	20	1
Bacteriodetes								
2%	555 (407-805)	42	6	2	13	28	-	0
6%	239 (184-346)	40	5	1	19	21	-	1
15%	115 (54-74)	41	43	4	18	20	-	1

this overlap based on the Chao1 diversity index. At the genus level and higher, the *Thermocrinis/Thermus* and the *Sulfurihydrogenibium* groups are subsets of the other groups. The bacteria in the *Thermocrinis/Thermus* group are nearly a complete subset of the organisms in the Phototroph group. The *Sulfurihydrogenibium* group is mostly a subset of *Thermocrinis/Thermus* and Phototroph, but also shares a quarter of its estimated diversity with the *Hydrogenobaculum* and Proteobacteria/Bacteriodetes groups.

Although there is significant overlap among the species detected in the five bacterial communities, the communities are distinct because the structures are distinct. The Phototroph group is the most diverse group of communities that we examined with an estimated richness of over 1000 species. Contrast that with the *Thermocrinis/Thermus* group with an estimate 290 species and the extremely low diversity present in the *Sulfurihydrogenibium* group, which only has a Chao1 estimate of 25 species. Clearly even though the members of the communities are similar between these groups, their organization is not. The very high diversity in the Phototroph group is likely due to the relatively lower temperature of their habitat and the physical structure of the mats that creates numerous niches (Ward et al. 1998). Most of these communities were found at 70°C or lower. Many, but not all, of the Phototroph communities have sequences of likely *Thermocrinis* and *Thermus* cells, however it is not possible to know if these generally higher temperature bacteria (Reysenbach et al. 2005) are alive and functioning in the lower temperature of the phototrophic region of the runoff channel or are simply dead, but still intact, cells washed in from the higher temperature source springs. Interestingly, none of the Phototroph communities had any *Sulfurihydrogenibium* sequences. Two of the Phototroph communities are from 78°C locations, above the

known limit for photosynthesis of 73°C (Madigan 2003); both of these samples were collected from springs that were downstream from many other pools. Whether the cells of the phototrophic organisms detected in these two higher temperature communities are still viable and contributing genetic material to the phototrophic mat downstream of the sampling site is unknown. However it is likely that the presence of the phototrophic sequences is due to the cells being washed in from cooler areas above the sampling site, rather than discovery of new phototrophic Bacteria that are capable of growth 5°C above the known limit. Such sites of interconnected streams of thermal features could be interesting locations for testing biogeography of microbes that are exposed to several fluctuations of temperature along a relatively short spatial and temporal gradient. Perhaps it is more correct to think of the *Thermocrinis/Thermus* and Phototroph communities not as separate communities, but rather temperature optimized end members of the community that inhabit near neutral and higher pH springs. The division between *Thermocrinis/Thermus* and Phototroph communities is the only one that can be attributed to temperature in these samples.

The *Sulfurihydrogenibium* group can be defined by extremely low diversity, however it is not clear what is driving that low diversity. These communities are all from springs above the pH 5.5, likely hot water dominated systems (Fournier 2005). But they span a very wide temperature range (53°-85°). Five of the seven samples that fall into this group form macroscopically visible filaments; whereas, the other two are from filtered water samples with no obvious visible biomass in the source pool or runoff channel. Even more confounding, one of these samples, 03YMAM02, was collected from a spring in the Mammoth Hot Springs area, geographically near and geochemically

very similar to another sample, 03YMAM01, that had visible filaments dominated by several closely related *Sulfurihydrogenibium* sequences, but that also had *Thermotoga* and *Geothermobacterium* sequences and therefore grouped with the *Thermocrinis/Thermus* communities. Whether these differences are reflective of the *in situ* community or biases in the processing of the samples is not clear at this time (Hall et al. 2008). The energetic modeling shows that the *Sulfurihydrogenibium* communities have energy available from most of the metabolisms that the Phototrophic and *Thermocrinis/Thermus* communities possess with the addition of a few reactions that were common in the Proteobacteria/Bacteriodetes and *Hydrogenobaculum* communities (Table 3.2). Even though the *Sulfurihydrogenibium* have been studied for the past decade (Reysenbach et al. 2000; Takacs et al. 2001; Takacs-Vesbach et al. 2008), it is clear from this research that there is still much to be discovered about their ecology.

Like the samples that compose the *Sulfurihydrogenibium* group, those that make up the *Hydrogenobaculum* group are also indicated by only one species, *Hydrogenobaculum*. However, unlike the *Sulfurihydrogenibium* group this is not because *Hydrogenobaculum* communities have low diversity, but rather that a wide range of other species can populate these communities without any other single species being common to the majority of the *Hydrogenobaculum* communities. Some of the organisms that inhabit the *Hydrogenobaculum* communities and are statistically significant, but weak indicators, are Actinobacteria (indicator value 20%), Proteobacteria (*Ralstonia* 32%, *Desulfurella* 21%, *Acetobacteria* 18%, and *Acidothiobacillus* 14%), Thermotoga (18%), and *Paenibacilla* (18%).

Table 3.2.

Community	Reaction	Terminal Electron Acceptor
All		
	2MAGNETITE+1/2 O_2+3H_2O→6GOETHITE (2)	O_2
	S+3/2 O_2+H_2O→SO_4^{-2}+2H^+ (6)	O_2
	2MAGNETITE+1/2 O_2→3HEMATITE (2)	O_2
	PYRITE+7/2 O_2+H_2O→2SO_4^{-2}+Fe^{+2}+2H^+ (14)	O_2
	3CH_4+4NO_2^-+5H^++H_2O→3HCO_3^-+4NH_4^+ (24)	NO_2
	3CH_4+4NO_2^-+8H^+→3CO_2+4NH_4^++2H_2O (24)	NO_2
	NH_4^++3/2 O2→NO_2^-+2H^++H_2O (6)	O_2
	NH_4^++2O_2→NO_3^-+2H^++H_2O (8)	O_2
	4S+4H_2O→SO_4^{-2}+3HS^-+2H^+ (6)	S
	6MAGNETITE+SO_4^{-2}+2H^++8H_2O→S+18GOETHITE (2)	SO_4
	6MAGNETITE+SO_4^{-2}+2H^+→S+9HEMATITE+H_2O (24)	Hematite
Hydrogenobaculum and Proteobacteria/Bacteriodetes		
	2HS^-+Fe^{+2}+1/2 O_2→PYRITE+2H^++H_2O (2)	O_2
	Fe^{+2}+1/4 O_2+3/2 H_2O→GOETHITE+2H^+ (1)	O_2
	2Fe^{+2}+1/2 O_2+2H_2O→HEMATITE+4H^+ (2)	O_2
	3Fe+2+1/2 O_2+3H_2O→Fe_3O_4+6H^+ (2)	O_2
	HS^-+1/2 O_2→S+H_2O (2)	O_2
	Fe^{+2}+2S+H_2O→PYRITE+2H^++1/2 O_2 (2)	O_2
	2MAGNETITE+2S+Fe^{+2}+4H_2O→PYRITE+6GOETHITE+2H^+ (2)	S
	3Fe^{+2}+7S+4H_2O→3PYRITE+SO_4^-2+8H^+ (6)	S
	2MAGNETITE+2S+Fe^{+2}+H_2O→PYRITE+3HEMATITE+2H^+ (2)	S
	9Fe^{+2}+NO_2^-+10H_2O→3MAGNETITE+NH_4^++16H^+ (6)	NO_2
	3Fe^{+3}+2S+4H_2O→PYRITE+2GOETHITE+6H^+ (2)	S
	3Fe^{+2}+2S+3H_2O→PYRITE+HEMATITE+6H^+ (2)	S
	4Fe^{+2}+2S+4H_2O→PYRITE+MAGNETITE+8H^+ (2)	S
	2MAGNETITE+S+4H_2O→6GOETHITE+HS^- (2)	S
	2MAGNETITE+S+H_2O→3HEMATITE+HS^- (2)	S
	9Fe^{+2}+SO_4^{-2}+8H_2O→S+3MAGNETITE+16H^+ (6)	Magnetite
	12Fe^{+2}+SO_4^{-2}+12H_2O→4MAGNETITE+HS^-+22H^+ (8)	Magnetite
	6Fe^{+2}+SO_4^{-2}+5H_2O→S+3HEMATITE+10H^+ (6)	Hematite
	6Fe^{+2}+SO_4^{-2}+8H_2O→S+6GOETHITE+10H^+ (6)	Goethite
	PYRITE+7MAGNETITE+40H^+→ 2SO_4^{-2}+22Fe^{+2}+20H_2O (14)	Magnetite
	14MAGNETITE+Fe^{+2}+2SO_4^{-2}+2H^++20H_2O→ PYRITE+42GOETHITE (14)	SO_4

Table 3.2 cont.

Community	Reaction	Terminal Electron Acceptor
	$CH_4+4PYRITE+8H^++2H_2O\rightarrow CO_2+4Fe^{+2}+8HS^-$ (8)	SO_4
	$14MAGNETITE+Fe^{+2}+2SO_4^{-2}+2h^+\rightarrow$ $PYRITE+21HEMATITE+H_2O$ (14)	SO_4
Hydrogenobaculum and Proteobacteria/Bacteriodetes cont.		
	$8Fe^{+2}+SO_4^{-2}+8H_2O\rightarrow 4HEMATITE+HS^-+14H^+$ (8)	Hematite
	$8Fe^{+2}+SO_4^{-2}+12H_2O\rightarrow 8GOETHITE+HS^-+14H^+$ (8)	Goethite
	$15Fe^{+2}+2SO_4^{-2}+13H_20\rightarrow PYRITE+7HEMATITE+26H^+$ (14)	Hematite
	$15Fe^{+2}+2SO_4^{-2}+20H_2O\rightarrow PYRITE+14GOETHITE+26H^+$ (14)	Goethite
Sulfurihydrogenibium and Hydrogenobaculum and Proteobacteria/Bacteriodetes		
	$2H^++SO_4^{-2}\rightarrow HS^-+2O_2$ (8)	O_2
	$MAGNETITE+PYRITE+2H_2O\rightarrow 2HEMATITE+2HS^-$ (2)	Hematite
Sulfurihydrogenibium		
	$HS^-+4CO_2\rightarrow SO4^{-2}+4CO+2H^+$ (8)	SO_4
	$CH_4^++SO_4^{-2}+H^+\rightarrow HCO_3^-+HS^-+H_2O$ (8)	SO_4
	$CH_4+SO_4^{-2}+2H^+\rightarrow CO_2+HS^-+2H_2O$ (24)	SO_4
	$CH_4+4S+2H_2O\rightarrow CO_2+4HS^-$ (8)	S
Thermocrinis/Thermus and Sulfurihydrogenibium		
	$NH_4^++3CO_2\rightarrow NO_2^-+3CO+2H^++H_2O$ (6)	NO_2
Thermocrinis/Thermus and Sulfurihydrogenibium and Phototrophs		
	$7CO(aq)+2SO_4^{-2}+2H^++Fe^{+2}\rightarrow PYRITE+7CO_2(aq)+H_2O$ (14)	SO_4
	$7CH_4+8SO_4^-+H^++4Fe^{+2}\rightarrow 4PYRITE+7HCO_3^-+11H_2O$ (56)	SO_4
	$3HEMATITE+CO(aq)\rightarrow 2MAGNETITE+CO_2(aq)$ (2)	Hematite
	$S+3CO_2+H_2O\rightarrow SO_4^{-2}+3CO+2H^+$ (6)	SO_4
	$7CH_4+8SO_4^{-2}+8H^++4Fe^{+2}\rightarrow 4PYRITE+7CO_2+18H_2O$ (56)	SO_4
	$6GOETHITE+CO(aq)\rightarrow 2MAGNETITE+CO_2(aq)+3H_2O$ (2)	Goethite
	$CH_4+24GOETHITE\rightarrow 8MAGNETITE+CO_2+14H_2O$ (8)	Goethite
	$CH_4+12HEMATITE\rightarrow 8MAGNETITE+CO_2+2H_2O$ (14)	Hematite
	$3CH_4+4SO_4^{-2}+8H^+\rightarrow 4S+3CO_2+10H_2O$ (8)	SO_4
	$SO_4^{-2}+3H_2+2H^+\rightarrow S+4H_2O$ (8)	SO_4
	$3CH_4+4SO_4^{-2}+5H^+\rightarrow 4S+3HCO_3^-+7H_2O$ (8)	Bicarb

The Proteobacteria/Bacteriodetes and the *Hydrogenobaculum* groups are different from each other in terms of the sequences present. As well, the sites that fall into each of these groups also differ from each other. These sites are fairly diverse, the samples in the Proteobacteria/Bacteriodetes group have as many species level OTUs as all but the most diverse Phototroph samples. The vast majority of this diversity is quite rare however, only being identified in a single sample. As groups, the Proteobacteria/Bacteriodetes communities are estimated to share only 8% of their total OTUs with any other group, while *Hydrogenobaculum* shares 13% of their OTUs. This is in stark contrast to the relationship between the circumneutral groups which, in general, appear to consist of subsets of a larger metacommunity. The relationship between the low pH groups is unclear. Although two groups best described these communities, it is not apparent what drives this division or even if this simple bifurcation is truly representative of these largely unexamined bacterial communities.

Geochemical diversity

What makes this study different from previous work in thermal environments, in addition to the number and range of samples collected, is the extensive geochemical analysis that was done concurrently with the microbial analysis. The geochemistry allowed us to examine the importance of a wide variety of abiotic factors that could potentially control the microbial communities in the springs. But, surprisingly, the raw geochemical concentrations were not useful in grouping the samples (Figure 3.12). These analyses were performed using both the entire geochemical dataset and many iterations of subsets of the data (e.g., just major anion and cations or just nutrients). Note that no signal in the geochemical concentrations was detected for the fundamental

division of pH seen in YNP thermal waters. Lack of clustering does not mean that geochemistry is unimportant to these communities, but that it is not possible to predict the community type that inhabits a particular spring based simply on the geochemistry of the water.

Indicator analysis on the raw geochemistry, which identifies the chemical species and compounds that are significantly higher or lower in certain groups, is shown in Figure 3.12. It should be noted that the geochemical indicator analysis showed the same general clustering of the samples as was seen in the sequence based analyses. The *Thermocrinis/Thermus* and the Phototroph communities share the most similar geochemical make up. These two communities also share some geochemical composition with the *Sulfurihydrogenibium* group. Likewise, the *Hydrogenobaculum* and Proteobacteria/Bacteriodetes groups share lower pH and higher concentrations of several metals. However, there is no overlap between the chemical species that are indicated between those two larger groups (*Thermocrinis/Thermus*, Phototroph, and *Sulfurihydrogenibium* vs. the *Hydrogenobaculum* and the Proteobacteria/Bacteriodetes). The same major divide in the relationship among samples that is present in the community structure is present in the bulk geochemistry of the springs and seems to be based on pH. The geochemistry suggests there may be two metacommunities in YNP hot springs. However, the clustering analysis of the sequence data supported five community groups rather than two. Further work is needed to determine how five community types are maintained in the environment when there seems to be only two geochemical settings amongst the springs.

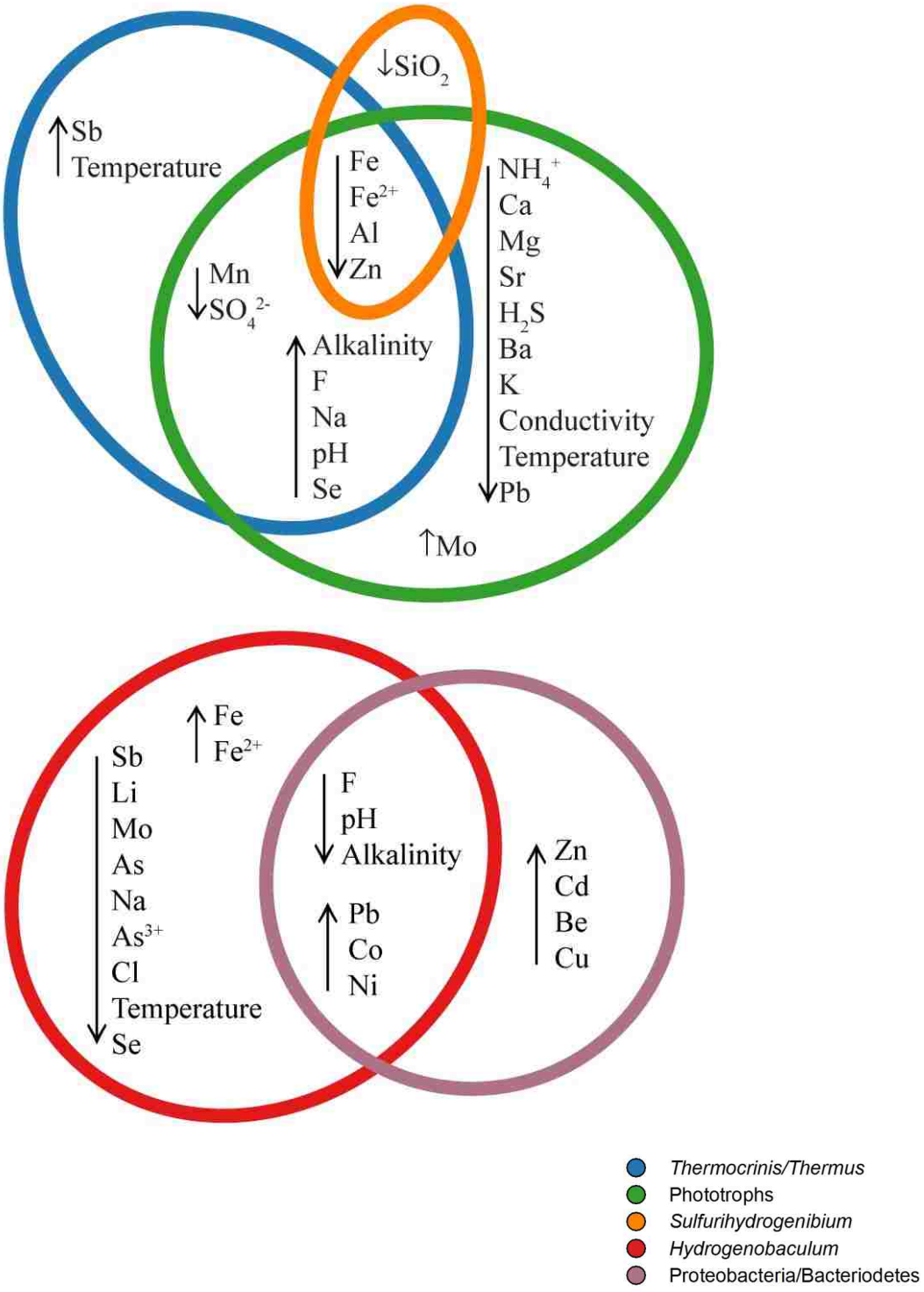


Figure 3.12. Indicator analysis of the geochemical parameters that are significantly different between community types.

In general, the *Thermocrinis/Thermus* and Phototroph communities share similar bulk geochemistry, i.e. higher alkalinity and fluoride and lower sulfate. The *Sulfurihydrogenibium* group is only indicated by lower concentrations of chemicals, most of which are also shared with the *Thermocrinis/Thermus* and Phototroph communities, i.e. iron, aluminum, and zinc, as well as lower silica. The lower concentration of silica is of interest as one possible reason for the lower diversity of *Sulfurihydrogenibium* communities. We have observed in YNP springs that *Thermocrinis/Thermus* communities nearly always form filaments in springs that have hard (putatively siliceous) sinter (Christiansen 2001) deposits while *Sulfurihydrogenibium* communities can be found on both hard deposits and soft bottomed runoff channels. This analysis gives some geochemical backing to our observation, and suggests that there may be a difference in how the members of the Aquificales attach to their environment. This difference could be investigated through growth experiments as well as mineralogical studies of geochemically similar springs that differ in the dominant genera of Aquificales.

The *Hydrogenobaculum* and Proteobacteria/Bacteriodetes samples have low pH and alkalinity and higher concentrations of lead, cobalt, and nickel. Additionally, the Proteobacteria/Bacteriodetes sites have high levels of zinc, cadmium, beryllium, and copper. While these metals may be involved in some metabolic processes, it is also possible that these communities do not obtain energy from, but simply have a higher tolerance for, these elements. With more intensive sampling efforts and a subsequent increase in our knowledge of the environmental interactions of these organisms, the significance of the metals will likely be discovered.

One of the major questions in microbial ecology is what controls community structure in natural environments. While forty-four variables were measured during this study, it was expected that a smaller number of parameters capable of reliably predicting community types would be determined. However, this did not prove to be the case, implying that the communities are not simply niche assembled because the strong pattern of separation between the samples based on sequences present is not similarly reflected in the geochemistry of the spring.

Geochemical modeling

The majority of the hot spring communities, especially those above the temperature limit of photosynthesis, are expected to have chemoautotrophs as the primary producers of the system (Shock et al. 2005). Chemoautotrophs gain energy by catalyzing reactions, generally reduction/oxidation coupled reactions or redox reactions, that are thermodynamically favorable but kinetically impeded (Amend and Shock 2001; Bach and Edwards 2003). Modeling of the energy available to a system based on calculations of energy available from likely biologically relevant redox reactions has been used to predict metabolisms that are likely significant sources of energy (Amend et al. 2003; Hayes et al. 2006). This type of modeling has shown some success in improving the applicability of one time geochemical measurements to the community (as opposed to multiple measurements across either short temporal or spatial distances measuring the production and utilization of compounds (Inskeep et al. 2004)). Our application of this type of modeling is one of the broadest completed to date. We model the O_2 and H_2 concentrations across the range of values previously found in YNP hot springs; O_2 from the detection limit of many methods (0.1 mg/L) to the highest found in typical

phototrophic mats in YNP (6 mg/L)(Pierson et al. 1999) and H_2 spanning the range reported in YNP, 2nM to 325 nM (Spear et al. 2005). Although the concentrations of modeled gasses spanned a wide range, very little difference in the amount of energy produced by the different models was detected (Appendix Table A2). It appears, that for the reactions modeled, as long as there is a small amount of O_2 or H_2 available, the reactions will proceed and yield essentially the same amount of energy. When we corrected the energy yielded for concentration of limiting reactant, the patterns in the data became more clearly defined. Figure 3.6 shows the amount of energy per kg of water (x axis) that the reactions yield for each sample (y axis), the intensity of the color from yellow to red represents available energy. There is very little difference in the amount of energy produced by any reactions across the entire range of H_2 concentrations found in YNP hot springs; however increasing the O_2 concentration does cause some reactions that utilize O_2 as the terminal electron acceptor to yield more energy. This difference in energy yield across O_2 concentrations has very little effect when comparing these samples, as the change in energy affects the samples relatively uniformly.

The raw data displayed in Figure 3.6 confirms that there is a pattern to the distribution of available energy. It is however, very difficult to consider over 100 variables (the reactions) simultaneously to identify the relationships among the samples. We therefore ordinated the energetic matrices using NMS, compressing the data into two axes (Figure 3.10). A similar pattern appears across the models, with the *Thermocrinis/Thermus*, Phototroph, and *Sulfurihydrogenibium* samples clustering together and separate from the *Hydrogenobaculum* and Proteobacteria/Bacteriodetes samples. Another pattern that is apparent in all seven models is that the

Hydrogenobaculum and Proteobacteria/Bacteriodetes group do not form an amorphic cluster, but rather a line regardless of which H_2 and O_2 model was used. Because of the striking and consistent nature of this feature in the ordinations, we regressed the NMS axes against the raw geochemistry of the springs. Four measured parameters seem to be driving this relationship, sulfate, aluminum, and lead concentrations increase as pH decreases across this ordination line. While sulfates are likely tied to the metabolic processes of the communities, either as substrates to be reduced or products of sulfur oxidation, the presence of aluminum and lead are best explained as a result of a greater tolerance for these metals by the communities at the lowest pH. Genes coding for metal transport ATPases have been discovered in a number of organisms including Proteobacteria (i.e. *Ralstonia* (Borremans et al. 2001)), *Desulfovibrio*, and *Comamonas* (Benyehuda et al. 2003)), Firmicutes (Staphylococcus (Rensing et al. 1998)), and Cyanobacteria (Thelwell et al. 1998), members of which are found in and are often indicators for the *Hydrogenobaculum* and Proteobacteria/Bacteriodetes communities. Additionally, these metal transport genes have been shown to be amenable to horizontal gene transfer (Coombs and Barkay 2005), which may explain several of the inconsistencies in the grouping of the low pH communities. Clearer differentiation of these communities may be possible with further exploration of the diversity of additional genes likely to be involved in protecting the organisms from the harsh conditions present in low pH environments.

In addition to providing insight into the metabolic processes important to these microbial communities, the energetic modeling is likely to advance culturing efforts by guiding media selection. Much of the ecological inference we can draw from

phylogenetic and energetic analyses of these communities will only be confirmed through further directed examination. Targeted sequencing of metabolic genes based on the reactions that were modeled to be high energy yielding and by targeted enrichment cultures and manipulation. However, that is not to diminish the importance of this type of diversity surveys. Without this type of broad discovery focused research we would not have shown that pH, not temperature, is the most significant factor controlling microbial communities in thermal areas. Also, where previous research has been focused on either a single spring or type of community, we examined the range of thermal springs found in YNP resulting in the discovery of a previously unrecognized community type, i.e. the Proteobacteria/Bacteriodetes.

That pH is a major controller of microbial community diversity is not unknown in the environment, however temperature was presumed to be more significant in thermal communities (Brock 1978, Skirnisdottir et al. 2000). Studies on microbial communities from a variety of environments have found that pH is the most significant factor determining bacterial diversity across continent scale (Fierer and Jackson 2006), diversity and transcriptional activity of ammonia oxidizing bacteria and archaea (Nicol et al. 2008), and aquatic community structure (Fierer et al. 2007). Other work has shown a combination of pH and another factor to be significant: pH and soil texture related to bacterial community composition invariant of land use types (Lauber et al. 2008) and pH and C to N ratio predicted fungal and bacterial community composition in boreal forest soil (Hogberg et al. 2007). However, temperature has also been found to be significant in controlling the organisms present in a system for *Prochlorococcus* in the Atlantic Ocean (Johnson et al. 2006) and ecotypes of cyanobacteria in YNP (Miller et al. 2009).

Temperature and sulfide combined have been used to explain differences between high temperature, near neutral communities inhabiting hot springs from Iceland (Skirnisdottir et al. 2000) and Thailand (Purcell et al. 2007). In the studies to date it seems that temperature may control which ecotypes are present within similar habitats while pH controls the whole community across variable habitats, this hypothesis requires further examination.

Exploration of bacterial diversity in natural communities is a rapidly evolving field. As such it should be expected that this type of broad diversity survey generates many more questions than it answers. Future ecological research that may integrate this study as an initial baseline include studies more closely examining the largely unexplored low pH, high temperature bacterial communities similar to those in YNP, those seeking to further elucidate those factors controlling diversity and those seeking to determine the extent of the diversity present in these environments. Also, the *Sulfurihydrogenibium* communities have been better studied than the low pH communities, but it is still not apparent why these habitats have such low diversity. Is it a result of yet unmeasured abiotic parameters that prevent other organisms from establishing, or is there something about the *Sulfurihydrogenibium* themselves that allow them to out compete other organisms? The ecosystems described in this study are ideal for further application of ecological theory, especially community assembly patterns, biogeographic theory, and macroecological experiments that take advantage of the high diversity of habitats and short generation time of thermal communities.

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Chapter 4

Evaluating the taxa-area and taxa-energy relationship in varied thermal springs

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Abstract

The taxa-area relationship (TAR) is regarded as one of the few laws in ecology. Although it has been investigated for decades in plants, animals, and insects, the taxa-area relationship has only begun to be examined in microbes. We evaluate the taxa-area relationship of bacterial diversity in terrestrial hot spring representing the range of environmental conditions found in Yellowstone National Park (YNP). The pH and temperature of the samples spanned the range of conditions found in YNP, 1.68 to 9.19 and 34.8°C to 94.7°C. Species richness was determined using two types of culture independent molecular analyses of the 16S rDNA: restriction fragment length polymorphism (RFLP) of whole gene clone libraries, n=18, and forward direction sequencing of whole gene clone libraries, n= 61. Island size, energy available, and temperature were determined for each sample. There was no significant relationship between species richness and either island size or energy available. It is impossible to completely sample microbial diversity and one sample per spring is unlikely to approximate total richness; therefore we also tested these relationships on estimated

diversity. There was also no relationship between either area or energy and the estimated richness. The finding of no relationship between either species richness and island size or energy available could be the first evidence of a small island effect in bacterial communities. This study is the first to examine a large number of natural isolated microbial communities, but it is still possible that more extensive sampling is needed to detect the relationship between richness and island size. The relationship between richness and size in bacteria may only exist at the extremes of size, either very large or very small areas, while we sampled the middle of that range. Also, given the broad range of physiochemical conditions sampled, a factor other than size could have much greater impact on the type and number of organisms that could inhabit a spring.

Introduction

Our understanding of the diversity and distribution of microorganisms in the natural environment was radically changed by the application of molecular biology techniques to microbial ecology. For example, entire phyla and domains super-kingdoms have been discovered and many of the underlying molecular similarities among living organisms have been revealed (Woese and Fox 1977; Barns et al. 1994). Furthermore, although perhaps only reflections of the metabolic potential of microbial communities, these methods have enabled us to bring these glimpses into better focus by facilitating the enrichment and culturing of otherwise transparent members of the microbial flora (Reysenbach et al. 2000; Reysenbach et al. 2006). Despite an ever-expanding database of newly discovered diversity, the application of ecological theory to microbial ecology is largely lacking (Prosser et al., 2007).

The taxa-area relationship (TAR) is regarded as one of the few laws in ecology (Zhou et al., 2008, Lomolino, 2001). Although it has been investigated for decades in plants, animals, and insects, the taxa-area relationship has only begun to be examined in microbes. Work in eukaryotic microorganisms has been divided, Artic benthic microfauna (Azovsky, 2002) and soil fungi (Peay et al., 2007) display a positive TAR, while flagellates show no biogeographic pattern at all (Fenchel, 2003, Finlay and Fenchel, 2004, Finlay, 2002). While some have suggested that flagellate ubiquity should logically apply to Bacteria (Fenchel and Finlay, 2005), experiments examining biogeography in microbial communities have found evidence for TAR in nested sampling schemes in soil (Noguez et al., 2005, Zhou et al., 2008), salt marsh (Horner-Devine et al., 2004), and alpine lakes (Reche et al., 2005). Contrasted with the connected habitats of

soil and marshes, to date, only artificial ecosystems have been used to examined bacterial TAR in isolated habitats: simulated tree holes (Bell et al., 2005), machine-wells (van der Gast et al., 2005), and membrane bioreactors (van der Gast et al., 2006). The tree hole and machine-well simulated islands studies removed much of the potential sampling bias by homogenizing the community before analyzing the sample, this action ensured that the sample analyzed represented the entire community rather than one portion of a spatially organized biomass.

Terrestrial thermal springs are particularly suited to addressing the existence of a species area relationship in microbes because the diversity is low relative to soils or more temperate habitats and, owing to the inability of thermophiles to thrive at lower temperatures, a single thermal spring represents an isolated island. The studies that best demonstrate that microbes can be biogeographically isolated have been done in thermal environments (Takacs-Vesbach et al., 2008, Papke et al., 2003, Whitaker et al., 2003). Given that little is known about the potential of thermophiles to disperse, examination of the taxa area relationship among thermal communities may provide clues about the dispersal and extinction rates of these organisms.

The controls of microbial diversity in thermal springs are not well understood. Previous work suggested that the most obvious parameter to consider is temperature, although the geochemistry of the spring is also likely to be important. For example, using thermodynamic calculations to model potential energy available from putative chemolithotrophic reactions (Shock et al., 2005, Amend and Shock, 2001), it was found that the energy available to communities is more dependent on the geochemistry of the water than the temperature of the spring (Amend et al., 2003) and that the energy

available has a stronger impact on microbial community composition than temperature or pH (Spear et al., 2005, Meyer-Dombard et al., 2005). Both of these studies examined a relatively small number of springs compared to the number included in this study, we found pH to be the strongest controller of both the community present in a spring and in the types of metabolisms that are likely to yield significant energy *in situ*. The contradictory results from studies examining the control of community by theoretical energetic calculations suggests that other factors that have not been examined, such as taxa-energy relationship, maybe influential among thermophilic communities.

We conducted the first baseline inventory of microbial diversity throughout Yellowstone National Park (YNP). Although the thermal features of YNP have been studied for over a century (Wondrak Biel, 2004), many investigations were limited to only a few springs, usually in the front country of the park (Ward et al., 1998, Barns et al., 1994, Hugenholtz et al., 1998a), or were focused on a specific lineage (Boomer et al., 2002, Fishbain et al., 2003). We have analyzed bacterial richness in 79 thermal springs in order to identify factors that determine species richness and community structure in these systems. Molecular analysis of such a diverse collection of samples enables us to explore relationships among species richness and environmental factors to determine patterns of diversity and distribution in terrestrial thermal systems. In extreme environments the community is restricted to microbes and often, except in the cases of very acidic springs, overwhelmingly dominated by Bacteria (Hugenholtz et al., 1998b). With the exception of invertebrates that inhabit the margins of springs, grazing may be unimportant; therefore abiotic factors are presumably critical biotic determinants. Specifically, we

determined if species richness in thermal systems is limited by temperature, island size, or energy available.

3. Methods and Materials

Site description and sampling

Samples were collected from thermal features throughout Yellowstone National Park, USA during the summers of 2003 and 2004. Seventy-nine samples were analyzed that encompassed the full range of pH, temperature, and biomass types found in the park. Temperature and pH were measured using a Thermo Orion 290A+ meter. Pool or spring size was measured in the field or digitized from color infrared aerial photos in ArcGIS 9.1 (ESRI) and was calculated as cm^2. The samples were also categorized by the type of biomass collected: sediment, photosynthetic mat, filaments, or water. Approximately 1-2.5 ml of sample was collected at each site with either sterile forceps or a syringe. Water samples were collected by filtering 0.6-1 L water through a 0.02 μm filter (Millipore) then preserving the filter as described below.

Sample preservation and extraction

Previously, we determined the most effective sample preservation and DNA extraction procedure to maximize the quality of DNA extracted and the diversity detected in the samples (Mitchell and Takacs-Vesbach, 2008). Samples were collected from each spring and were preserved in an equal volume of sucrose lysis buffer (20 mM EDTA, 200 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 9.0) (Giovannoni et al., 1990). Samples were held at ambient temperature for up to five days before they were stored at –80 ºC.

DNA was extracted using a modified CTAB extraction (Zhou et al., 1996). Briefly: 2 volumes of 1% CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10

mM EDTA) and proteinase K (final concentration 100 μg/ml) were added to the SLB preserved samples; incubated for one hour at 60°C, sodium dodecyl sulphate (SDS) (final concentration 2%) was added and incubated one hour at 60°C, extracted once with phenol/chloroform then twice with chloroform, finally the DNA was precipitated with ethanol.

Dilutions of the environmental genomic DNA were used as template DNA for PCR amplification of the 16S rDNA. The reaction included 1X Promega buffer with 1.5 mM $MgCl_2$, bovine serum albumin (0.04 % final, 2.5 U Taq DNA polymerase (Promega U.S.) 2.5% Igepal CA-630 (Sigma-Aldrich), 10 μM each dATP, dGTP, dCTP, dTTP (BioLine USA, Inc.), 20 μM Bacterial specific primers, 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACACTT). The PCR reaction (50 μl) was incubated in a thermocycler (ABI GeneAmp 2700) for 5 minutes at 94.0°C then for 30 cycles of 30 seconds at 94.0°C, 30 seconds at 50.0°C and 30 seconds at 72.0°C. The reaction was incubated at 72.0°C for 7 minutes for final extension. The PCR products were ligated and cloned using the TOPO TA pCR2.1 kit (Invitrogen). Species richness was determined for each 96-clone library by restriction fragment length polymorphism (RFLP) and sequencing of the unique clones (n=18) or by sequencing from the 5' end (n=62).

Sequences were discarded if they were less than 250 bases long and were aligned using GreenGenes (DeSantis et al., 2006). A distance matrix of the aligned sequences was calculated in ARB (Ludwig et al., 2003). Dotur (Schloss et al., 2003) analysis on the distance matrix was used to determine 2% divergent OTUs, which we have used as a proxy for species (Stackebrandt, 2006), and Chao1 estimator of diversity.

Energetic Modeling

Total energy available to the communities was modeled based on the activities of chemical species and compounds measured from the environment (unpublished, available upon request) as described in Amend et al. (2003), Amend and Shock (2001), Shock et al. (2005). Briefly: the raw geochemical concentrations were speciated using the EQ3 program (Lawrence Livermore National Laboratories). The activities from this program were used to calculate the Gibbs free energy available in each spring for 179 reactions that may be microbially mediated and likely to occur in thermal springs (Shock et al., 2005). This calculation gave the amount of energy per mole of electron transferred. We multiplied this value by the modeled concentration of the limiting reactant for each reaction, resulting in energy per kg of water. Evaluating the energy available from a particular reaction by kJ per mole of electron transferred allows comparison of reactions by removing the impact of the size of the compound being reduced (e.g. directly comparing the energy from reduction of a small molecule, such as H_2 by O_2, which involves the transfer of two electrons, with the reduction of methane and iron II by sulfate where 56 electrons are transferred). However, this study is concerned with comparing not reactions but springs. In order to compare springs, it was necessary to express the energy available in kilograms of water rather that moles of electrons.

Statistical Analysis

Statistical analyses were performed using SPSS 11 (for Mac OS X). The relationship between richness and environmental parameters was tested by general linear model (GLM) univariate analysis of variance, partial correlations, and linear regression analysis. GLM univariate analysis was used to detect statistical differences in species

richness (dependent variable) by pH, temperature, biomass type, spring area, and total energy available (fixed factors).

Results

The springs included in this study ranged in size from $3.5x10^1$ to $2.4x10^6$ cm^2. Diversity in the springs ranged from 1 to 41 species. Total energy available to the communities based on our modeling ranged from 0.59 kJ – 31.6 kJ per kg water. All possible combinations of partial correlations between species richness and island area, energy available, temperature, and pH were calculated. None of these correlations were significant. Single and multiple regressions were also run using species richness as the dependent variable and all possible combinations of area, energy available, temperature and pH as the independent variables. The r^2 for these regressions ranged from 0 to 0.05 (Fig. 4.1 and 4.2). GLM univariate analysis was run with the same variables to confirm the lack of relationship. Since samples collected from the hot springs represent very different sample and community types, we also ran the regressions species richness against area and energy available on subsets of the data by sample type (Fig.4.3) and community type as determined in Chapter 3 of this dissertation (Fig. 4.4). Again there was no relationship between richness and either area or energy available. Finally, since there were no relationships between the measured diversity and any of the parameters considered, we analyzed Chao1 estimators and area and energy. There was still no positive taxa-area or taxa-energy pattern, even when attempting to correct for undersampling.

Discussion

While there is still debate whether ecosystem size influences microbial species richness (Fenchel and Finlay, 2005), the consensus from the few previous field and experimental studies is that significant TAR relationships exist (Bell et al., 2005, Reche et al., 2005, Horner-Devine et al., 2004). There are a number of inferences that can be made from these results, because they imply that extinction, immigration, and dispersal rates are not consistent among the sites sampled and that variability exists in the availability of niches. This study is unique from the previous studies in several aspects; most importantly, the samples were collected from naturally separated sites opposed to the contiguous sampling or experimental microcosms of previous work. Regardless of how the data were partitioned (whole data set, only high or low pH or temperature, community type, or sample type) we were unable to find any evidence for a taxa-area or taxa-energy relationship. While it is possible that we didn't detect taxa-area and taxa-energy relationships because area and energy are not dominant controls on thermal communities, there are also a number of methodological constraints that could explain this.

Gene surveys of microbial communities have greatly increased our knowledge of the extent of microbial diversity, however they are still only snapshots of the community. There is certainly a portion of the community that we did not detect because of the biases inherent in sample preservation, DNA extraction (Mitchell and Takacs-Vesbach, 2008), PCR and cloning bias (von Wintzingerode et al., 1997), and the sheer abundance of

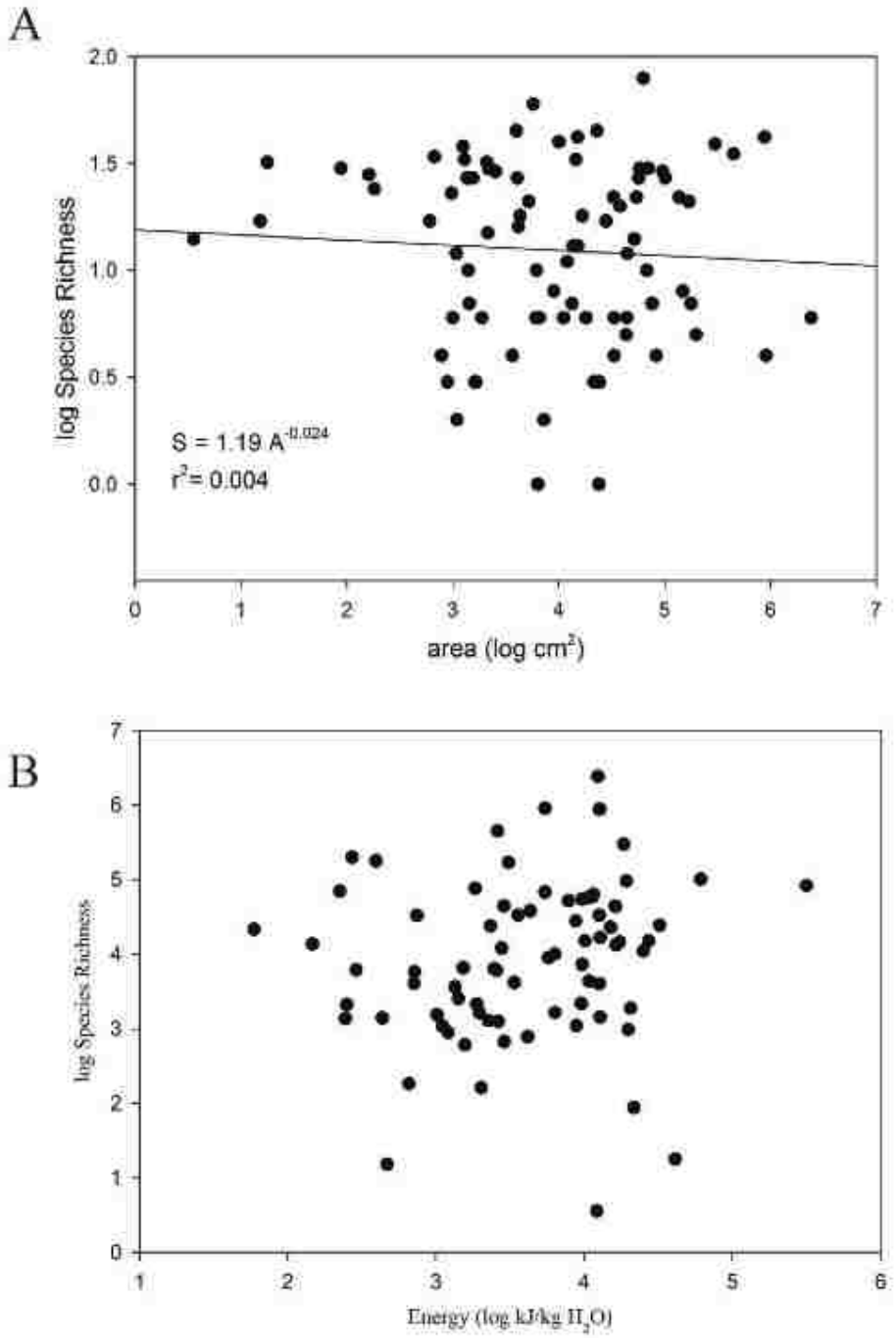

Figure 4.1. Scatter plots of species richness vs. A) island size and B) energy available for all samples.

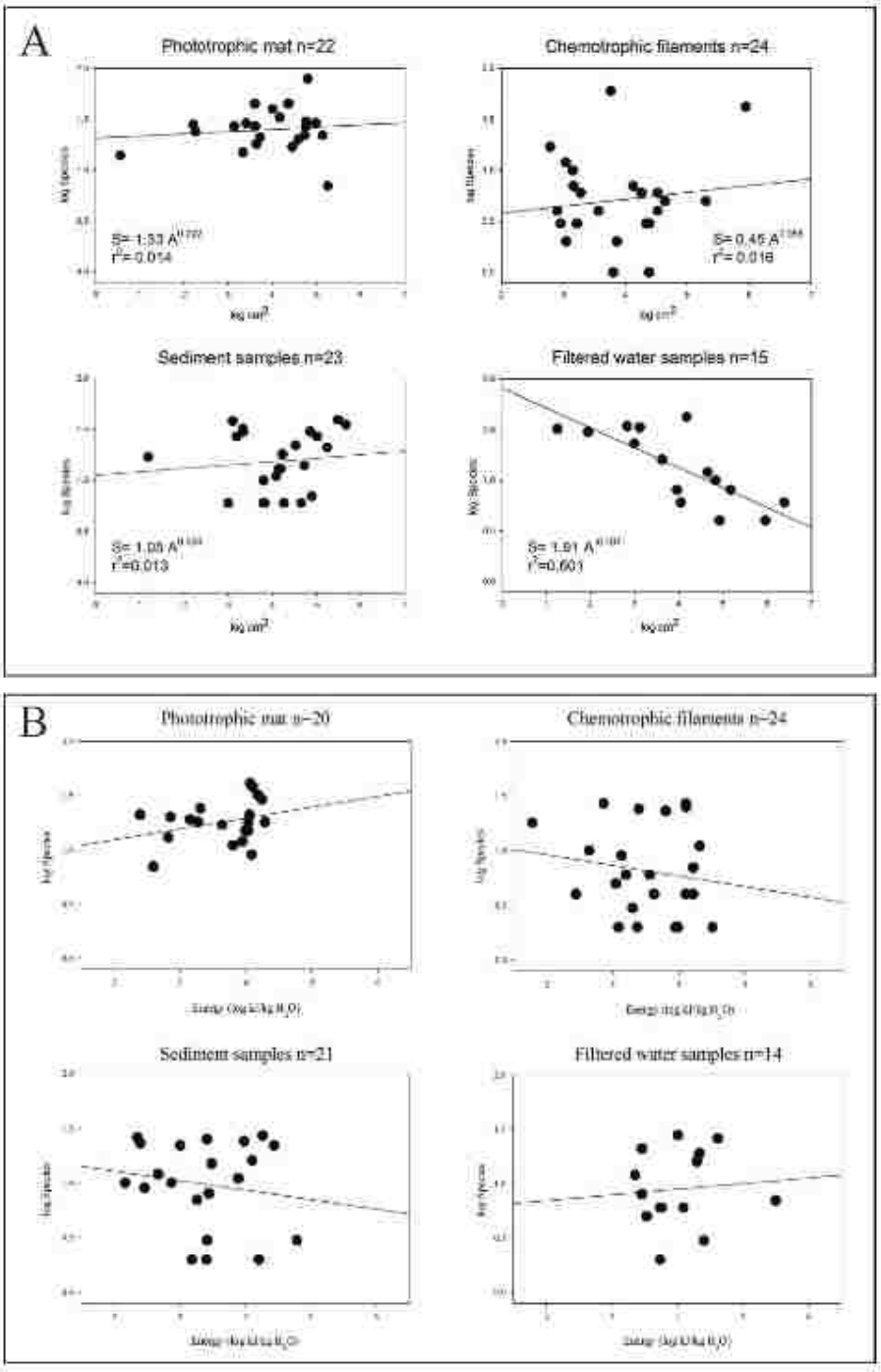

Figure 4.2. Scatter plots of species richness vs. A) island size and B) energy available for each sample type.

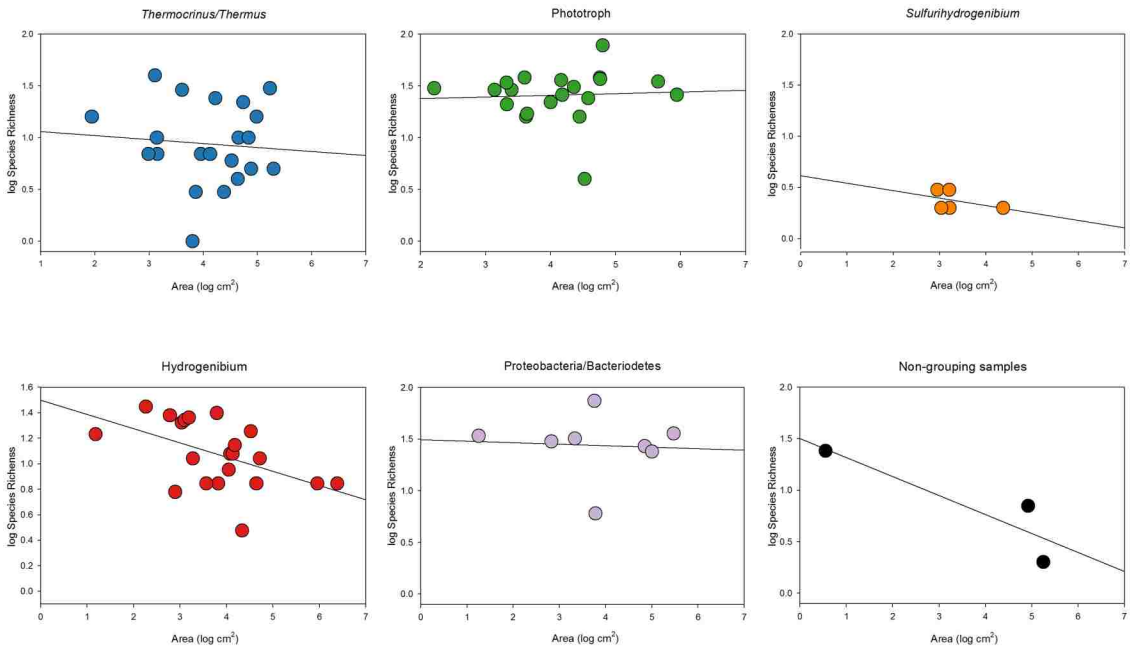

Figure 4.3. Scatter plots of species richness vs. island size for each community type

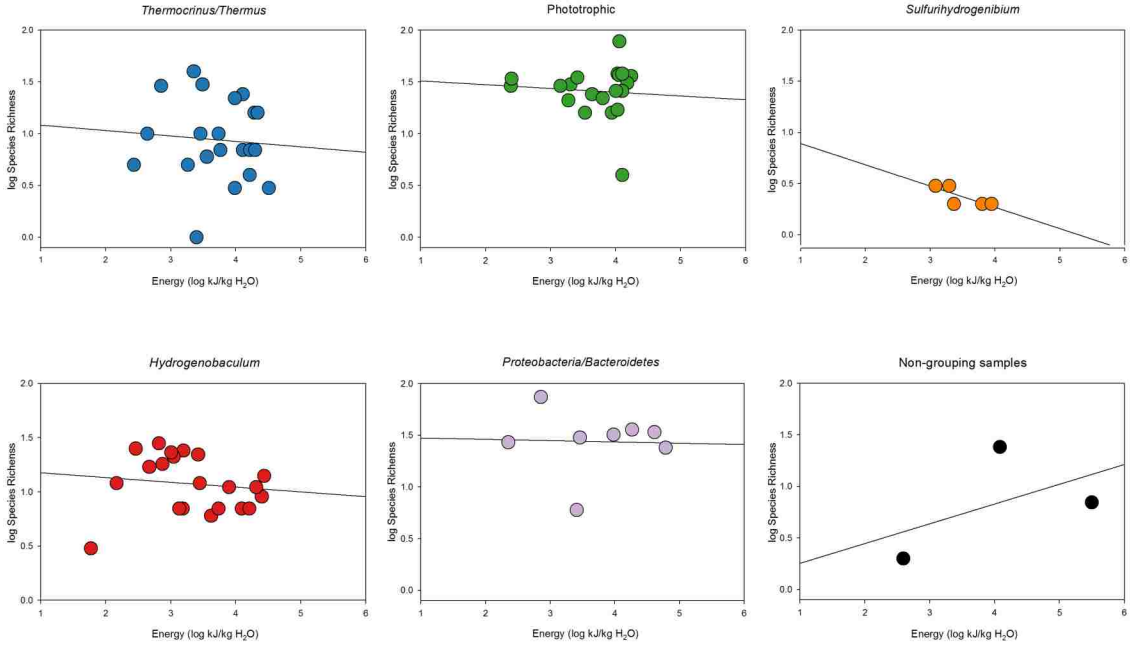

Figure 4.4. Scatter plots of species richness vs. energy available for each community type.

microbes in the environment (Sogin et al., 2006). Theoretical simulations of microbial communities (Woodcock et al., 2006) showed that it is mathematically unlikely to detect a TAR by sampling a community using techniques such as clone libraries or fingerprinting techniques, which either grossly undersample the community, or have a high detection limit, respectively. A recent study utilizing a microarray chip to sample soil communities, a technique which samples more individuals than either clone libraries or fingerprinting, found positive, but variable, TAR across phylogenetic groups (Zhou et al., 2008). Future technological developments are likely to make it possible to gain a much more in depth view of many communities. To date however, methods such as whole community sequencing are not practical for the large numbers of samples needed to examine the species area relationship.

Another consideration that may explain our results is the sampling scheme. While we collected samples that spanned the range of temperature and pH found in each thermal area, approximating the range of pool sizes proved more difficult. We compared the median size of the pools that we included in this study with the median size of pools selected by a stratified (by geographic area then pH) random sampling scheme of the 14,000 springs previously inventoried by the park service (A. Rodman personal communication). The median size of pools in this study is 9.8×10^3 cm^2, while the median size of the randomly selected pools was 6.6×10^3 cm^2. If the results described here are caused by the sampled pools being too large, i.e. only mainlands were included, it would be possible to survey more springs with the specific aim of reflecting the size range of pools not just the temperature and pH.

Finally, the work presented here represents the widest survey for bacteria that inhabit the hot springs in Yellowstone. Even so, each pool is represented by a single sample collected at a single point in time. The hot springs are generally thought to be relatively homogenous communities due to convective mixing. Interestingly, the sample type that should be the best mixed and represents the largest volume sampled was the filtered water samples (0.6 – 1 L water was filtered compared to 1-2.5 ml of the other sample types), which exhibited a strong negative relationship between richness and area ($r^2 = 0.451$). More extensive sampling of each pool is likely to reveal more diversity. Increased sampling of the pools by taking samples at several different time points would also be useful in determining if the small island effect is what we are seeing, to determine how stable the communities are across time, and if they are at equilibrium or experience increases in diversity followed by mass extinction of most of the community. While it is always desirable to have more samples across more spatial and temporal scales, current methods are still too expensive and time consuming.

Undersampling and uneven sampling of the communities present likely explain the lack of a TAR in these thermal springs. However, even if we were able accurately measure the richness of the springs, a taxa-energy relationship may not have been detectable. The direction and mechanism of the relationship between richness and energy are not known. A meta-analysis of productivity and richness found a mix of positive, negative, unimodal, and no relationship in plants and animals (Waide et al., 1999). The few microbial studies are equally mixed, unimodal in grassland fungal communities (Waldrop et al., 2006), increase in richness with the addition of benzoate in soil (Langenheder and Prosser, 2008), and no relationship between richness of sulfate

reducing bacteria or methanogens with the addition of carbon in coastal sediment (Edmonds et al., 2008). One mesocosm experiment found different relationships depending on the phyla; unimodal response for CFB, U-shaped for α-Proteobacteria, and no relationship for β-Proteobacteria (Horner-Devine et al., 2003).

It is also possible that we failed to detect any strong correlations among our parameters because we are comparing very different systems. For example, our samples included acidic springs, which are likely dominated by Archaea (Reysenbach et al., 1994), and compared them to photosynthetic mats, and chemoautotrophic communities. To control for this possibility we performed regression analyses on portions of the dataset, i.e. only including the samples with temperatures above 70°C and partial correlation analysis, i.e. correlation between richness and temperature controlling for pH. However, results of subset regressions and partial correlations did not differ from the whole dataset analysis.

Thermal springs are inhabited by species that are by necessity, specifically adapted to high temperature ecosystems. Presumably, these populations are adapted to the conditions of a particular spring type and have a competitive advantage over other potential invaders. Because of the extreme temperatures and pH of these systems, combined with their geographic separation, colonization would be restricted and thus mortality or extinction would be high. Furthermore, any traces of inferior competitors presumably are degraded rapidly. The application of taxa-area and –energy theory to microbial populations is very intriguing, although how appropriate it is to microorganisms is not yet clear. The power of this theory in studying thermal areas may

be its ability to help us identify and understand non-equilibrium conditions in these environments.

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Chapter 5

Summary

This study is the first Yellowstone (YNP) wide survey for microbial diversity undertaken using molecular techniques. The most striking result of this work is that pH is shown to be much more important in determining the resident microbial community than temperature. In addition to expanding the known distribution of many organisms, we found sequences belonging to 14 new candidate phyla.

The structure of thermophilic microbial communities depends on the type of community. The *Thermocrinis/Thermus* communities are a higher temperature subset of the Phototroph communities, which is not a very surprising finding because the two communities can be observed, macroscopically, inhabiting the same spring. The *Sulfurihydrogenibium* community is unique in its very low species level diversity. However, we have shown using these communities, that there is a strong biogeographical pattern to the diversity of *Sulfurihydrogenibium* at finer phylogenetic scales across YNP. This could indicate that the *Sulfurihydrogenibium* has high ecotype diversity, allowing them to fill many of the niches present in a spring and out competing other organisms. The structure of *Hydrogenobaculum* and Proteobacteria/Bacteriodetes communities are not as clear. Although, given the high levels of metals common in those springs, it is possible that the structure of those groups is controlled by tolerance of their environment rather than rapid growth or resource utilization that may be displayed by the *Sulfurihydrogenibium*.

The geochemistry has allowed us to examine the importance of a wide variety of abiotic factors that could potentially control the microbial communities in the springs. By examining a wide range of geochemistries from this large set of springs, we expected to find correlations between the microbial inhabitants and the abiotic conditions of the sites. Surprisingly, the raw geochemical concentrations were not useful in grouping the samples. Therefore we modeled the energy available to a system based on Gibbs free energy calculations of energy available from putatively biological relevant redox reactions. Oxygen and hydrogen concentrations are necessary for most of the reactions that we modeled, however we do not have measured O_2 or H_2 so we modeled the range of concentrations found in YNP springs. It appears, that for the reactions modeled, as long as there is a small amount of O_2 or H_2 available the reactions will proceed and yield essentially the same amount of energy. The *Thermocrinis/Thermus*, Phototroph, and *Sulfurihydrogenibium* samples clustering together and separate from the *Hydrogenobaculum* and Proteobacteria/Bacteriodetes samples. Another pattern that is apparent in all seven models is that the *Hydrogenobaculum* and Proteobacteria/Bacteriodetes group do not form an amorphic cluster, but rather a line. Four measured parameters seem to be driving this relationship, sulfate, aluminum, and lead concentrations increase as pH decreases across this ordination line.

Finally, I found no evidence for a taxa-area or taxa-energy relationship in these communities. However, potential undersampling of the extremely diverse sample types preclude us from making strong conclusions from this result. Thermal springs are inhabited by species that are, by necessity, specifically adapted to high temperature ecosystems. Presumably, these populations are adapted to the conditions of a particular

spring type and have a competitive advantage over other potential invaders. Because of the extreme temperatures and pH of these systems, combined with their geographic separation, colonization would be restricted and thus mortality or extinction would be high. Furthermore, any traces of inferior competitors presumably are degraded rapidly. The application of taxa-area and taxa-energy theory to microbial populations is very intriguing, although how appropriate it is to microorganisms is not yet clear. The power of this theory in studying thermal areas may be its ability to help us identify and understand non-equilibrium conditions in these environments.

Table A1. Geochemical measurements

Sample ID	03YMAM001	03YMAM002	03YWSH003	03YWSH004	03YWSH005	03YNOR006
Date	6/2/03	6/2/03	6/3/03	6/3/03	6/3/03	6/5/03
Temperature	73.5	70.0	84.8	84.5	69.0	82.9
pH	6.43	6.64	6.50	6.61	3.04	3.31
Conductivity	2230	2260	2150	2050	4460	1015
DO	0	0	0	0	0	0
DOC	0	0.0	16.8	20.8	10.1	0
Ca	290.00	280.00	19.00	19.00	38.40	4.01
K	56.00	55.00	14.00	13.00	14.20	45.70
Na	110.00	120.00	39.99	38.63	28.60	223.00
Mg	65.00	67.00	9.60	11.00	18.90	24.30
Alkalinity as HCO3-	781.5	709.4	168.2	144.6	BD	BD
SO4	546.0	564.0	830.0	783.0	2049.0	153.7
Cl	163.00	163.00	0.86	0.82	5.81	329.71
Al	0.003	0.003	0.039	0.098	BD	4.000
As	0.1860	0.4920	0.0028	0.0028	0.0004	0.0500
As(III)	0.1860	0.4920	0.0025	0.0030	0.0000	0.0500
Ba	0.0550	0.0530	0.0670	0.0630	BD	0.0100
Be	0.00140	0.00110	BD	BD	BD	0.00300
Cd	BD	BD	BD	BD	BD	BD
Co	BD	BD	BD	BD	BD	BD
Cu	0.00220	0.00220	0.00270	0.00210	BD	BD
Fe	0.008	0.018	BD	0.012	14.900	0.010
Fe(II)	0.007	0.018	BD	0.010	14.900	0.010
Li	1.6000	1.6000	0.0170	0.0160	0.0400	0.9000
Mn	0.0190	0.0150	0.1100	0.1300	0.7120	BD
Ni	BD	BD	BD	BD	0.0050	BD
P	BD	BD	1.0000	1.1000	3.0000	BD
Pb	BD	BD	BD	0.0001	BD	BD
Sb	0.0004	0.0002	0.0003	0.0003	BD	0.0010
Se	0.0023	0.0021	0.0002	0.0002	BD	0.0001
SiO2	51.0	50.0	178.0	152.0	233.0	390.0
Sr	1.6000	1.4000	0.1500	0.1600	0.2010	0.0800
V	BD	BD	0.0036	0.0130	0.0330	BD
Zn	0.0023	0.0022	0.0038	0.0042	0.0810	0.0010
NH4	0.71	0.73	281.00	263.00	571.00	1.00
NO3	BD	BD	BD	0.14700	BD	BD
H2S	BD	BD	2.5250	2.0708	4.5625	0.0219
F	3.2200	2.6500	0.5240	0.8700	0.3220	1.0000
Br	0.6100	0.5880	0.1020	0.1020	0.1020	0.0100
NO2	0.0128	0.0106	0.1215	0.0204	0.0005	ND
Cr	BD	BD	0.0016	0.0018	BD	BD
Cs	0.1800	0.1700	0.0027	0.0020	BD	BD
Mo	0.0015	0.0006	0.0007	0.0007	BD	BD
Northing	4979165.3	4979335.9	4956962.3	4956993.1	4956978.2	4951893.9
Easting	522886.6	523209.3	545084.6	4956993.1	545101.3	523198.8

Sample ID	03YNOR007	03YNOR008	03YNOR009	03YNOR010	03YMUD011	03YMUD012
Date	5/31/03	5/31/03	6/5/03	6/1/03	6/5/03	6/5/03
Temperature	73.0	71.7	90.5	36.7	74.3	34.8
pH	3.61	7.46	6.10	3.31	1.68	1.89
Conductivity	1175	2370	2150	1469	6725	5350
DO	0	0	0	0	0	0
DOC	0	0	0	0.3	8.1	4.3
Ca	4.30	3.10	ND	2.58	53.00	40.00
K	54.00	65.00	BD	18.20	26.00	28.00
Na	190.13	398.01	ND	211.00	16.00	74.00
Mg	0.50	0.05	ND	0.05	14.00	22.00
Alkalinity as HCO3-	BD	49.1	ND	BD	BD	BD
SO4	267.0	65.5	ND	71.4	3172.0	1323.0
Cl	144.00	559.00	ND	411.00	2.72	100.00
Al	2.000	0.750	BD	BD	120.000	37.000
As	0.0844	2.5500	ND	0.0760	0.0034	0.0166
As(III)	0.0112	2.4200	ND	0.0760	0.0031	0.0124
Ba	0.0650	0.0180	BD	BD	0.0860	0.0490
Be	0.00790	0.00340	BD	BD	0.00890	0.00120
Cd	BD	BD	BD	BD	0.00039	0.00009
Co	0.00003	BD	BD	BD	0.00480	0.00600
Cu	0.00250	0.00180	BD	BD	0.00280	0.00340
Fe	1.270	0.261	ND	0.271	24.800	21.100
Fe(II)	0.972	0.259	ND	0.268	24.600	15.000
Li	0.9500	5.6000	BD	BD	0.0300	0.0320
Mn	0.2600	0.0160	BD	BD	0.7800	1.4000
Ni	BD	BD	BD	BD	0.0180	0.0190
P	BD	0.0064	BD	BD	0.2400	0.2000
Pb	0.0001	0.0001	BD	BD	0.0150	0.0014
Sb	0.0029	0.1100	BD	BD	0.0002	0.0002
Se	0.0018	0.0067	BD	BD	BD	0.0012
SiO2	309.0	389.0	400.0	152.0	456.0	247.0
Sr	0.0083	0.0092	BD	BD	0.2500	0.3900
V	BD	BD	BD	BD	0.0460	0.0280
Zn	0.0140	0.0065	BD	BD	0.2000	0.0520
NH4	1.05	0.64	ND	0.86	0.89	4.51
NO3	BD	6.15700	ND	0.11000	0.27900	BD
H2S	BD	BD	0.1537	BD	BD	0.0016
F	4.8000	4.7100	ND	2.1600	2.7900	0.6240
Br	0.5130	1.8200	ND	1.2000	0.1020	0.3700
NO2	0.0022	0.0016	ND	BD	BD	BD
Cr	BD	BD	BD	BD	0.0510	0.0450
Cs	0.0830	0.4200	BD	BD	0.0095	0.0038
Mo	0.0063	0.1100	BD	BD	0.0005	0.0006
Northing	4952118	4952119	4952350	4952901	4941783	4941233
Easting	523592	523114	523266	522783	544980	545083

Sample ID	03YLOW013	03YLOW014	03YLOW015	03YLOW016	03YLOW017	03YLOW018
Date	6/6/03	6/6/03	6/6/03	6/6/03	6/6/03	6/6/03
Temperature	94.7	84.0	75.9	67.6	62.0	57.6
pH	8.84	8.91	8.96	9.05	9.09	9.14
Conductivity	1350	1391	1422	1431	1454	1481
DO	0	0	0	0	0	0
DOC	0.4	0	0	0	0	0
Ca	0.34	ND	BD	BD	BD	BD
K	12.40	BD	9.80	10.00	BD	10.10
Na	305.42	ND	302.00	302.00	281.00	302.00
Mg	BD	ND	BD	BD	BD	BD
Alkalinity as HCO3-	376.5	306.8	310.1	313.0	314.8	319.3
SO4	19.5	15.3	15.7	16.2	16.3	16.5
Cl	235.00	266.00	266.00	262.00	256.00	266.00
Al	0.290	BD	BD	BD	BD	BD
As	1.0400	1.0600	1.0800	1.1400	1.1400	1.1600
As(III)	0.4760	1.0600	1.0800	1.1400	1.1400	1.1600
Ba	0.0003	BD	BD	BD	BD	BD
Be	0.00110	BD	BD	BD	BD	BD
Cd	BD	BD	BD	BD	BD	BD
Co	BD	BD	BD	BD	BD	BD
Cu	0.00110	BD	BD	BD	BD	BD
Fe	0.006	0.006	0.006	0.004	0.004	0.004
Fe(II)	0.004	0.005	0.005	0.004	0.003	0.003
Li	1.6000	BD	BD	BD	1.6200	1.6800
Mn	BD	BD	BD	BD	BD	BD
Ni	BD	BD	BD	BD	0.0030	0.0020
P	BD	BD	BD	BD	BD	BD
Pb	BD	BD	BD	BD	BD	BD
Sb	0.0370	BD	BD	BD	BD	BD
Se	0.0024	BD	BD	BD	BD	BD
SiO2	354.0	276.0	304.0	249.0	244.0	294.0
Sr	0.0003	BD	BD	BD	BD	BD
V	0.0002	BD	BD	BD	BD	BD
Zn	0.0008	BD	BD	BD	BD	BD
NH4	0.63	0.36	0.40	0.58	0.36	0.35
NO3	0.27000	BD	BD	BD	BD	BD
H2S	0.4595	ND	0.2303	0.1841	0.1438	0.1044
F	25.2000	ND	26.7000	28.0000	28.2000	28.7000
Br	0.8370	ND	0.8770	0.8930	0.8750	0.9100
NO2	0.2391	ND	0.0010	0.0013	0.0010	0.0012
Cr	BD	ND	BD	BD	BD	BD
Cs	0.0660	ND	BD	BD	BD	BD
Mo	0.0200	ND	BD	BD	BD	BD
Northing	4934624	4934624	4934624	4934624	4934624	4934624
Easting	511119	511119	511119	511119	511119	511119

Sample ID	03YLOW019	03YNOR020	03YNOR021	03YNOR022	03YGIB023	03YGIB024
Date	6/6/03	6/12/03	6/13/03	6/13/03	6/16/03	6/17/03
Temperature	54.0	70.5	84.5	59.2	73.8	62.4
pH	9.19	6.76	4.34	3.00	8.51	6.73
Conductivity	1481	1947	2130	1972	2230	875
DO	0	0	0	0	0	0
DOC	0	0	0.3	0.6	0.0	0.3
Ca	BD	2.10	4.30	2.20	2.80	3.20
K	10.50	40.00	44.00	50.00	16.00	20.00
Na	309.00	328.93	382.24	265.79	457.34	160.54
Mg	BD	0.11	0.02	0.06	BD	0.01
Alkalinity as HCO3-	317.9	20.6	BD	BD	115.3	202.3
SO4	16.6	43.8	73.3	139.0	107.0	90.6
Cl	263.00	488.00	599.00	399.00	537.00	69.80
Al	BD	0.086	0.910	2.300	0.079	0.160
As	1.1600	2.1000	2.1600	0.2000	2.4560	0.1800
As(III)	1.1600	0.6100	2.1600	0.2000	0.9210	0.0110
Ba	BD	0.0130	0.0190	0.0750	0.0028	0.0044
Be	BD	0.00360	0.00380	0.00370	0.00390	0.00100
Cd	BD	BD	BD	BD	BD	BD
Co	BD	BD	BD	BD	BD	BD
Cu	BD	0.00230	0.00140	0.00200	0.00210	0.00300
Fe	0.005	0.243	0.033	0.743	0.020	0.002
Fe(II)	0.003	0.100	0.031	0.738	0.017	BD
Li	1.7000	3.4000	3.8000	3.9000	6.1000	0.4800
Mn	BD	0.0400	0.0031	0.0072	0.0012	0.0094
Ni	0.0020	BD	BD	0.0002	BD	BD
P	BD	BD	0.0020	0.0085	0.0040	0.0066
Pb	BD	0.0001	BD	0.0002	0.0001	0.0001
Sb	BD	0.0600	0.0600	0.0034	0.1200	0.0120
Se	BD	0.0052	0.0072	0.0056	0.0069	0.0017
SiO2	308.0	409.0	396.0	237.0	222.0	253.0
Sr	BD	0.0088	0.0140	0.0140	0.0028	0.0041
V	BD	BD	BD	BD	BD	BD
Zn	BD	0.0071	0.0160	0.0470	0.0820	0.0800
NH4	0.47	0.34	7.19	0.73	0.58	0.20
NO3	BD	BD	BD	BD	BD	BD
H2S	0.0753	0.0312	0.3266	6.3083	0.0227	0.0065
F	28.6000	6.4000	8.7000	4.6400	18.9000	11.0000
Br	0.9050	1.7600	2.0100	1.3800	1.7600	0.3020
NO2	0.0012	0.0095	0.0029	0.0050	0.0050	0.1503
Cr	BD	BD	BD	BD	BD	BD
Cs	BD	0.3100	0.4300	0.3000	0.4800	0.0400
Mo	BD	0.1100	0.1000	0.0023	0.0870	0.0034
Northing	4934624	4952739	4953268	4953213	4948812	4950750
Easting	511119	523505	522980	523295	520790	525170

Sample ID	03YLOW025	03YLOW026	03YLOW027	03YLOW028	03YVIO029	03YGAB030
Date	6/6/03	6/6/03	6/18/03	6/18/03	6/23/03	6/24/03
Temperature	83.4	91.0	86.2	64.2	68.5	54.2
pH	8.68	8.83	8.43	3.20	2.18	7.12
Conductivity	1512	1520	1487	494	4220	1010
DO	0	0	0	0	0	0
DOC	0	0	0.1	1.6	3.1	0
Ca	1.15	0.86	1.20	2.60	3.30	13.00
K	14.31	12.16	12.00	25.00	16.00	38.00
Na	337.00	316.00	323.20	45.16	9.10	>400
Mg	BD	BD	0.01	0.90	2.30	2.80
Alkalinity as HCO3-	210.0	220.0	210.1	BD	BD	78.2
SO4	36.7	27.7	28.8	126.0	1350.0	191.0
Cl	301.82	297.12	320.00	34.50	2.86	98.30
Al	BD	BD	0.120	2.400	36.000	0.038
As	1.5093	1.1598	1.1200	0.4090	0.0100	0.2240
As(III)	1.5093	1.1440	1.1100	0.1970	0.0080	0.0100
Ba	BD	BD	0.0044	0.0850	0.0670	0.0360
Be	BD	BD	0.00160	0.00370	0.00200	0.00130
Cd	BD	BD	BD	0.00004	0.00009	0.00007
Co	BD	BD	BD	0.00008	0.00300	0.00002
Cu	BD	BD	0.00260	0.00300	0.00300	0.00190
Fe	0.037	0.036	0.002	6.440	19.000	0.017
Fe(II)	0.037	0.036	0.002	6.420	18.900	0.017
Li	2.8845	3.1133	2.0000	0.4100	0.0100	0.8000
Mn	BD	BD	0.0004	0.1400	0.1300	0.3400
Ni	BD	BD	BD	0.0004	0.0110	BD
P	BD	BD	0.0099	0.0280	0.1400	0.0200
Pb	0.0011	BD	0.0001	0.0006	0.0038	0.0001
Sb	BD	BD	0.0780	0.0064	0.0002	0.0009
Se	BD	BD	0.0043	0.0007	0.0000	0.0016
SiO2	253.1	244.8	265.0	263.0	213.0	153.0
Sr	BD	BD	0.0085	0.0220	0.1300	0.0400
V	BD	BD	BD	0.0014	0.0330	0.0007
Zn	BD	BD	0.1000	0.1500	0.0520	0.0380
NH4	0.16	0.30	0.32	0.73	7.87	BD
NO3	BD	BD	BD	BD	BD	BD
H2S	BD	BD	0.0789	0.9250	2.6333	ND
F	BD	BD	35.6000	4.0000	0.5156	9.7800
Br	BD	BD	1.1000	0.2030	BD	0.3810
NO2	BD	BD	0.0026	0.0010	0.0019	0.0300
Cr	BD	BD	BD	BD	0.0320	BD
Cs	BD	BD	0.2900	0.1400	0.0060	0.1100
Mo	BD	BD	0.0630	0.0034	0.0007	0.0160
Northing	4934192	4934123	4934078	4934044	4944381	4940181
Easting	513275	513196	513246	513372	534054	532693

Sample ID	03YGAB031	03YHHS032	03YVIO033	03YCOF034	03YCOF035	03YJOS036
Date	6/24/03	6/24/03	6/25/03	7/3/03	7/3/03	7/4/03
Temperature	79.4	77.1	57.9	66.2	65.2	52.4
pH	6.29	2.16	7.21	6.26	5.75	6.15
Conductivity	1273	311	1185	831	555	152
DO	0	0	0	0	0	0
DOC	0.0	2.0	0.1	0.9	10.4	0.0
Ca	19.00	2.30	13.00	1.40	5.60	14.00
K	41.00	27.00	59.00	8.30	10.00	46.00
Na	>400	23.76	286.02	5.06	26.76	108.84
Mg	1.10	0.37	6.70	0.13	2.60	0.76
Alkalinity as HCO3-	269.5	BD	368.3	75.3	7.8	379.5
SO4	136.0	1080.0	319.0	252.0	195.0	27.6
Cl	192.00	2.69	92.80	1.04	0.93	2.86
Al	0.016	23.000	0.005	0.150	0.360	0.004
As	0.3000	0.0020	0.3740	BD	0.0020	0.0010
As(III)	0.1780	0.0010	0.2200	BD	0.0010	BD
Ba	0.0530	0.0300	0.1300	0.0360	0.0350	0.2300
Be	0.00730	0.00370	0.00340	0.00005	0.00078	0.00350
Cd	0.00024	0.00007	BD	BD	BD	BD
Co	0.00000	0.00032	BD	BD	0.00004	BD
Cu	0.00250	0.00400	0.00180	0.00220	BD	BD
Fe	0.232	6.430	0.708	0.021	5.420	0.008
Fe(II)	0.155	6.130	0.043	0.020	5.420	0.007
Li	0.8800	0.0120	1.1000	0.0022	0.0470	0.2700
Mn	0.8800	0.1400	0.2600	0.0320	0.2000	0.2500
Ni	BD	0.0010	BD	BD	0.0004	BD
P	0.0056	0.0240	0.0320	0.0000	0.0140	0.0079
Pb	0.0002	0.0042	BD	BD	BD	BD
Sb	0.0003	0.0001	0.0006	0.0001	0.0001	BD
Se	0.0029	0.0000	0.0011	BD	BD	BD
SiO2	188.0	292.0	275.0	111.0	91.0	159.0
Sr	0.0290	0.0170	0.0630	0.0099	0.0460	0.1800
V	BD	0.0010	BD	BD	0.0005	BD
Zn	0.0280	0.0760	0.1100	0.0130	0.0570	0.0026
NH4	BD	0.88	0.78	103.00	39.00	7.64
NO3	BD	BD	BD	BD	BD	BD
H2S	ND	0.1074	BD	0.3918	0.0921	10.2917
F	12.1000	0.3310	13.2000	0.7440	0.9020	7.1000
Br	0.6310	BD	0.3380	BD	BD	BD
NO2	0.0107	0.0011	0.0150	0.0040	0.0033	0.0026
Cr	BD	BD	BD	BD	BD	BD
Cs	0.0620	0.0025	0.0380	0.0014	0.0024	0.0200
Mo	0.0660	0.0013	0.0070	0.0006	0.0003	0.0002
Northing	4940631	4939241	4944084	4955829	4955984	4954215
Easting	532910	530202	535431	554762	554771	552568

Sample ID	03YJOS037	03YJOS038	03YHSB039	03YHSB040	03YHSB041	03YHSB042
Date	7/5/03	7/5/03	7/8/03	7/8/03	7/9/03	7/9/03
Temperature	89.6	67.3	55.4	80.0	82.4	64.5
pH	2.66	6.04	3.42	2.63	6.11	5.90
Conductivity	1136	1680	337	2220	786	1678
DO	0	0	0	0	0	0
DOC	1.4	0.0	2.5	1.4	3.4	0.6
Ca	7.20	46.00	2.00	10.00	8.50	20.00
K	30.00	62.00	10.00	66.00	11.00	53.22
Na	26.99	225.42	14.32	86.46	10.28	139.01
Mg	1.50	4.70	0.92	2.80	0.94	3.90
Alkalinity as HCO3-	BD	144.0	BD	BD	100.7	39.9
SO4	505.0	242.0	101.0	1070.0	232.0	701.0
Cl	1.87	299.00	0.90	2.49	1.65	1.97
Al	5.100	0.006	1.100	4.400	0.890	0.067
As	0.0290	1.2400	0.0020	0.0510	0.0930	BD
As(III)	0.0250	1.2400	0.0010	0.0350	0.0910	BD
Ba	0.0340	0.0590	0.0610	0.0420	0.2700	0.0640
Be	0.00450	0.00220	0.00054	0.00350	0.00016	0.00052
Cd	0.00020	BD	BD	0.00006	0.00002	BD
Co	0.00027	BD	0.00003	0.00010	0.00100	BD
Cu	0.00054	0.00160	0.00280	0.00084	0.00480	0.00320
Fe	6.250	0.023	0.433	9.450	0.020	0.007
Fe(II)	6.230	0.022	0.431	9.440	0.019	0.007
Li	0.0150	1.8000	0.0066	0.0160	0.0070	0.0340
Mn	0.2500	0.8400	0.0320	0.1600	0.0900	0.2400
Ni	0.0014	BD	0.0003	0.0007	0.0028	BD
P	0.0120	0.0070	0.0010	0.0270	0.1300	0.0640
Pb	0.0003	BD	0.0002	0.0008	0.0049	BD
Sb	0.0004	0.0004	BD	0.0002	0.0008	BD
Se	BD	0.0035	BD	BD	0.0023	BD
SiO2	197.0	134.0	155.0	223.0	171.0	234.0
Sr	0.0850	0.3500	0.0300	0.0900	0.1300	0.1800
V	0.0016	BD	0.0008	0.0034	0.0009	0.0005
Zn	0.1300	0.0370	0.0093	0.0790	0.0130	0.0390
NH4	19.20	24.80	2.80	95.80	91.30	49.10
NO3	BD	BD	BD	BD	BD	BD
H2S	0.2842	BD	4.0750	BD	4.6083	4.6500
F	0.6310	2.6500	0.2690	0.7810	0.4120	1.1700
Br	BD	1.0100	BD	BD	BD	BD
NO2	0.0007	0.0006	0.0004	0.0005	0.0228	0.0006
Cr	BD	BD	BD	BD	BD	BD
Cs	0.0062	0.0810	0.0016	0.0150	0.0046	0.0270
Mo	0.0002	0.0001	0.0002	0.0002	0.0013	0.0001
Northing	4954164	4954349	4954641	4955418	4955955	4955653
Easting	553377	553828	559724	558907	558511	558889

Sample ID	03YRNB043	03YRNB044	03YMRY045	03YMRY046	03YMRY047	03YMKL048
Date	7/10/03	7/10/03	8/6/03	8/7/03	8/7/03	8/10/03
Temperature	73.3	54.5	69.3	86.6	80.7	48.9
pH	6.23	3.23	7.68	6.80	4.32	2.00
Conductivity	1500	1415	2230	434	1260	5660
DO	0	0	0	0	0	0
DOC	0.4	0.6	0.0	4.9	0	31.3
Ca	21.00	16.00	6.20	11.00	6.00	34.00
K	59.00	78.00	19.00	12.00	4.20	37.00
Na	277.02	102.58	463.46	66.32	4.60	37.85
Mg	8.60	5.20	0.01	6.70	4.00	13.00
Alkalinity as HCO3-	296.1	BD	82.6	53.7	BD	BD
SO4	340.0	638.0	157.0	130.0	542.0	2611.0
Cl	99.80	6.73	588.00	9.03	3.67	1.35
Al	0.004	16.000	0.057	0.260	0.047	110.000
As	0.2190	0.1980	1.8700	0.0050	0.0380	0.0240
As(III)	0.2110	0.0390	0.0900	BD	0.0250	0.0240
Ba	0.0730	0.0360	0.0044	0.0940	0.0400	0.0550
Be	0.00120	0.01000	0.00310	BD	0.00022	0.00220
Cd	BD	0.00025	BD	BD	0.00006	0.00018
Co	BD	0.00140	BD	BD	BD	0.00780
Cu	BD	0.00350	BD	0.00094	0.00098	0.00170
Fe	0.009	BD	0.001	0.003	1.400	48.200
Fe(II)	0.009	BD	BD	0.003	0.359	48.200
Li	0.5500	0.2700	6.2000	0.0730	0.0047	0.0220
Mn	0.2300	0.2800	0.0006	0.0170	0.1300	0.5000
Ni	BD	0.0070	BD	BD	0.0005	0.0310
P	0.0190	0.0190	0.0010	0.1100	0.0170	1.6000
Pb	BD	0.0001	BD	BD	0.0005	0.0071
Sb	BD	0.0001	0.0910	0.0005	0.0017	BD
Se	0.0015	BD	0.0083	0.0006	BD	0.0006
SiO2	393.0	259.0	276.0	148.0	237.0	200.0
Sr	0.1300	0.2800	0.2700	0.1200	0.0190	1.1000
V	0.0003	0.0140	0.0028	0.0007	0.0009	0.1000
Zn	0.0500	0.1300	0.0330	0.0250	0.0670	0.0950
NH4	13.46	49.70	BD	2.69	BD	184.24
NO3	BD	BD	0.15000	0.16000	0.23000	0.18000
H2S	0.1035	0.0019	BD	0.5926	1.1750	0.2602
F	2.5700	2.1600	18.2800	0.9100	0.2500	0.3000
Br	0.3500	BD	2.3500	0.1000	BD	BD
NO2	0.0205	0.0014	BD	0.0377	BD	0.0025
Cr	BD	0.0050	BD	BD	BD	0.1500
Cs	0.0440	0.0510	0.6200	0.0029	0.0071	0.0180
Mo	0.0009	0.0002	0.0810	0.0021	0.0006	0.0003
Northing	4957515	4957520	4929959	4933541	4933572	4942771
Easting	557702	557675	557393	555331	555234	561100

Sample ID	03YMKL049	04YAPT001	04YGIB002	04YWNR003	04YWNR004	04YCRT005
Date	8/10/03	6/3/04	6/7/04	6/8/04	6/8/04	6/9/04
Temperature	72.3	70.8	62.3	80.0	57.0	77.1
pH	4.35	2.20	7.17	2.76	7.07	2.59
Conductivity	1170	2250	1772	890	1821	3550
DO	0	0	0	0	0	0
DOC	1.7	0	0	0	0	0
Ca	46.00	7.24	3.95	6.83	10.10	13.90
K	23.00	33.00	21.90	11.90	9.73	85.10
Na	137.64	52.57	310.06	16.75	339.48	355.25
Mg	19.00	2.40	0.09	1.87	0.10	10.40
Alkalinity as HCO3-	BD	BD	55.0	BD	110.0	BD
SO4	571.0	684.0	107.0	241.0	41.0	840.0
Cl	1.82	8.12	385.00	1.26	405.00	440.00
Al	0.430	17.200	0.302	3.890	0.136	22.900
As	0.0020	0.0600	2.0500	BD	1.4900	2.7400
As(III)	0.0010	0.0360	0.0500	BD	0.3080	2.3520
Ba	0.0830	0.0168	0.0119	0.0823	0.0121	0.0185
Be	0.00025	0.00190	0.00100	0.00170	0.00320	0.00770
Cd	BD	0.00004	0.00009	0.00008	0.00023	0.00034
Co	0.00003	0.00023	0.00004	0.00007	0.00003	0.00020
Cu	0.00063	0.00060	BD	BD	BD	0.00091
Fe	0.264	3.720	0.147	0.582	0.008	2.140
Fe(II)	0.252	3.720	0.010	0.582	0.008	2.140
Li	0.0720	0.0450	3.0700	0.0286	0.3960	2.6300
Mn	0.2900	0.1300	0.0187	0.2410	0.0631	0.8060
Ni	BD	0.0012	BD	0.0006	BD	0.0023
P	0.1300	BD	BD	BD	BD	BD
Pb	0.0001	0.0006	0.0001	0.0004	BD	0.0004
Sb	BD	0.0013	0.1180	BD	0.0535	0.0073
Se	BD	BD	0.0048	BD	0.0046	0.0066
SiO2	282.0	313.0	200.0	130.0	263.0	409.0
Sr	1.1000	0.0228	0.0054	0.0213	0.0161	0.1600
V	0.0007	0.0040	BD	BD	BD	0.0151
Zn	0.0780	0.0475	0.0142	0.0294	0.0069	0.0824
NH4	33.06	3.29	BD	4.45	0.59	13.83
NO3	BD	BD	0.61000	BD	0.12000	BD
H2S	1.6250	2.0333	BD	0.6485	BD	0.7500
F	0.5000	0.7830	13.6000	0.7200	23.1000	15.4000
Br	BD	BD	1.2400	BD	1.3600	1.3700
NO2	0.0013	BD	BD	BD	BD	BD
Cr	BD	BD	BD	BD	BD	0.0042
Cs	0.0091	0.0116	0.4820	0.0013	0.0725	0.4330
Mo	0.0003	BD	0.0798	BD	0.2130	0.0038
Northing	4942444	4960849	4948840	4954627	4953947	4944663
Easting	531365	521453	520782	520321	520343	540925

Sample ID	04YCRT006	04YUPP007	04YLST008	04YLST009	04YLST010	04YLST011
Date	6/9/04	6/14/04	6/15/04	6/15/04	6/15/04	6/16/04
Temperature	79.9	53.2	56.3	79.0	73.6	60.5
pH	2.47	8.23	5.69	3.08	7.90	3.04
Conductivity	2310	1059	1025	562	1253	914
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	32.20	2.87	6.69	2.90	3.86	2.50
K	20.40	31.50	27.30	16.50	10.80	27.00
Na	38.91	205.99	188.94	10.38	208.17	57.68
Mg	13.40	0.07	0.34	0.66	0.15	0.44
Alkalinity as HCO3-	BD	311.0	121.0	BD	55.0	BD
SO4	813.0	9.4	35.3	140.0	16.5	142.0
Cl	2.99	126.00	200.00	2.22	277.00	73.60
Al	11.400	0.029	0.087	1.960	0.249	3.060
As	0.0170	0.6510	0.3180	0.0060	0.8740	0.0470
As(III)	0.0130	0.0140	0.2740	0.0030	0.2550	0.0300
Ba	0.0686	0.0027	0.0384	0.0980	0.0094	0.0924
Be	0.00270	0.00050	0.00430	0.00200	0.00050	0.00160
Cd	0.00012	0.00002	BD	0.00008	0.00012	0.00003
Co	0.00432	BD	BD	0.00034	0.00007	0.00006
Cu	0.00083	BD	BD	0.00054	BD	BD
Fe	12.400	BD	0.051	0.691	0.015	1.320
Fe(II)	12.400	BD	0.050	0.641	0.004	1.200
Li	0.0410	1.1600	0.8930	0.0555	1.1000	0.0779
Mn	0.7040	0.0463	0.2250	0.1750	0.0308	0.0822
Ni	0.0231	BD	BD	0.0006	BD	BD
P	BD	BD	BD	BD	BD	BD
Pb	0.0014	BD	BD	0.0052	0.0002	0.0013
Sb	BD	0.0213	0.0007	BD	0.0775	BD
Se	BD	0.0023	0.0026	BD	0.0037	BD
SiO2	391.0	269.0	226.0	167.0	184.0	222.0
Sr	0.0870	0.0021	0.0178	0.0135	0.0088	0.0153
V	0.0104	BD	BD	BD	0.0010	BD
Zn	0.1280	0.0060	0.0034	0.0629	0.0057	0.0394
NH4	8.81	BD	0.16	2.16	BD	0.67
NO3	0.12700	0.10700	BD	0.16200	0.16300	BD
H2S	BD	BD	2.5833	BD	0.0213	0.0863
F	0.5060	20.6000	11.0000	0.3900	16.0000	1.3500
Br	BD	0.4390	0.6630	BD	0.9270	0.3190
NO2	BD	BD	BD	BD	BD	BD
Cr	0.0062	BD	BD	BD	BD	BD
Cs	0.0128	0.0559	0.1150	0.0210	0.2660	0.0320
Mo	BD	0.0240	BD	BD	0.1180	0.0020
Northing	4944569	4925316	4918176	4917990	4918086	4919243
Easting	540856	511887	515216	514898	515156	515189

Sample ID	04YUPP012	04YUPP013	04YMID014	04YMID015	04YMID016	04YFOR017
Date	6/17/04	6/17/04	6/22/04	6/22/04	6/22/04	6/23/04
Temperature	78.4	60.3	74.6	78.7	67.6	45.9
pH	8.80	9.02	8.47	4.28	6.48	2.30
Conductivity	2260	2260	1475	298	363	2690
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	0.71	0.60	1.00	1.80	0.60	17.30
K	24.10	24.60	14.30	22.90	14.40	15.40
Na	413.81	459.98	330.60	38.66	72.92	26.07
Mg	BD	BD	BD	0.09	0.04	10.80
Alkalinity as HCO3-	407.0	610.0	380.0	BD	53.0	BD
SO4	19.1	17.7	13.8	113.0	103.0	678.0
Cl	379.00	303.00	260.00	1.66	1.78	1.80
Al	0.554	0.212	0.142	1.340	0.023	11.500
As	1.8600	1.5000	1.5000	0.0020	0.0060	0.0340
As(III)	0.2200	0.0340	0.4500	0.0010	0.0010	0.0290
Ba	0.0026	0.0027	0.0048	0.0190	0.0094	0.0514
Be	0.00140	0.00160	0.00160	0.00220	0.00060	0.00290
Cd	0.00006	0.00005	0.00004	0.00003	BD	0.00007
Co	0.00002	0.00002	0.00002	0.00009	BD	0.00344
Cu	BD	BD	BD	BD	BD	BD
Fe	BD	0.005	0.004	0.271	0.003	8.890
Fe(II)	BD	BD	0.004	0.219	BD	8.890
Li	3.3000	2.4600	2.8900	0.0531	0.1630	0.0933
Mn	0.0005	0.0012	0.0052	0.0800	0.0182	0.5010
Ni	BD	BD	BD	BD	BD	0.0118
P	BD	BD	BD	BD	0.0001	0.0002
Pb	BD	0.0001	0.0001	0.0004	0.0001	0.0024
Sb	0.1130	0.0607	0.0886	BD	0.0008	0.0033
Se	0.0052	0.0035	0.0034	BD	BD	BD
SiO2	416.0	370.0	239.0	253.0	202.0	205.0
Sr	0.0038	0.0025	0.0030	0.0029	0.0017	0.1890
V	0.0019	0.0007	0.0007	BD	BD	0.0123
Zn	0.0097	0.0092	0.0074	0.0313	0.0505	0.0518
NH4	0.00	0.00	0.43	5.28	0.06	0.68
NO3	BD	BD	0.11300	0.15100	BD	0.16100
H2S	0.2555	0.0032	0.0339	0.0379	0.0066	BD
F	36.0000	34.8000	26.2000	4.3000	6.4100	0.8900
Br	1.2500	0.9740	0.8200	BD	BD	BD
NO2	BD	BD	BD	BD	BD	BD
Cr	BD	BD	BD	BD	BD	0.0073
Cs	0.4780	0.1540	0.3500	0.0039	0.0185	0.0595
Mo	0.0568	0.0542	0.0308	0.0032	0.0097	BD
Northing	4923398	4923083	4928875	4928382	4929579	4951164
Easting	513006	511515	514754	515181	515456	541478

Sample ID	04YFOR018	04YSMH019	04YSMH020	04YSMH021	04YSMH022	04YSMH023
Date	6/23/04	6/29/04	6/29/04	6/29/04	6/29/04	6/30/04
Temperature	79.4	72.7	55.8	82.7	48.2	71.1
pH	2.61	3.08	4.05	8.00	8.62	2.91
Conductivity	892	1528	1538	1798	2170	1101
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	9.98	55.40	88.80	0.82	7.56	5.66
K	10.80	44.40	30.00	58.60	59.20	78.40
Na	13.75	132.60	152.53	362.51	411.04	73.35
Mg	10.10	20.60	29.10	BD	0.02	3.36
Alkalinity as HCO3-	BD	BD	BD	207.0	88.0	BD
SO4	408.0	399.0	306.0	109.0	191.0	317.0
Cl	44.10	176.00	272.00	380.00	470.00	57.80
Al	17.100	2.140	0.263	0.117	0.106	2.730
As	0.0370	0.1710	0.2000	1.5300	2.2300	0.1840
As(III)	0.0310	0.0040	0.0050	1.5300	0.6610	0.0650
Ba	0.1230	0.0413	0.0730	0.0223	0.0160	0.0568
Be	0.00310	0.00200	0.00090	0.00060	0.00060	0.00430
Cd	0.00051	0.00005	0.00008	0.00005	0.00004	0.00006
Co	0.00279	0.00120	0.00293	BD	0.00004	0.00056
Cu	0.00056	0.00065	0.00090	BD	BD	BD
Fe	9.070	0.263	0.009	0.026	0.021	4.429
Fe(II)	9.070	0.039	0.003	0.026	0.021	2.723
Li	0.0458	0.4670	0.6110	2.6200	3.4700	0.4640
Mn	0.3530	0.7340	1.0700	0.0003	0.0020	0.1230
Ni	0.0112	0.0044	0.0100	BD	BD	0.0022
P	0.0001	BD	BD	BD	BD	BD
Pb	0.0006	0.0001	BD	BD	0.0001	0.0003
Sb	0.0011	0.0031	0.0006	0.1080	0.1240	0.0084
Se	BD	0.0021	0.0035	0.0049	0.0062	0.0016
SiO2	239.0	220.0	190.0	530.0	180.0	227.0
Sr	0.0735	0.5570	1.0100	0.0302	0.2690	0.0682
V	0.0373	BD	BD	0.0028	BD	BD
Zn	0.2000	0.0478	0.0308	0.0146	0.0042	0.0417
NH4	6.86	14.63	16.90	2.20	1.74	11.84
NO3	0.30300	3.66000	37.00000	BD	0.44900	0.13500
H2S	1.2500	0.0144	BD	3.1667	0.0589	0.0068
F	0.9170	1.3500	0.5180	13.9000	14.1000	1.9400
Br	BD	0.6380	0.9070	1.4200	1.5600	0.2830
NO2	BD	BD	0.0530	0.0010	0.0230	0.0010
Cr	0.0093	BD	BD	BD	BD	BD
Cs	0.0130	0.0407	0.0480	0.2680	0.4460	0.0456
Mo	BD	BD	BD	0.0542	0.0412	BD
Northing	4951135	4955776	4955891	4955514	4955139	4955678
Easting	541480	546216	546235	546142	546463	546136

Sample ID	04YSMH024	04YLEW025	04YLEW026	04YSMJ027	04YSMJ028	04YSMJ029
Date	6/30/04	7/7/04	7/7/04	7/13/04	7/13/04	7/13/04
Temperature	69.7	59.0	77.0	56.2	87.8	67.7
pH	7.21	7.68	7.51	3.20	2.39	1.94
Conductivity	2050	0	0	567	1264	4470
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	47.10	5.04	3.41	2.80	0.91	0.72
K	26.10	14.00	20.20	34.80	10.20	7.89
Na	335.39	108.19	136.67	29.70	3.89	5.81
Mg	9.93	0.09	0.04	0.28	0.17	0.19
Alkalinity as HCO3-	172.0	172.0	170.0	BD	BD	BD
SO4	161.0	11.5	40.0	158.0	427.0	1930.0
Cl	457.00	47.00	78.00	1.91	1.65	2.96
Al	0.006	0.013	0.048	1.340	11.400	153.000
As	3.7000	BD	BD	BD	0.0070	BD
As(III)	3.6450	BD	BD	BD	0.0050	BD
Ba	0.0473	0.0227	0.0038	0.0899	0.0320	0.0352
Be	0.00010	0.00330	0.00430	0.00380	0.00110	0.00120
Cd	BD	BD	BD	0.00002	0.00005	0.00013
Co	0.00005	BD	BD	BD	0.00009	0.00075
Cu	BD	BD	BD	BD	BD	0.00190
Fe	0.017	0.016	0.012	2.272	1.456	2.088
Fe(II)	0.017	0.005	0.003	0.539	1.417	0.990
Li	1.8900	0.4740	0.5300	0.0178	0.0060	0.0063
Mn	0.2130	0.5640	0.0940	0.1580	0.0489	0.0134
Ni	0.0009	BD	BD	BD	0.0004	0.0013
P	BD	BD	BD	BD	BD	BD
Pb	BD	0.0001	BD	0.0008	0.0059	0.0059
Sb	0.0142	0.0007	0.0066	BD	BD	0.0003
Se	0.0047	BD	0.0016	0.0011	BD	0.0010
SiO2	187.0	177.0	254.0	271.0	226.0	332.0
Sr	0.9600	0.0068	0.0040	0.0064	0.0032	0.0037
V	BD	BD	BD	BD	0.0006	0.0104
Zn	0.0044	0.0164	0.0081	0.0423	0.0427	0.0296
NH4	21.89	0.06	BD	0.51	4.82	10.30
NO3	0.12400	0.14900	0.12200	0.81700	BD	BD
H2S	1.1667	0.0031	0.0035	0.0030	0.1489	0.0663
F	1.9800	17.4000	15.2000	1.9700	0.4640	BD
Br	1.5300	BD	0.3340	BD	BD	BD
NO2	0.0020	0.0010	0.0490	0.0010	0.0010	BD
Cr	BD	BD	BD	BD	BD	0.0111
Cs	0.2020	0.0319	0.0740	0.0029	0.0012	0.0008
Mo	BD	0.0095	BD	BD	BD	BD
Northing	4955775	4906902	4906562	4917244	4918771	4918667
Easting	547166	527874	527512	503295	503338	503762

Sample ID	04YSMJ030	04YVMS031	04YVMS032	04YWST033	04YWST034	04YHRT035
Date	7/13/04	7/15/04	7/15/04	7/25/04	7/25/04	7/27/04
Temperature	54.6	50.2	59.7	67.5	77.5	50.9
pH	2.43	2.69	2.64	8.14	7.66	3.03
Conductivity	2130	1073	1056	1190	1912	503
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	0.57	2.62	2.49	0.54	0.68	0.82
K	12.50	14.00	14.50	12.20	19.40	12.70
Na	34.77	15.81	24.45	261.31	425.69	8.81
Mg	0.08	1.08	0.99	0.04	0.03	0.28
Alkalinity as HCO3-	BD	BD	BD	400.0	526.0	BD
SO4	72.1	331.0	299.0	24.6	49.6	112.0
Cl	1.33	13.00	25.20	148.00	291.00	0.75
Al	1.190	33.400	24.600	0.109	0.159	0.769
As	BD	0.2260	0.1520	1.0000	1.7500	0.0040
As(III)	BD	0.0540	0.1320	0.2510	0.0530	0.0030
Ba	0.0097	0.0271	0.0146	0.0027	0.0046	0.1680
Be	0.00040	0.00080	0.00070	0.00330	0.00230	0.00020
Cd	BD	0.00005	0.00002	0.00005	0.00010	0.00004
Co	BD	0.00044	0.00007	BD	0.00004	0.00032
Cu	BD	BD	BD	BD	BD	0.00180
Fe	0.042	7.776	5.293	0.009	0.005	0.815
Fe(II)	0.041	0.068	5.252	0.002	0.003	0.350
Li	0.0446	0.2070	0.2880	1.6700	2.5700	0.0246
Mn	0.0128	0.0364	0.0352	0.0174	0.0027	0.0221
Ni	BD	0.0023	0.0008	BD	BD	0.0008
P	BD	BD	BD	0.0001	BD	BD
Pb	BD	BD	0.0001	BD	BD	0.0007
Sb	BD	0.0016	0.0013	0.0561	0.1320	0.0006
Se	0.0010	BD	0.0014	0.0021	0.0036	BD
SiO2	223.0	177.0	190.0	251.0	288.0	135.0
Sr	0.0019	0.0260	0.0180	0.0010	0.0054	0.0084
V	BD	0.0081	0.0086	BD	0.0008	BD
Zn	0.0038	0.0420	0.0382	0.0084	0.0144	0.0209
NH4	0.12	2.24	2.63	0.39	0.74	5.63
NO3	BD	BD	BD	BD	0.80400	BD
H2S	2.4167	BD	1.3333	0.0075	0.0115	BD
F	3.1300	0.9270	1.1700	17.4000	35.4000	0.1680
Br	BD	BD	BD	0.5460	0.9010	BD
NO2	0.0010	BD	BD	0.0010	0.0160	0.0020
Cr	BD	0.0036	0.0113	BD	BD	BD
Cs	0.0020	0.0191	0.0244	0.0736	0.3610	0.0037
Mo	BD	BD	0.0033	0.0511	0.1130	BD
Northing	4918098	4936869	4936772	4918651	4918148	4905990
Easting	503550	554437	554318	534025	534155	537694

Sample ID	04YHRT036	04YHRT037	04YHRT038	04YHRT039	04YHRT040	04YSHO041
Date	7/27/04	7/27/04	7/27/04	7/27/04	7/28/04	8/3/04
Temperature	84.8	61.1	57.8	69.3	67.4	77.0
pH	9.06	9.15	8.63	8.93	3.05	6.49
Conductivity	1660	900	1522	1500	464	1122
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	1.37	1.93	1.35	0.89	0.38	0.70
K	30.60	11.30	8.56	12.00	2.34	21.10
Na	343.88	194.73	334.58	329.81	2.90	242.58
Mg	BD	0.02	0.01	0.02	0.12	0.02
Alkalinity as HCO3-	251.0	128.0	279.0	254.0	BD	251.0
SO4	146.0	82.5	127.0	104.0	125.0	50.2
Cl	271.00	145.00	250.00	196.00	0.81	165.00
Al	0.159	0.074	0.172	0.206	2.390	0.050
As	1.0600	0.5910	1.1000	0.4460	0.0010	0.4000
As(III)	1.0520	0.2480	0.0380	0.1380	BD	0.4220
Ba	0.0016	0.0022	0.0018	0.0025	0.0541	0.0170
Be	0.00130	0.00030	0.00090	0.00090	0.00020	0.00260
Cd	0.00007	0.00004	0.00006	0.00006	BD	BD
Co	BD	BD	BD	BD	0.00010	BD
Cu	BD	BD	BD	BD	BD	BD
Fe	0.008	0.003	0.010	0.009	0.419	0.018
Fe(II)	0.006	0.002	0.007	0.005	0.256	0.016
Li	3.1600	2.0200	2.6700	3.4800	0.0014	0.5330
Mn	0.0004	0.0003	0.0005	0.0010	0.0100	0.0209
Ni	BD	BD	BD	BD	BD	BD
P	BD	BD	BD	BD	BD	BD
Pb	BD	BD	BD	BD	0.0004	BD
Sb	0.0556	0.0276	0.0436	0.0415	BD	0.0031
Se	0.0024	0.0016	0.0024	0.0020	BD	0.0039
SiO2	308.0	160.0	235.0	177.0	116.0	256.0
Sr	0.0177	0.0144	0.0285	0.0107	0.0027	0.0013
V	0.0020	0.0008	0.0045	0.0031	BD	BD
Zn	0.0105	0.0102	0.0125	0.0115	0.0225	0.0038
NH4	BD	BD	BD	BD	4.52	0.41
NO3	BD	0.39100	1.35000	0.25700	BD	0.10200
H2S	0.6542	0.0751	BD	0.0345	0.0451	4.5833
F	28.0000	16.0000	21.3000	18.6000	0.1690	25.3000
Br	1.0400	0.5460	0.8380	0.6620	BD	0.5490
NO2	0.0130	0.0060	0.1170	0.0220	0.0020	0.0010
Cr	BD	BD	BD	BD	BD	BD
Cs	0.3130	0.1390	0.3290	0.2860	0.0008	0.0295
Mo	0.0800	0.0447	0.0706	0.0731	BD	BD
Northing	4905827	4905824	4905025	4904299	4906448	4910921
Easting	538071	538066	538545	539046	537266	516276

Sample ID	04YSHO042	04YSHO043	04YSHO044	04YSHO045	04YSHO046	04YBEC047
Date	8/3/04	8/3/04	8/3/04	8/3/04	8/3/04	8/5/04
Temperature	53.8	91.5	72.5	59.4	80.9	80.4
pH	3.78	7.84	8.49	8.39	8.32	6.82
Conductivity	1397	1273	1402	1316	1365	1276
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	0.35	0.71	0.62	0.69	0.71	4.82
K	15.60	12.30	13.80	14.20	12.10	10.20
Na	276.77	302.14	326.21	299.26	314.30	317.94
Mg	0.04	BD	BD	0.01	BD	0.08
Alkalinity as HCO3-	BD	443.0	460.0	427.0	382.0	640.0
SO4	238.0	42.0	35.9	32.6	34.4	21.9
Cl	244.00	141.00	174.00	156.00	200.00	77.40
Al	4.340	0.195	0.311	0.250	0.098	0.011
As	0.5480	0.5550	0.6000	0.5050	0.8240	0.2000
As(III)	0.5150	0.5510	0.4820	0.0330	0.8200	0.0060
Ba	0.0474	0.0038	0.0008	0.0016	0.0029	0.0095
Be	0.00030	0.00190	0.00160	0.00170	0.00140	0.00210
Cd	0.00002	0.00003	0.00011	0.00005	0.00006	0.00004
Co	0.00004	BD	BD	BD	BD	BD
Cu	0.00063	0.00390	0.00058	BD	BD	BD
Fe	0.386	0.013	0.005	0.026	0.010	0.006
Fe(II)	0.385	0.004	0.001	0.018	0.007	0.002
Li	0.9530	1.2300	1.3800	1.2900	0.9360	1.0400
Mn	0.0075	0.0047	0.0012	0.0051	0.0119	0.0192
Ni	BD	BD	BD	BD	BD	BD
P	BD	BD	BD	BD	BD	BD
Pb	0.0001	0.0001	BD	0.0001	BD	BD
Sb	0.0015	0.0176	0.0269	0.0229	0.0347	0.0007
Se	0.0041	0.0035	0.0042	0.0049	0.0076	0.0078
SiO2	248.0	248.0	275.0	256.0	292.0	189.0
Sr	0.0018	BD	BD	0.0008	0.0006	0.0084
V	BD	BD	BD	BD	BD	BD
Zn	0.0072	0.0086	0.0060	0.0074	0.0070	0.0172
NH4	2.07	0.41	0.40	BD	0.48	BD
NO3	BD	0.12200	1.05000	BD	BD	BD
H2S	5.8333	0.3982	0.0716	BD	0.2426	0.0200
F	21.8000	21.9000	23.9000	22.1000	26.3000	19.9000
Br	0.7860	0.5180	0.5160	0.5380	0.6600	0.3280
NO2	0.0010	0.0010	0.0030	0.0050	0.0030	0.0190
Cr	BD	BD	BD	BD	BD	BD
Cs	0.0094	0.1180	0.1580	0.1380	0.0838	0.0695
Mo	0.0126	0.0246	0.0536	0.0452	0.0641	0.0250
Northing	4911458	4911373	4910969	4910990	4910962	4903817
Easting	516234	516107	515602	515628	515877	508033

Sample ID	04YBEC048	04YBEC049	04YBEC050	04YBEC051	04YBEC052	04YCAS053
Date	8/5/04	8/6/04	8/6/04	8/6/04	8/6/04	8/13/04
Temperature	51.7	59.6	80.6	90.8	51.6	78.3
pH	6.75	8.37	7.81	7.72	8.28	8.08
Conductivity	528	1152	1003	1148	706	841
DO	0	0	0	0	0	0
DOC	0	0	0	0	0	0
Ca	4.61	1.88	3.81	4.69	3.71	2.89
K	9.96	11.90	13.10	9.89	6.12	7.90
Na	112.00	256.91	223.36	279.54	163.24	177.06
Mg	0.08	0.03	0.02	0.04	0.11	0.08
Alkalinity as HCO3-	269.0	518.0	454.0	584.0	334.0	274.0
SO4	7.7	25.0	21.2	19.4	12.3	9.9
Cl	24.60	79.50	69.90	63.20	35.50	96.20
Al	0.003	0.050	0.045	0.032	0.042	0.041
As	0.0420	0.2120	0.1780	0.2000	0.0920	0.3030
As(III)	0.0010	0.0200	0.1540	0.1510	0.0010	0.0060
Ba	0.0198	0.0018	0.0013	0.0039	0.0025	0.0010
Be	0.00160	0.00190	0.00410	0.00370	0.00250	0.00020
Cd	0.00005	BD	BD	BD	BD	0.00004
Co	BD	BD	BD	BD	BD	BD
Cu	BD	BD	BD	BD	BD	BD
Fe	0.003	0.012	0.037	0.021	0.009	0.004
Fe(II)	0.001	0.009	0.027	0.013	0.004	0.002
Li	0.3500	1.0900	0.9580	0.8660	0.5050	0.9810
Mn	0.0003	0.0119	0.0269	0.0469	0.0192	0.0047
Ni	BD	BD	BD	BD	BD	BD
P	BD	BD	BD	BD	BD	BD
Pb	BD	0.0001	BD	BD	BD	BD
Sb	0.0005	0.0116	0.0090	0.0065	0.0035	0.0014
Se	0.0075	0.0097	0.0105	0.0106	0.0094	0.0096
SiO2	147.0	195.0	171.0	162.0	103.0	223.0
Sr	0.0108	0.0045	0.0021	0.0092	0.0099	0.0014
V	BD	BD	BD	BD	BD	0.0034
Zn	0.0227	0.0053	0.0191	0.0054	0.0064	0.0037
NH4	0.05	BD	BD	BD	BD	0.04
NO3	0.19900	0.13100	BD	BD	BD	BD
H2S	0.0180	0.0248	0.0654	0.2656	BD	0.0285
F	10.7000	16.9000	14.9000	16.9000	11.0000	21.1000
Br	BD	0.3130	BD	BD	BD	0.3890
NO2	0.0010	0.0020	0.0060	0.0020	0.0030	0.0010
Cr	BD	BD	BD	BD	BD	0.0113
Cs	0.0116	0.1040	0.1000	0.0865	0.0471	0.1120
Mo	0.0072	0.0037	0.0035	0.0024	BD	0.0513
Northing	4904047	4903652	4903642	4903776	4903753	4902337
Easting	508687	509668	509696	509756	509770	498469

Sample ID	04YCAS054	04YCAS055
Date	8/13/04	8/14/04
Temperature	55.9	52.6
pH	8.44	5.63
Conductivity	871	160
DO	0	0
DOC	0	0
Ca	3.65	6.77
K	7.60	6.89
Na	193.47	22.21
Mg	0.09	1.00
Alkalinity as HCO3-	281.0	66.0
SO4	10.0	11.2
Cl	102.00	1.38
Al	0.031	0.295
As	0.3000	BD
As(III)	0.0080	BD
Ba	0.0014	0.0139
Be	0.00020	0.00270
Cd	0.00005	BD
Co	BD	BD
Cu	0.00053	BD
Fe	0.010	0.086
Fe(II)	0.004	0.075
Li	0.9750	0.1220
Mn	0.0235	0.8860
Ni	BD	BD
P	BD	BD
Pb	BD	BD
Sb	0.0011	BD
Se	0.0113	BD
SiO2	235.0	94.8
Sr	0.0020	0.0088
V	BD	BD
Zn	0.0095	0.0072
NH4	0.07	BD
NO3	BD	BD
H2S	BD	BD
F	22.5000	6.2000
Br	0.3860	BD
NO2	0.0090	BD
Cr	BD	BD
Cs	0.1170	0.0061
Mo	0.0482	BD
Northing	4902339	4905566
Easting	498484	496746

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$CO(aq)+1/2\ O_2(aq)+H_2O \rightarrow HCO_3^-+H^+$ (2)	41.6	41.6	41.6	41.6	43.2	43.2	43.2	43.2	44.4	44.4	44.7	44.7
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CO+1/2\ O_2 \rightarrow CO_2$ (2)	41.6	41.6	41.6	41.6	43.2	43.2	43.2	43.2	44.4	44.4	44.7	44.7
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2HS^-+Fe^{+2}+1/2\ O_2 \rightarrow PYRITE+2H^++H_2O$ (2)	159.1	159.1	159.1	159.1	160.7	160.7	160.7	160.7	162.0	162.0	162.4	162.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$H_2+1/2\ O_2 \rightarrow H_2O$ (2)	96.6	96.6	101.6	103.1	98.1	100.2	103.1	104.6	99.3	101.3	99.6	101.6
	88.0	88.0	93.9	95.7	89.7	92.2	95.7	97.5	91.1	93.6	91.5	93.9
$2MAGNETITE+1/2\ O_2+3H_2O \rightarrow 6GOETHITE$ (2)	102.1	102.1	102.1	102.1	103.6	103.6	103.6	103.6	104.7	104.7	105.1	105.1
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$S+3/2\ O_2+H_2O \rightarrow SO_4^-2+2H^+$ (6)	103.4	103.4	103.4	103.4	105.2	105.2	105.2	105.2	106.6	106.6	107.0	107.0
	85.4	85.4	85.4	85.4	87.0	87.0	87.0	87.0	88.2	88.2	88.5	88.5
$2MAGNETITE+1/2\ O_2 \rightarrow 3HEMATITE$ (2)	98.7	98.7	98.7	98.7	100.2	100.2	100.2	100.2	101.3	101.3	101.6	101.6
	94.3	94.3	94.3	94.3	96.0	96.0	96.0	96.0	97.4	97.4	97.8	97.8
$CH_4+2O_2 \rightarrow HCO_3^-+H^++H_2O$ (8)	41.7	41.7	41.7	41.7	43.2	43.2	43.2	43.2	44.4	44.4	44.8	44.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CH_4+2O_2 \rightarrow CO_2+2H_2O$ (8)	41.7	41.7	41.7	41.7	43.2	43.2	43.2	43.2	44.4	44.4	44.8	44.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CH_4+3/2\ O_2 \rightarrow CO+2H_2O$ (6)	41.7	41.7	41.7	41.7	43.2	43.2	43.2	43.2	44.5	44.5	44.8	44.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2H^++SO_4^{-2} \rightarrow HS^-+2O_2$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-102.0	-102.4	-102.4	-102.4	-104.1	-104.1	-104.1	-104.1	-105.5	-105.5	-105.9	-105.9
$PYRITE+7/2\ O_2+H_2O \rightarrow 2SO_4^{-2}+Fe^{+2}+2H^+$ (14)	95.7	95.7	95.7	95.7	97.5	97.5	97.5	97.5	98.9	98.9	99.3	99.3
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$Fe^{+2}+1/4\ O_2+3/2\ H_2O \rightarrow GOETHITE+2H^+$ (1)	105.7	105.7	105.7	105.7	107.2	107.2	107.2	107.2	108.4	108.4	108.7	108.7
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2Fe^{+2}+1/2\ O_2+2H_2O \rightarrow HEMATITE+4H^+$ (2)	104.8	104.8	104.8	104.8	106.3	106.3	106.3	106.3	107.5	107.5	107.8	107.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3Fe+2+1/2\ O_2+3H_2O \rightarrow Fe_3O_4+6H^+$ (2)	108.3	108.3	108.3	108.3	109.8	109.8	109.8	109.8	111.0	111.0	111.4	111.4
	-24.4	-24.4	-24.4	-24.4	-22.7	-22.7	-22.7	-22.7	-21.4	-21.4	-21.0	-21.0
$HS^-+1/2\ O_2 \rightarrow S+H_2O$ (2)	106.8	106.8	106.8	106.8	108.3	108.3	108.3	108.3	109.5	109.5	109.9	109.9
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CO(aq)+NO_3^-+H_2O \rightarrow HCO_3^-+NO_2^-+H^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6
$CO(aq)+NO_3^- \rightarrow CO_2(aq)+NO_2^-$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7	-63.7

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$2HS^-+NO_3^-+Fe^{+2}\rightarrow PYRITE+NO_2^-+2H^++H_2O$ (2)	102.9	102.9	102.9	102.9	102.9	102.9	102.9	102.9	102.9	102.9	102.9	102.9
	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6	-83.6
$4CO(aq)+NO_3^-+5H_20\rightarrow 4HCO_3^-+NH_4^++2H^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5	-29.5
$2MAGNETITE+NO_3^-+H_2O\rightarrow 6GOETHITE+NO_2^-$ (2)	286.8	286.8	286.8	286.8	286.8	286.8	286.8	286.8	286.8	286.8	286.8	286.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$S+3NO_3^-+H_2O\rightarrow SO_4^{-2}+3NO_2^-+2H^+$ (6)	52.2	52.2	52.2	52.2	52.2	52.2	52.2	52.2	52.2	52.2	52.2	52.2
	-100.0	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5
$2MAGNETITE+NO_3^-\rightarrow 3HEMATITE+NO_2^-$ (2)	45.3	45.3	45.3	45.3	45.3	45.3	45.3	45.3	45.3	45.3	45.3	45.3
	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2	-88.2
$3NO_2^-+CO+2H_2O\rightarrow 3NO_3^-+CH_4$ (6)	63.5	63.5	63.5	63.5	63.5	63.5	63.5	63.5	63.5	63.5	63.5	63.5
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CH_4+4NO_3^-\rightarrow HCO_3^-+4NO_2^-+H^++H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5	-63.5
$NH_4^++4CO_2\rightarrow NO_3^-+4CO+2H^++H_2O$ (8)	29.6	29.6	29.6	29.6	29.6	29.6	29.6	29.6	29.6	29.6	29.6	29.6
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$NO_3^-+H_2\rightarrow NO_2^-+H_2O$ (2)	41.4	41.4	46.8	48.5	41.4	43.6	46.8	48.5	41.4	43.6	41.4	43.6
	-91.2	-91.2	-86.0	-84.4	-91.2	-89.0	-86.0	-84.4	-91.2	-89.0	-91.2	-89.0
$CH_4+4NO_3^-\rightarrow CO_2+4NO_2^-+2H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6	-63.6
$3CO(aq)+NO2^-+4H_2O\rightarrow 3HCO_3^-+NH4^++H^+$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-29.4	-29.1	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4
$8HS^-+NO_3^-+4Fe^{+2}\rightarrow 4PYRITE+NH_4^++6h^++3H_2O$ (8)	105.8	105.8	105.8	105.8	105.8	105.8	105.8	105.8	105.8	105.8	105.8	105.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$PYRITE+7NO_3^-+H_2O\rightarrow Fe^{+2}+2SO_4^{-2}+7NO_2^-+H_2O$ (14)	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5
	-102.0	-101.7	-101.7	-101.7	-101.7	-101.7	-101.7	-101.7	-101.7	-101.7	-101.7	-101.7
$HS^-+4NO_3^-\rightarrow SO_4^{-2}+4NO_2^-+2H^+$ (8)	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2
	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4	-99.4
$NH_4^++3CO_2\rightarrow NO_2^-+3CO+2H^++H_2O$ (6)	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$NO_3^-+4H_2+2H^+\rightarrow NH_4^++3H_2O$ (8)	43.3	43.3	48.5	50.1	43.3	45.4	48.5	50.1	43.3	45.4	43.3	45.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$8MAGNETITE+NO_3^-+2H^++13H_2O\rightarrow 24GOETHITE+NH_4^+$ (8)	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$6HS^-+NO_2^-+3Fe^{+2}\rightarrow 3PYRITE+NH_4^++4H^++2H_2O$ (6)	114.5	114.5	114.5	114.5	114.5	114.5	114.5	114.5	114.5	114.5	114.5	114.5
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$4S+3NO_3^-+7H_2O \rightarrow 4SO_4^-+3NH_4^++2H^+$ (24)	56.6	56.6	56.6	56.6	56.6	56.6	56.6	56.6	56.6	56.6	56.6	56.6
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$8MAGNETITE+NO_3^-+2H^++H_2O \rightarrow 12HEMATITE+NH_4^+$ (8)	47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$Fe^{+2}+2S+H_2O \rightarrow PYRITE+2H^++1/2\ O_2$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-89.9	-89.9	-89.9	-89.9	-91.5	-91.5	-91.5	-91.5	-92.8	-92.8	-93.2	-93.2
$2Fe^{+2}+NO_3^-+3H_2O \rightarrow 2GOETHITE+NO_2^-+4H^+$ (2)	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0
	-140.0	-140.3	-140.3	-140.3	-140.3	-140.3	-140.3	-140.3	-140.3	-140.3	-140.3	-140.3
$CH_4+NO_3^-+H^+ \rightarrow HCO_3^-+NH_4^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4	-29.4
$NO_2^-+3H_2+2H^+ \rightarrow NH_4^++2H_2O$ (6)	58.3	58.3	63.5	65.1	58.3	60.4	63.5	65.1	58.3	60.4	58.3	60.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2Fe^{+2}+NO_3^-+2H_2O \rightarrow HEMATITE+NO_2^-+4H^+$ (2)	50.4	50.4	50.4	50.4	50.4	50.4	50.4	50.4	50.4	50.4	50.4	50.4
	-141.0	-141.2	-141.2	-141.2	-141.2	-141.2	-141.2	-141.2	-141.2	-141.2	-141.2	-141.2
$NH_4^++CO_2+H_2O \rightarrow NO_3^-+CH_4+2H^+$ (8)	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$6MAGNETITE+NO_2^-+2H^++10H_2O \rightarrow 18GOETHITE+NH_4^+$ (6)	63.9	63.9	63.9	63.9	63.9	63.9	63.9	63.9	63.9	63.9	63.9	63.9
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$S+NO_2^-+2H_2O \rightarrow SO_4^{-2}+NH_4^+$ (6)	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$6MAGNETITE+NO_2^-+2H^++H_2O \rightarrow 9HEMATITE+NH_4^+$ (6)	61.8	61.8	61.8	61.8	61.8	61.8	61.8	61.8	61.8	61.8	61.8	61.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3NH_4^++4CO+5H_2O \rightarrow 3NO_3^-+4CH_4+6H^+$ (24)	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4	29.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3CH_4+4NO_2^-+5H^++H_2O \rightarrow 3HCO_3^-+4NH_4^+$ (24)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-29.3	-29.0	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3
$2S+CO(aq)+Fe^{+2}+2H_2O \rightarrow PYRITE+HCO_3^-+3H^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2
$3CH_4+4NO_2^-+8H^+ \rightarrow 3CO_2+4NH_4^++2H_2O$ (24)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-29.3	-29.1	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3	-29.3
$HS^-+NO_3^-+H_2O \rightarrow SO_4^{-2}+NH_4^+$ (8)	55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$4PYRITE+7NO_3^-+6H^++11H_2O \rightarrow 4Fe^{+2}+8SO_4^{-2}+7NH_4^+$ (56)	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9	48.9
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3Fe^{+2}+NO_3^-+3H_2O \rightarrow MAGNETITE+NO_2^-+6H^+$ (2)	52.9	52.9	52.9	52.9	52.9	52.9	52.9	52.9	52.9	52.9	52.9	52.9
	-168.0	-167.7	-167.7	-167.7	-167.7	-167.7	-167.7	-167.7	-167.7	-167.7	-167.7	-167.7

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$CH_4+NO_3^-+2H^+ \rightarrow CO_2+NH_4^++H_2O$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3	-39.3
$3HS^-+4NO_2^-+2H^++4H_2O \rightarrow 3SO_4^{-2}+4NH_4^+$ (24)	57.0	57.0	57.0	57.0	57.0	57.0	57.0	57.0	57.0	57.0	57.0	57.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3PYRITE+7NO_2^-+8H^++10H_2O \rightarrow 3Fe^{+2}+6SO_4^{-2}+7NH_4^+$ (42)	52.0	52.0	52.0	52.0	52.0	52.0	52.0	52.0	52.0	52.0	52.0	52.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2S+CO(aq)+Fe^{+2}+H2O \rightarrow PYRITE+CO2(aq)+2H^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3	-32.3
$8Fe^{+2}+NO_3^-+13H_2O \rightarrow 8GOETHITE+NH_4^++14H^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0	-76.0
$8Fe^{+2}+NO_3^-+9H_2O \rightarrow 4HEMATITE+NH_4^++14H^+$ (8)	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3
	-56.8	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*	*-56.8*
$6Fe^{+2}+NO_2^-+10H_2O \rightarrow 6GOETHITE+NH_4^++10H^+$ (6)	59.0	59.0	59.0	59.0	59.0	59.0	59.0	59.0	59.0	59.0	59.0	59.0
	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6	-9.6
$HS^-+Fe^{+2}+S \rightarrow PYRITE+2H^+$ (1)	110.4	110.4	110.4	110.4	110.4	110.4	110.4	110.4	110.4	110.4	110.4	110.4
	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4
$6Fe^{+2}+NO_2^-+7H_2O \rightarrow 3HEMATITE+NH_4^++10H^+$ (6)	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1	58.1
	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7	-9.7
$HS^-+NO_3^- \rightarrow S+NO_2^-+H_2O$ (2)	49.5	49.5	49.5	49.5	49.5	49.5	49.5	49.5	49.5	49.5	49.5	49.5
	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3	-96.3
$H_2+2S+Fe^{+2} \rightarrow PYRITE+2H^+$ (2)	47.9	47.9	53.1	54.7	47.9	50.0	53.1	54.7	47.9	50.0	47.9	50.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$NH_4^++3/2\ O2 \rightarrow NO_2^-+2H^++H_2O$ (6)	48.2	48.2	48.2	48.2	49.8	49.8	49.8	49.8	51.0	51.0	51.4	51.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2MAGNETITE+2S+Fe^{+2}+4H_2O \rightarrow PYRITE+6GOETHITE+2H^+$ (2)	53.5	53.5	53.5	53.5	53.5	53.5	53.5	53.5	53.5	53.5	53.5	53.5
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$12Fe^{+2}+NO_3^-+13H_2O \rightarrow 4MAGNETITE+NH_4^++22H^+$ (8)	56.8	56.8	56.8	56.8	56.8	56.8	56.8	56.8	56.8	56.8	56.8	56.8
	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9	-96.9
$3Fe^{+2}+7S+4H_2O \rightarrow 3PYRITE+SO_4^-2+8H^+$ (6)	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4
	-3.8	-3.7	-3.7	-3.7	-3.7	-3.7	-3.7	-3.7	-3.7	-3.7	-3.7	-3.7
$2MAGNETITE+2S+Fe^{+2}+H_2O \rightarrow PYRITE+3HEMATITE+2H^+$ (2)	50.8	50.8	50.8	50.8	50.8	50.8	50.8	50.8	50.8	50.8	50.8	50.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$NH_4^++2O_2 \rightarrow NO_3^-+2H^++H_2O$ (8)	73.8	73.8	73.8	73.8	75.3	75.3	75.3	75.3	76.5	76.5	76.8	76.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CH_4+8S+4Fe^{+2}+3H_2O \rightarrow 4PYRITE+HCO_3^-+9H^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$9Fe^{+2}+NO_2^-+10H_2O \rightarrow 3MAGNETITE+NH_4^++16H^+$ (6)	61.7	61.7	61.7	61.7	61.7	61.7	61.7	61.7	61.7	61.7	61.7	61.7
	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4	-44.4
$CH_4+8S+4Fe^{+2}+2H_2O \rightarrow 4PYRITE+CO_2+8H^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2	-32.2
$6S+CH_4(aq)+3Fe^{+2}+H_2O \rightarrow 3PYRITE+CO(aq)+6H^+$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$2H^++4HS^-+NO_3^- \rightarrow 4S+NH_4^++3H_2O$ (8)	52.4	52.4	52.4	52.4	52.4	52.4	52.4	52.4	52.4	52.4	52.4	52.4
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$NH_4^++3S+2H_2O \rightarrow 3HS^-+NO_2^-+2H^+$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3	-63.3
$3Fe^{+3}+2S+4H_2O \rightarrow PYRITE+2GOETHITE+6H^+$ (2)	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8
	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8	-73.8
$NO_2^-+1/2\ O_2 \rightarrow NO_3^-$ (2)	185.9	185.9	185.9	185.9	187.4	187.4	187.4	187.4	188.6	188.6	189.0	189.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3Fe^{+2}+2S+3H_2O \rightarrow PYRITE+HEMATITE+6H^+$ (2)	58.0	58.0	58.0	58.0	58.0	58.0	58.0	58.0	58.0	58.0	58.0	58.0
	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1	-74.1
$4Fe^{+2}+2S+4H_2O \rightarrow PYRITE+MAGNETITE+8H^+$ (2)	62.1	62.1	62.1	62.1	62.1	62.1	62.1	62.1	62.1	62.1	62.1	62.1
	-114.0	-114.2	-114.2	-114.2	-114.2	-114.2	-114.2	-114.2	-114.2	-114.2	-114.2	-114.2
$S+CO(aq)+2H_2O \rightarrow HCO_3^-+HS^-+H^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8	-83.8
$PYRITE+NO_3^-+2H^+ \rightarrow 2S+Fe^{+2}+NO_2^-+H_2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0	-109.0
$HS^-+CO_2 \rightarrow S+CO+H_2O$ (2)	83.9	83.9	83.9	83.9	83.9	83.9	83.9	83.9	83.9	83.9	83.9	83.9
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$S+H_2 \rightarrow HS^-$ (2)	12.4	12.4	17.4	19.0	12.4	14.5	17.4	19.0	12.4	14.5	12.4	14.5
	-13.8	-13.8	-8.1	-6.4	-13.8	-11.5	-8.1	-6.4	-13.8	-11.5	-13.8	-11.5
$2MAGNETITE+S+4H_2O \rightarrow 6GOETHITE+HS^-$ (2)	17.9	17.9	17.9	17.9	17.9	17.9	17.9	17.9	17.9	17.9	17.9	17.9
	-7.9	-7.8	-7.8	-7.8	-7.8	-7.8	-7.8	-7.8	-7.8	-7.8	-7.8	-7.8
$4S+4H_2O \rightarrow SO_4^{-2}+3HS^-+2H^+$ (6)	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3	-11.3
$2MAGNETITE+S+H_2O \rightarrow 3HEMATITE+HS^-$ (2)	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5
	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3
$MAGNETITE+CO(aq)+5H^+ \rightarrow HCO_3^-+3Fe+2+2H_2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6	-88.6
$CH_4+4S+3H_2O \rightarrow HCO_3^-+4HS^-+H^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$2Fe^{+2}+PYRITE+4H_2O \rightarrow MAGNETITE+2HS^-+4H^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-101.0	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5	-100.5
$4PYRITE+NO_3^-+10H^+ \rightarrow 8S+4Fe^{+2}+NH_4^++3H_2O$ (8)	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4
	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6	-4.6
$CH_4+4S+2H_2O \rightarrow CO_2+4HS^-$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7	-83.7
$Fe_3O_4+H_2+6H^+ \rightarrow 3Fe_2^++4H_2O$ (2)	115.4	115.4	121.0	122.7	115.4	117.7	121.0	122.7	115.4	117.7	115.4	117.7
	-17.9	-17.9	-12.1	-10.3	-17.9	-15.5	-12.1	-10.3	-17.9	-15.5	-17.9	-15.5
$HEMATITE+CO(aq)+3H^+ \rightarrow HCO_3^-+2Fe^{+2}+H^2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6
$2GOETHITE+CO(aq)+3H^+ \rightarrow HCO_3^-+2Fe^{+2}+2H_2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2
$7CO(aq)+2SO_4^{-2}+Fe^{+2}+6H_2O \rightarrow PYRITE+7HCO_3^-+5H^+$ (14)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4
$3HS^-+CO \rightarrow 3S+CH_4+H_2O$ (6)	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CO+Fe_3O_4+6H^+ \rightarrow CO_2+3Fe^{+2}+3H_2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7	-88.7
$9Fe^{+2}+SO_4^{-2}+8H_2O \rightarrow S+3MAGNETITE+16H^+$ (6)	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6
	-110.0	-110.5	-110.5	-110.5	-110.5	-110.5	-110.5	-110.5	-110.5	-110.5	-110.5	-110.5
$4CO(aq)+5H_2O \rightarrow CH_4(aq)+3HCO_3^-+3H^+$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$4CO(aq)+SO_4^{-2}+4H_2O \rightarrow 4HCO_3^-+HS^-+2H^+$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1
$Fe^{+2}+PYRITE+3H_2O \rightarrow HEMATITE+2HS^-+2H^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9
$3PYRITE+NO_2^-+8H^+ \rightarrow 6S+3Fe^{+2}+NH_4^++2H_2O$ (6)	49.6	49.6	49.6	49.6	49.6	49.6	49.6	49.6	49.6	49.6	49.6	49.6
	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2	-1.2
$2HS^-+2GOETHITE+2H^+ \rightarrow PYRITE+Fe^{+2}+4H_2O$ (2)	77.1	77.1	77.1	77.1	77.1	77.1	77.1	77.1	77.1	77.1	77.1	77.1
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CH_4+4MAGNETITE+23H^+ \rightarrow HCO_3^-+12Fe^{+2}+13H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5
$H_2+HEMATITE+4H^+ \rightarrow 2Fe^{+2}+3H_2O$ (2)	75.3	75.3	81.0	82.7	75.3	77.6	81.0	82.7	75.3	77.6	75.3	77.6
	-14.0	-14.0	-8.1	-6.3	-14.0	-11.5	-8.1	-6.3	-14.0	-11.5	-14.0	-11.5
$1/2\ H^2+GOETHITE+2H^+ \rightarrow Fe^{+2}+2H_2O$ (1)	75.0	75.0	80.6	82.3	75.0	77.3	80.6	82.3	75.0	77.3	75.0	77.3
	-14.0	-14.0	-8.1	-6.4	-14.0	-11.6	-8.1	-6.4	-14.0	-11.6	-14.0	-11.6

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
HEMATITE+CO(aq)+4H$^+$→CO2(aq)+2Fe^{+2}+2H$_2$O (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7	-84.7
2GOETHITE+CO(aq)+4H$^+$→CO$_2$(aq)+2Fe^{+2}+3H$_2$O (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2	-85.2
CH$_4$+4Fe$_3$O$_4$+24H$^+$→CO$_2$+12Fe^{+2}+14H$_2$O (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5
7CO(aq)+2SO$_4^{-2}$+2H$^+$+Fe^{+2}→PYRITE+7CO$_2$(aq)+H$_2$O (14)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5	-78.5
NH$_4^+$+3NO$_3^-$→2H$^+$+4NO$_2^-$+H2O (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-149.0	-149.5	-149.5	-149.5	-149.5	-149.5	-149.5	-149.5	-149.5	-149.5	-149.5	-149.5
2Fe^{+2}+S+4H$_2$O→2GOETHITE+HS$^-$+4H$^+$ (2)	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3	-62.3
2Fe^{+2}+S+3H$_2$O→HEMATITE+HS$^-$+4H$^+$ (2)	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4
	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8	-62.8
4H$_2$O+4PYRITE+6H$^+$→SO$_4^{-2}$+7HS$^-$+4Fe^{+2} (7)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3	-68.3
CO(aq)+3Fe^{+2}+6HS$^-$→3PYRITE+CH$_4$(aq)+6H$^+$+H$_2$O (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12Fe^{+2}+SO$_4^{-2}$+12H$_2$O→4MAGNETITE+HS$^-$+22H$^+$ (8)	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6	-99.6
6Fe^{+2}+SO$_4^{-2}$+5H$_2$O→S+3HEMATITE+10H$^+$ (6)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4	-70.4
3HEMATITE+CO(aq)+H$_2$O→2MAGNETITE+HCO$_3^-$+H$^+$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0
4CO+2H$_2$O→3CO$_2$+CH$_4$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2
3CO(aq)+SO$_4^{-2}$+2H^{2}O→S+3HCO$_3^-$+H$^+$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1
6Fe^{+2}+SO$_4^{-2}$+8H$_2$O→S+6GOETHITE+10H$^+$ (6)	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9
	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0	-70.0
7H^2+2SO$_4^{-2}$+Fe^{+2}+2H$^+$→PYRITE+8H$_2$O (14)	11.7	11.7	16.8	18.3	11.7	13.8	16.8	18.3	11.7	13.8	11.7	13.8
	-7.8	-7.8	-1.8	0.0	-7.8	-5.3	-1.8	0.0	-7.8	-5.3	-7.8	-5.3
HS$^-$+4CO$_2$→SO4^{-2}+4CO+2H$^+$ (8)	85.2	85.2	85.2	85.2	85.2	85.2	85.2	85.2	85.2	85.2	85.2	85.2
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6GOETHITE+CO(aq)→2MAGNETITE+HCO$_3^-$+H$^+$+2H$_2$O (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
MAGNETITE+PYRITE+2H_2O→2HEMATITE+2HS^- (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8	-65.8
CH_4+4HEMATITE+15H^+→HCO_3^-+8Fe^{+2}+9H_2O (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5	-84.5
CH_4+8GOETHITE+15H^+→HCO_3^-+8Fe^{+2}+13H_2O (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1
PYRITE+7MAGNETITE+40H^+→2SO_4^{-2}+22Fe^{+2}+20H_2O (14)	111.0	111.0	111.0	111.0	111.0	111.0	111.0	111.0	111.0	111.0	111.0	111.0
	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1	-16.1
MAGNETITE+PYRITE+4H_2O→4GOETHITE+2HS^- (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2	-64.2
14MAGNETITE+Fe^{+2}+2SO_4^{-2}+2H^++20H_2O→PYRITE+42GOETHITE (14)	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2
	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6	-1.6
SO_4^{-2}+4H_2+2H^+→HS^-+4H_2O (8)	11.1	11.1	16.1	17.6	11.1	13.2	16.1	17.6	11.1	13.2	11.1	13.2
	-14.4	-14.4	-8.4	-6.6	-14.4	-12.0	-8.4	-6.6	-14.4	-12.0	-14.4	-12.0
CO+3H_2→CH_4+H_2O (6)	74.5	74.5	79.8	81.4	74.5	76.7	79.8	81.4	74.5	76.7	74.5	76.7
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CH_4+4HEMATITE+16H^+→CO_2+8Fe^{+2}+10H_2O (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6	-84.6
CH_4+8GOETHITE+16H^+→CO_2+8Fe^{+2}+14H_2O (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1	-85.1
CO(aq)+2H_2O→H_2(g)+$HCO3^-$+H^+ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-74.6	-74.6	-79.9	-81.5	-74.6	-76.8	-79.9	-81.5	-74.6	-76.8	-74.6	-76.8
14MAGNETITE+Fe^{+2}+2SO_4^{-2}+2h^+→PYRITE+21HEMATITE+H_2O (14)	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8
	-14.6	-1.5	-1.5	-1.5	-1.5	-1.5	-1.5	-1.5	-1.5	-1.5	-1.5	-1.5
CH_4+4PYRITE+8H^++2H_2O→CO_2+4Fe^{+2}+8HS^- (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	137.0	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1
8MAGNETITE+SO_4^{-2}+2H^++12H_2O→24GOETHITE+HS^- (8)	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6
	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3	-8.3
CH_4+3Fe3O_4+18H^+→CO+9Fe^{+2}+11H_2O (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5	-88.5
3Fe^{+2}+S+4H_2O→MAGNETITE+HS^-+6H^+ (2)	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3
	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5	-99.5
6MAGNETITE+CO(aq)+11H_2O→18GOETHITE+CH_4(aq) (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8MAGNETITE+SO_4^{-2}+2H^+→12HEMATITE+HS^- (8)	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2
	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1	-8.1

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$7CH_4+8SO_4^{-}+H^{+}+4Fe^{+2}\rightarrow 4PYRITE+7HCO_3^{-}+11H_2O$ (56)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3	-78.3
$CH_4+4PYRITE+7H^{+}+3H_2O\rightarrow 4Fe^{+2}+HCO_3^{-}+8HS^{-}$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-137.0	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1	-137.1
$S+CO+3H_2O\rightarrow SO_4^{-2}+CH_4+2H^{+}$ (6)	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$6MAGNETITE+CO(aq)+2H_2O\rightarrow 9HEMATITE+CH_4(aq)$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$8Fe^{+2}+SO_4^{-2}+8H_2O\rightarrow 4HEMATITE+HS^{-}+14H^{+}$ (8)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9	-62.9
$CO_2+4H_2\rightarrow CH_4+2H_2O$ (8)	74.6	74.6	79.9	81.5	74.6	76.7	79.9	81.5	74.6	76.7	74.6	76.7
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$3HEMATITE+CO(aq)\rightarrow 2MAGNETITE+CO_2(aq)$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0	-78.0
$S+3CO_2+H_2O\rightarrow SO_4^{-2}+3CO+2H^{+}$ (6)	86.2	86.2	86.2	86.2	86.2	86.2	86.2	86.2	86.2	86.2	86.2	86.2
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$8Fe^{+2}+SO_4^{-2}+12H_2O\rightarrow 8GOETHITE+HS^{-}+14H^{+}$ (8)	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3
	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4	-62.4
$PYRITE+CO(aq)+H^{+}+2H_2O\rightarrow Fe^{+2}+HCO_3^{-}+2HS^{-}$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-137.0	-137.2	-137.2	-137.2	-137.2	-137.2	-137.2	-137.2	-137.2	-137.2	-137.2	-137.2
$7CH_4+8SO_4^{-2}+8H^{+}+4Fe^{+2}\rightarrow 4PYRITE+7CO_2+18H_2O$ (56)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4	-78.4
$6GOETHITE+CO(aq)\rightarrow 2MAGNETITE+CO_2(aq)+3H_2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3
$CH_4^{+}+SO_4^{-2}+H^{+}\rightarrow HCO_3^{-}+HS^{-}+H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0
$4H_2(aq)+HCO_3^{-}+H^{+}\rightarrow CH_4(aq)+3H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$CH_4+24GOETHITE\rightarrow 8MAGNETITE+CO_2+14H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3
$15Fe^{+2}+2SO_4^{-2}+13H_20\rightarrow PYRITE+7HEMATITE+26H^{+}$ (14)	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6
	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9	-70.9
$15Fe^{+2}+2SO_4^{-2}+20H_2O\rightarrow PYRITE+14GOETHITE+26H^{+}$ (14)	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5
	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6	-70.6
$CH_4+12HEMATITE\rightarrow 8MAGNETITE+CO_2+2H_2O$ (14)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9

Table 2. Summary of energetic calculations kJ per electron transferred. The range of energy available across springs is shown for each reaction, maximum (grey) and minimum (white).

	0.1 mg/L O_2 2 nM H_2	0.1 mg/L O_2 10 nM H_2	0.1 mg/L O_2 100 nM H_2	0.1 mg/L O_2 325 nM H_2	0.5 mg/L O_2 2 nM H_2	0.5 mg/L O_2 10 nM H_2	0.5 mg/L O_2 100 nM H_2	0.5 mg/L O_2 325 nM H_2	3 mg/L O_2 2 nM H_2	3 mg/L O_2 10 nM H_2	6 mg/L O_2 2 nM H_2	6 mg/L O_2 10 nM H_2
$3CH_4+4SO_4^{-2}+8H^+ \rightarrow 4S+3CO_2+10H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1	-86.1
$CH_4+SO_4^{-2}+2H^+ \rightarrow CO_2+HS^-+2H_2O$ (24)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0	-85.0
3HEMATITE+CH_4(aq)+$12H^+ \rightarrow$CO(aq)+$6Fe^{+2}+8H_2O$ (24)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6GOETHITE+CH_4(aq)+$12H^+ \rightarrow$CO(aq)+$6Fe^{+2}+11H_2O$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CH_4+24GOETHITE$\rightarrow$8MAGNETITE+H^++HCO_3^-+$13H_2O$ (6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3	-80.3
$SO_4^{-2}+3H_2+2H^+ \rightarrow S+4H_2O$ (8)	10.7	10.7	15.7	17.2	10.7	12.8	15.7	17.2	10.7	12.8	10.7	12.8
	-15.5	-15.5	-9.5	-7.7	-15.5	-13.0	-9.5	-7.7	-15.5	-13.0	-15.5	-13.0
H_2+3HEMATITE$\rightarrow$2MAGNETITE+H_2O (6)	-2.0	-2.0	3.0	4.5	-2.0	0.0	3.0	4.5	-2.0	0.0	-2.0	0.0
	-6.3	-6.3	-0.3	1.5	-6.3	-3.9	-0.3	1.5	-6.3	-3.9	-6.3	-3.9
$7CH_4$(aq)+$6SO_4^{-2}$+$6H^+$+$3Fe^{+2}\rightarrow$3PYRITE+7CO(aq)+$17H_2O$ (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CO_2+$H_2\rightarrow$CO+H_2O (42)	74.6	74.6	79.9	81.5	74.6	76.8	79.9	81.5	74.6	76.8	74.6	76.8
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CO_2(aq)+Fe^{+2}+$2HS^-\rightarrow$PYRITE+CO(aq)+$2H^+$+H_2O (2)	137.2	137.2	137.2	137.2	137.2	137.2	137.2	137.2	137.2	137.2	137.2	137.2
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CH_4+12HEMATITE$\rightarrow$8MAGNETITE+HCO_3^-+H^++H_2O (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9	-77.9
$3CH_4+4SO_4^{-2}+5H^+ \rightarrow 4S+3HCO_3^-+7H_2O$ (8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0	-86.0
H_2+6GOETHITE$\rightarrow$2MAGNETITE+$4H_2O$ (24)	0.0	0.0	0.0	1.6	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0
	-6.1	-6.1	-0.5	0.0	-6.1	-3.7	-0.5	0.0	-6.1	-3.7	-6.1	-3.7
H_2+PYRITE+$2H^+\rightarrow Fe^{+2}+2HS^-$ (2)	7.7	7.7	13.2	14.9	7.7	9.9	13.2	14.9	7.7	9.9	7.7	9.9
	-67.5	-67.5	-61.9	-60.2	-67.5	-65.2	-61.9	-60.2	-67.5	-65.2	-67.5	-65.2
6MAGNETITE+SO_4^{-2}+$2H^+$+$8H_2O\rightarrow$S+18GOETHITE (2)	16.2	16.2	16.2	16.2	16.2	16.2	16.2	16.2	16.2	16.2	16.2	16.2
	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3	-9.3
$3HS^-$+4CO+$8H_2O\rightarrow3SO_4^{-2}$+$4CH_4$+$6H^+$ (6)	85.0	85.0	85.0	85.0	85.0	85.0	85.0	85.0	85.0	85.0	85.0	85.0
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6MAGNETITE+SO_4^{-2}+$2H^+\rightarrow$S+9HEMATITE+H_2O (24)	12.7	12.7	12.7	12.7	12.7	12.7	12.7	12.7	12.7	12.7	12.7	12.7
	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2	-9.2