On the energetics of chemolithotrophy in nonequilibrium systems: case studies of geothermal springs in Yellowstone National Park

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ABSTRACT

Chemolithotrophic micro-organisms are important primary producers in high-temperature geothermal environments and may catalyse a number of different energetically favourable redox reactions as a primary energy source. Analysis of geochemical constituents followed by chemical speciation and subsequent calculation of reaction free energies (ΔG_{rxn}) is a useful tool for evaluating the thermodynamic favourability and potential energy available for microbial metabolism. The primary goal of this study was to examine relationships among geochemical gradients and microbial population distribution, and to evaluate the utility of energetic approaches for predicting microbial metabolism from free-energy calculations, utilizing as examples, several geothermal habitats in Yellowstone National Park where thorough geochemical and phylogenetic analyses have been performed. Acidic (pH ∼ 3) and near-neutral (pH ∼ 6–7) geothermal springs were chosen for their range in geochemical properties. Aqueous and solid phase samples obtained from the source pools and the outflow channels of each spring were characterized for all major chemical constituents using laboratory and field methods to accurately measure the concentrations of predominant oxidized and reduced species. Reaction free energies (ΔG_{rxn}) for 33 oxidation–reduction reactions potentially important to chemolithotrophic micro-organisms were calculated at relevant spring temperatures after calculating ion activities using an aqueous equilibrium model. Free-energy values exhibit significant variation among sites for reactions with pH dependence. For example, free-energy values for reactions involving Fe^{3+} are especially variable across sites due in large part to the pH dependence of Fe^{3+} activity, and exhibit changes of up to 40 kJ mol^{-1} electron from acidic to near neutral geothermal springs. Many of the detected 16S rRNA gene sequences represent organisms whose metabolisms are consistent with exergonic processes. However, sensitivity analyses demonstrated that reaction free energies do not generally represent the steep gradients in local geochemical conditions resulting from air–water gas exchange and solid phase deposition that are important in defining microbial habitats and 16S rRNA gene sequence distribution within geothermal outflow channels.

INTRODUCTION

In many volcanic, hydrothermal and geothermal environments, chemolithoautotrophs represent the primary producers, capable of metabolism on the simplest and among the most common inorganic compounds in the universe (e.g. CO₂, H₂, CH₄, S, Fe, O₂). The oxidation–reduction reactions mediated by chemoautotrophic micro-organisms are central to biogeochemical cycling and represent a direct link among adapted microbial populations and geochemical processes (Newman & Banfield, 2002; Reysenbach & Shock, 2002). There has been significant interest in utilizing thermodynamic approaches and energetic calculations to evaluate the favourability and amount of energy potentially available for chemolithotrophic metabolism for numerous electron donor–acceptor combinations (Amend & Shock, 2001; Amend et al., 2003; Shock et al., 2005). Recent efforts include a significant compilation of temperature-dependent standard state Gibbs free-energy values (ΔG_{rxn}°) for redox couples potentially important for chemotrophic metabolism (Amend & Shock, 2001). Subsequent calculation of actual
free-energy values corresponding to specific environmental conditions (i.e. activities of relevant chemical species) provides a mechanism for determining which reactions are exergonic in a particular environment. Moreover, it is often inferred that the redox couples yielding the greatest amounts of energy will translate to a competitive biological advantage, or that the most thermodynamically favoured reactions are the most important reactions for microbial metabolism (e.g. Spear et al., 2005a).

The thermodynamic favourability of some redox couples will vary among different environments or across geochemical gradients common in soil–water–sediment systems. Consequently, an energetic profile of environmental systems is certainly useful for correlating geochemical energy availability with the distribution of specific, uniquely adapted populations (e.g. Ward et al., 1998), and in customizing appropriate cultivation strategies for specific microbial metabolisms. However, not all exergonic redox couples will necessarily be utilized by micro-organisms in a given environment. Furthermore, the most thermodynamically favoured redox couple may not necessarily correlate with the predominant microbial metabolism in a particular environment (Macur et al., 2004a). While energetic calculations are useful for inventorying the energy available from various redox couples, interpretations regarding predominant microbial metabolisms in dynamic environments should rely on multiple lines of evidence, not solely on the absolute magnitude of free energy available in an exergonic reaction. One of the long-term goals of our research program in geothermal systems is to determine how mass transfer rates, reaction kinetics and microbial specialization have been integrated in time and space. Although exergonic reactions are required for microbial energy yield, it is critical to explore other important factors that determine which of the many exergonic redox reactions are actually used by micro-organisms and why. As a part of this larger goal, the specific objectives of the current study were to (i) examine biogeochemical processes controlling redox cycling in geothermal springs of YNP, (ii) establish the predominant geochemical gradients that occur down gradient of geothermal discharge, (iii) compare observed changes in geochemistry and microbial population distribution with changes in the free-energy distribution among sites and across a suite of redox couples, and (iv) evaluate the utility and sensitivity of energetic approaches for assessing possible variations in microbial metabolism across observed geochemical gradients. The natural systems employed in this study include several geothermal habitats in Yellowstone National Park where thorough geochemical and phylogenetic analyses have been performed.

**METHODS**

**Geothermal sites**

The sites chosen for this study include geothermal springs located in the One Hundred Springs Plain of Norris Geyser Basin (NGB), the Rainbow Springs (RS) group and the Joseph’s Coat (JC) springs group of Yellowstone National Park (YNP). The coordinates of the subject springs are as follows: NGB-D (44°43’54.8”N, 110°42’39.9”W), NGB-B (44°43’53.4”N, 110°42’40.9”W), NGB-S (44°43’75.7”N, 110°42’74.7”W), NGB-PS (44°43’36.0”N, 110°42’29.8”W), RS2 (44°45’59.6”N, 110°16’08.2”W) and JC3 (44°44’21.4”N, 110°19’28.2”W). The geothermal springs in Norris Geyser Basin have also been analysed periodically for a full suite of chemical constituents by the US Geological Survey as part of geothermal discharge inventory programs in YNP (Ball et al., 2002). All springs exhibit source water temperatures ranging from 75 to 90 °C and flow rates ranging from approximately 10–50 L min⁻¹. During the last 3 years (2003–05), aqueous and solid phase samples have been subjected to detailed chemical characterization and molecular analyses to identify microbial populations (16S rDNA sequence analysis) present as a function of distance from spring discharge. Transects of four to six sampling points originating at the source pool and continuing down-gradient until approximately 50 °C were sampled quarterly for 2 years at NGB-B and NGB-PS (2003–05). Due to the remote locations of Rainbow and Joseph’s Coat Springs, transects at RS2 and JC3 were sampled annually for 3 years during the summers of 2003–05.

**Aqueous geochemistry and dissolved gases**

Two aqueous samples at each location were filtered (0.2 µm) on site directly into sterile 50 mL screw-cap tubes. One sample was preserved in 0.1 M HNO₃, then analysed using inductively coupled plasma spectrometry (ICP) for total dissolved Ca, Mg, Na, K, Si, Al, Fe and B, and trace elements including Cd, Cr, Cu, Mn, Ni, Pb, Sb, Se and Zn. One sample was left unacidified and analysed for predominant inorganic anions (F⁻, Cl⁻, SO₄²⁻, NO₃⁻, CO₃²⁻, S₂O₅²⁻, AsO₃³⁻) using ion exchange chromatography (IC) (Dionex Corp. (Sunnyvale, CA, USA) AS16-4 mm column), and aqueous NH₄⁺ using the phenolate colourimetric (A₆30 nm) procedure on a flow injection analyser (APHA, 1998). Dissolved inorganic C (DIC) and dissolved organic C (DOC) were determined on separate samples taken in closed headspace serum bottles using a C analyser (Dohrmann DC-80; Teledyne, Mason, OH, USA). In addition, several redox sensitive species were analysed on site including Fe²⁺ and Fe³⁺ using the ferrozine method (To et al., 1999) employing a filtered (0.2 µm) sample, and total dissolved sulfide (DS) using the amine sulfuric acid method (APHA, 1998) with unfiltered samples (to avoid rapid degassing of H₂S(aq) upon filtration). The amount of As³⁺ and As⁵⁺ was determined using atomic absorption spectrometry (hydride generation) on samples untreated and treated with Na borohydride in the field (Langner et al., 2001), where As(III) is then calculated as the difference between arsenate As(V) and total soluble As (As₅⁺). Aqueous pH and temperature values were obtained on site using a Mettler-Toledo portable ion meter (MA130) equipped with a temperature
probe, where pH 1.68 and 4.01 buffers were calibrated at spring temperatures.

Dissolved gas species including H₂, CH₄ and CO₂ were determined using headspace gas chromatography. Closed headspace aqueous geothermal samples were collected using a peristaltic pump with an in-line 0.2 µm filter. After a 4–5 pore-volume purge, 100-mL serum bottles were capped with zero headspace using butyl-acetate stoppers. A known volume of liquid was withdrawn and replaced with an equivalent volume of N₂(g) and the bottles incubated with intermittent shaking for 60 min. Preliminary analyses determined that an equilibration time of approximately 60 min was required for gas–water partitioning. Samples of the headspace were injected into a portable Varian gas chromatograph (Model CP2900) using Ar and N₂ as carrier gases and analysed for H₂, CH₄ and CO₂ on a dual-channel thermal conductivity detector system.

Values of headspace gas concentrations were then used to calculate original dissolved gas concentrations using temperature-corrected Henry’s Law constants for each gas (Amend & Shock, 2001), and a mass balance equation for total dissolved gas prior to headspace equilibration:

\[
\text{Henry’s Law: } K_H = \frac{c(\text{aq})}{c(g)} \quad (1)
\]

\[
\text{Mass balance: } [c(\text{aq})_{\text{sample}} V_{\text{L}}] = [c(g)_{\text{eq}} V_{\text{g,eq}}] + [c(\text{aq})_{\text{eq}} V_{\text{L,eq}}] \quad (2)
\]

Where \( K_H \) = Henry’s Law constant at temperature of incubation (temperature adjusted values of \( K_H \) determined from thermodynamic constants reported in Amend & Shock, 2001), \( c \) = concentration in either aqueous or gas phases (mol L⁻¹), and \( V \) = volume of liquid or gas. Concentrations of dissolved O₂ were determined in the field using the Winkler method (APHA, 1998).

**Solid phase geochemistry**

Samples of microbial mats and associated solid phases were subjected to a suite of analytical procedures to determine the composition, structure and mineralogy of solid phases deposited within the outflow channels including: (i) X-ray diffraction (XRD), (ii) scanning electron microscopy and energy dispersive analysis of X-rays (SEM/EDAX), (iii) transmission electron microscopy (TEM) of glutaraldehyde-fixed and epoxy-infiltrated thin sections, (iv) X-ray absorption near edge structure (XANES) spectroscopy, extended X-ray absorption fine structure (EXAFS) spectroscopy using a synchrotron light source at the Stanford Radiation Laboratory (SSRL), and (v) total chemical analysis of mat samples via acid digestion (HNO₃–HClO₄–HF at 110 °C). Although it is beyond the scope of this manuscript to report all information on solid phase structure and composition, some data are discussed as they relate to the energetic analyses of these systems.

**Chemical speciation and thermodynamic calculations**

Chemolithotrophic thermophiles may derive energy from a wide variety of inorganic electron transfer reactions. Recent literature has outlined details of energetic calculations necessary to evaluate the favourability of redox reactions under specific environmental conditions (Amend & Shock, 2001; Amend et al., 2003). Briefly, the free energies (\( \Delta G_{\text{rxn}} \), kJ mol⁻¹) of 33 potentially important redox couples were calculated using the expression:

\[
\Delta G_{\text{rxn}} = \Delta G_{\text{rxn}}^\circ + RT \ln(Q_{\text{rxn}}) \quad (3)
\]

where \( \Delta G_{\text{rxn}}^\circ \) is the standard state free energy of reaction (temperature dependent) and \( Q_{\text{rxn}} \) is the reaction quotient calculated using actual activities of chemical species (Stumm & Morgan, 1996). Temperature-dependent values of \( \Delta G_{\text{rxn}}^\circ \) were obtained from Amend & Shock (2001). Values of \( \Delta G_{\text{rxn}} \) not available in the literature were calculated using temperature corrected standard free energies of formation (\( \Delta G_{\text{rxn}}^\circ \)) (Amend & Shock, 2001). Activities of chemical species were calculated with the aqueous equilibrium model, Visual MINTEQ (Allison et al., 1991), using measured values of total soluble chemical constituents as input values. As discussed in Amend et al. (2003), solid phase, gas and redox equilibria were suppressed during chemical speciation calculations to accurately represent the solution as sampled.

**Microbial community analysis: DNA extraction, amplification and sequencing**

Microbial mat samples were analysed using molecular methods to assess the distribution of 16S rDNA sequences detectable across different geochemical and temperature gradients observed in the source pools and outflow channels (Jackson et al., 2001; Inskeep et al., 2004; Macur et al., 2004a). Briefly, solid phase microbial mats were sampled using sterile tools and tubes, and immediately placed on dry ice for transport to a –80 °C freezer. Total DNA was extracted from the samples using the FastDNA SPIN Kit for Soil (Q-Biogene, Irvine, CA, USA). Primers for near full-length polymerase chain reaction (PCR) of 16S rRNA genes consisted of the Bacteria-specific primer Bac8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal primer Univ1392r (5′-ACGG-GCGGTGTGTAC-3′). The Archaeal domain was targeted using Archaea-specific primer Arc2f (5′-TTCGGGTGATCAG-3′) and universal primer Univ1392r. Purified PCR products were cloned using the pGEM-T Vector System from Promega Corp. (Madison, WI) and the inserts were sequenced using T7 and SP6 primers (TGEN, Phoenix, AZ, USA). 16S rDNA diversity and distribution was also examined in several springs using denaturing gradient gel electrophoresis to separate short 16S rDNA fragments (322–461 bp; see Macur et al., 2004a and Inskeep et al.,
2004). Resultant sequences were edited and deposited in GenBank (Benson et al., 2005). Although beyond the scope of the current manuscript to discuss all phylogenetic data from these YNP geothermal systems, reference is made to specific 16S rDNA sequences found in these environments using GenBank Accession numbers.

RESULTS

Source water geochemistry

The geothermal springs featured in this energetics analysis include acid–sulfate–chloride (ASC) springs of Norris Geyser Basin (pH ∼ 3), an acid–sulfate (AS) spring from the Rainbow Spring group (pH ∼ 2.6) and two near-neutral springs containing significant levels of total soluble arsenic (Fig. 1). Previous reports have discussed geochemical and 16S rDNA analysis of the two ASC springs from Norris Geyser Basin (NGB-B = Beowulf Spring, Inskeep et al., 2004; NGB-S = Succession Spring; Macur et al., 2004a). The ASC springs of NGB have exhibited tightly constrained Cl–/ ratios of 9–10 over the course of our sampling program since 1999 (Langner et al., 2001; Inskeep et al., 2004; Macur et al., 2004a). The ASC geothermal source waters yield ionic strengths of near 0.016 M, comprised primarily of Na+, H+, Cl– and SO4^2– (Table 1). More importantly for chemolithotrophic metabolism, the source waters of these springs contain significant quantities of reduced inorganic constituents including H2, H2S, FeII, AsIII, NH4 and CH4 (Table 1).

The source waters of Rainbow Spring (RS2) are also acidic (pH 2.6), but otherwise are significantly different from the ASC springs of Norris Basin (Table 1). Most notably, the dominant anion in RS2 is sulfate rather than chloride, yielding Cl–/ SO4^2– ratios of ~0.02 consistently over 3 years of sampling (2003–05). Furthermore, total soluble concentrations of NH4^+ and Fe^{2+} are fairly high at 1.7 mM and 100 µM, respectively. The source waters contain significant quantities of dissolved H2, CH4 and CO2; however, total soluble sulfide has not exceeded 10 µM and total soluble As levels are low relative to many YNP geothermal springs (Stauffer & Thompson, 1984; Langner et al., 2001; Ball et al., 2002).

Perpetual Spouter (NGB-PS) and a hot spring from Joseph’s Coat Springs (JC3) both exhibit near-neutral pH values, and contain low levels of total soluble Fe (Table 1). However, there are significant differences in the source water chemistry of these two near-neutral pH springs, which result in different geochemical and microbiological processes within the source pools and subsequent outflow channels. The dominant cations and anions in JC3 are Na+ (12 mM), NH4^+ (5 mM), Cl– (13 mM) and SO4^2– (4 mM), and the source waters contain high concentrations of total soluble As (130 µM), B (5 mM) and Sb (1.5 µM). Total dissolved sulfide is fairly high ranging from 18 to 32 µM over the last 3 years. Concentrations of other dissolved gases including H2 (90–170 nM) and CH4 (100–200 nM) are significant and are likely important to chemotrophs inhabiting the source pool and outflow channel. The chemical composition of this spring is stable based on three annual sampling efforts (2002–05), and concentrations of As and Sb are nearly identical to values reported in Stauffer & Thompson (1984).

Perpetual Spouter (NGB-PS) is dominated simply by Na+ and Cl–, but contains notable concentrations of B (1.1 mM).
and As (40 µM). The concentrations of dissolved sulfide and other dissolved gases including CH4 and H2 are considerably lower than the other springs sampled (sulfide ∼ 3 µM, H2 − 27 nM and CH4 is near detection at 10 nM).

All springs contain significant concentrations of dissolved inorganic C (DIC) necessary for supporting autotrophic microbial growth. Values of DIC range from 0.15 to 4 mM in the source waters (Table 1), which correspond to CO2 supersaturation indices (log[CO2(aq)/CO2(aq, 0.00035 atm)]) of 0.63 for NGB-PS to nearly 3 for the ASC springs. Consequently, the source waters of these geothermal springs contain from approximately 1–3 orders of magnitude higher CO2(aq) than in equilibrium with atmospheric CO2 (~0.00035 atm).

### Free-energy distribution diagrams

The oxidation–reduction reactions considered in this case study analysis are but a small subset of the possible exergonic reactions that may be important in chemolithotrophic metabolism (Amend & Shock, 2001; Amend et al., 2003). However, the reactions covered here represent a comprehensive range in energy availability from the primary electron donors and acceptors measured in the selected geothermal springs (Table 2), and provide an energetics data set necessary for comparing direct geochemical and microbial observations with the energy availability of exergonic reactions.
Free-energy values (kJ per mol electron) for the exergonic reactions given in Table 2 are examined in energy distribution diagrams for each spring (Fig. 2). The distribution of ΔG_{rxn} values over these selected redox couples exhibits the common shape of a theoretical electron titration. For example, the oxidation of H₂ is most exergonic when O₂ is the electron acceptor, followed in general order by NO³⁻, Fe³⁺, As⁵⁺, S⁰, SO₄²⁻ and CO₃²⁻ (Fig. 2B). With O₂ as a constant electron acceptor, the calculated free-energy values are highest when H₂ serves as the electron donor followed by reactions with C⁴⁺, S⁻¹, As⁵⁺, Fe⁴⁺ and N⁵⁺ (Fig. 2C). These trends are generally as expected across a relevant range of earth-surface temperatures (0–100 °C) (Stumm & Morgan, 1996; Amend & Shock, 2001; Madigan et al., 2003), because the redox couples are poised at discrete free energies defined in large part by the standard state free-energy value and the pH. The specific spring conditions have an important modifying effect on calculated free-energy values, but as will be shown in sensitivity analyses (Figs 6, 7), the range in calculated ΔG_{rxn} values is often tightly constrained by the standard state value and a plausible range in the activities of oxidized and reduced species.

Free-energy values for the acidic springs (RS2 and NGB-B) track closely across the 33 redox reactions considered (Fig. 2A). Variation in reaction free-energy values between acidic and near-neutral springs was significant for reactions with pH dependence. For example, reactions involving H⁺ as a product were often considerably more favourable at higher pH due primarily to the fact that calculated Fe³⁺ activities drop considerably (6–8 orders of magnitude) at pH 6–7.

It is clear from the energy distribution profile of these springs that despite differences in chemistry and subsequent geochemical processes, variations in reaction free energies for many reactions do not exceed 5–10 kJ mol⁻¹ electron.
Reactions involving H⁺ or species highly dependent on H⁺ (e.g. Fe³⁺ activity) exhibit greater differences of up to 40 kJ mol⁻¹ electron between acidic and near-neutral sites. For example, reactions with Fe often exhibit the greatest variation in free-energy values among sites, and local geochemical conditions can more likely have an impact on whether a specific Fe reaction is exergonic vs. endergonic (Fig. 2A). The absolute magnitude and ranges in free-energy values observed in the YNP geothermal systems (Fig. 2) are similar to those reported by Amend et al. (2003) for the hydrothermal systems of Vulcano Island. In part, this is due to the fact that the reactions are poised by their standard state free-energy values, and due to the similar pH range of the 10 Vulcano Island sites (pH range ~ 2–6.3). Both studies show that the energetic calculations do not change the outcome that a similar set of redox reactions are highly exergonic in all sites and may provide energy for chemotrophic metabolism. Examination of the actual geochemical processes and microbial populations occurring in the springs can further elucidate which exergonic reactions may be most relevant.

Observed geochemical gradients and microbial populations

Nonequilibrium systems

The YNP geothermal source waters discussed here are nonequilibrium systems in two important respects. First, the source waters generally exhibit significant oversaturation of dissolved gas species such as CO₂, H₂, CH₄ and H₂S, whereas dissolved oxygen values are generally undersaturated with respect to earth surface conditions (e.g. ρO₂ ~ 0.2 atm). Second, the geothermal source waters exhibit significant redox nonequilibrium and contain various amounts of reduced constituents dependent in part on the degree of meteoric water mixing and oxygenation prior to discharge (Fournier, 1989).

The redox nonequilibrium of geothermal source waters can be illustrated by comparing values of pe (−log electron activity) calculated using individual half-cell reactions as a function of distance throughout the outflow channels (Fig. 3). Although temperature effects are accounted for in these calculations, the temperature changes occurring within these sampling zones (e.g. 80–55 °C) do not have a significant impact on the calculated pe values, or the free-energy values to be discussed further. This point has been noted in Amend & Shock (2001) and in Amend et al. (2003). The calculated pe values for a given half-cell reaction are poised by their respective standard state pe° values, and by a plausible window of observation ranging from analytical detection to realistic upper concentration limits. For example, the pe° value for the hydrogen half-cell (H⁺ + e⁻ = 1/2H₂(aq)) ranges from −1 to −1.25 from 85 to 55 °C; the calculated pe values are poised at −0 for the acidic systems (pH ~ 3) and at −3 for the near-neutral systems (Fig. 3). Conversely, the oxygen half-cell (0.25O₂(aq) + H⁺ + e⁻ = 0.5 H₂O) is poised near pe values of 10–15 (Fig. 3) over the same pH range. The chemical component that is most singly responsible for differences in calculated pe values among springs is H⁺ activity, which explains the 3–4 order-of-magnitude shift in electron activity observed comparing low pH (NGB-B, RS2, JC3, NGB-PS) with high pH (JC3, NGB-PS, pH ~ 7) (Fig. 2A).
Geochemical transformations occurring in the outflow channels of these springs (discussed below) are not readily apparent when examining changes in individually calculated pe values as a function of distance. The pe calculations also clarify that there is no ‘system’ pe that would adequately describe the ratios of all oxidized to reduced species. For example, the range of pe values observed (Fig. 3) for the predominant redox pairs (O2/H2O, H+/H2, SO4^{2-}/H2S, H2AsO4^-/H3AsO3, Fe^{3+}/Fe^{2+}, CO2/CH4, H+/H2) spans nearly 14 orders of magnitude throughout the outflow channel of each geothermal spring considered! Given the lack of redox equilibrium among half-cell reactions, platinum Eh values are of limited use in describing or representing the redox status of geothermal waters, or for that matter, any natural system. These points have been emphasized in previous literature (Stumm, 1984; Stumm & Morgan, 1996), but it should be reiterated that measurements of oxidized and reduced species are far more insightful than measurements of Eh for evaluating chemolithotrophic processes, and for determining the potential importance of specific redox couples in geothermal systems.

**Geochemical gradients resulting from air–water gas exchange**

The rates of degassing of H2S, CO2, CH4 and H2, combined with the rate of O2 ingassing are among the most important processes defining geochemical gradients and diverse microbial habitats as a function of distance within the outflow channels (Fig. 4). The rates of degassing or ingassing at specific distances within the outflow channels are dependent on physical factors including stream velocity, air–water surface area and boundary layer turbulence (Choi et al., 1998; Jähne & Haussbecker, 1998). For some components (H2S and CO2), pH plays a major role in defining the distribution of aqueous species, which can have a major effect on subsequent degassing rates. For example, in low pH systems such as RS2, NGB-B and NGB-S, H2S(aq) is the dominant chemical species comprising total dissolved sulfide. Consequently, the rates of degassing are rapid and within 4–6 m of discharge, dissolved sulfide values decline to less than 5% of source water values (also see Nordstrom et al., 2005). Conversely, at a pH of 6.1 (close to the pKa of H2S(aq)), dissolved sulfide values in JC3 only decline to 50% of source water values by 10 m. This is due in large part to the fact that HS^- becomes an
important soluble species at pH ~ 6. Likewise, the disappearance rates of dissolved inorganic C (DIC) are highly dependent on pH. In the acidic springs (NGB-B, NGB-S and RS2), > 99% of the DIC is present as CO₂(aq) and disappearance rates are rapid (Fig. 4). Conversely, the loss in DIC as a function of distance is less rapid for JC3 (pH ~ 6) and not significant at all across a 3-m interval in Perpetual Spouter (NGB-PS) at a pH near 7 (Fig. 4). In the low pH springs (pH ~ 3), the disappearance rates are similar for all dissolved gases including H₂S, CO₂, CH₄ and H₂, suggesting that mass transfer across the air–water interface is likely the dominant process controlling the distribution of these chemical species down gradient.

**Arsenic oxidation**

Arsenite (As³⁺) is oxidized to arsenate (As⁵⁺) within the outflow channels of all springs studied (Fig. 4). The oxidation of As³⁺ in ASC springs (NGB-B, NGB-S, NGB-D) is extremely rapid (first-order rate constants ranging from 1 to 5 min⁻¹) and has been shown to be microbiologically mediated where 40–100% of the soluble As³⁺ is oxidized to As⁵⁺ across a temperature range of ∼75–55 °C (Langner et al., 2001; Inskeep et al., 2004; Macur et al., 2004a). At least one isolate (a *Hydrogenobaculum acidophilum*-like organism) capable of oxidizing As³⁺ has been obtained from the ASC springs of Norris Geyser Basin and dissolved sulfide inhibits enzymatic oxidation of As³⁺ by this organism (Donahoe-Christiansen et al., 2004). This agrees with in situ observations that As³⁺ oxidation is not significant in the ASC springs until H₂S(aq) levels drop below ∼5 µM. The extent and rate of As³⁺ oxidation in RS2 and NGB-PS are also significant and generally correlate with O₂ ingassing (Fig. 4). Finally, As³⁺ oxidation is important even in JC3 where total dissolved sulfide drops from approximately 30 µM in the source waters to ∼10–12 µM within 10–11 m down gradient (Fig. 4).

**Solid phase deposition**

Solid phases are deposited in the source pools and throughout the outflow channels of all springs studied (Table 3). In the acid–sulfate (RS2) and acid-sulfate–chloride (NGB-B, NGB-S, NGB-D) springs, deposition of S⁰ occurs at the source and within the outflow channel until H₂S levels drop below ∼3–5 µM. Given the higher levels of total dissolved sulfide in the ASC springs, S⁰ is more abundant in the outflow channels.
Iron solid phases figure prominently in all springs studied. In the ASC springs, hydrous ferric oxide (HFO) phases form an abrupt boundary down gradient of elemental S deposition (Fig. 1) at approximately 5–12 m in NGB-B and NGB-S (Inskeep et al., 2004; Macur et al., 2004a). The Fe microbial mats are comprised primarily of filaments encrusted with poorly ordered ferrihydrite-like phases containing arsenate (0.7 mol As: mol Fe) complexed to FeIII-octahedral sites (Inskeep et al., 2004). Amorphous FeIII oxides also form in RS2, just down gradient of each of the shorter H2S zones corresponding to source water contributions at 0 cm and at 230–250 cm. Elemental S also accumulates within the softer sediments of JC3, where dissolved sulfide levels remain above 10 µM throughout the region of outflow channel studied (Fig. 1B). No elemental S has been observed within the neutral spring (NGB-PS) containing only 3 µM dissolved sulfide at the source.

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Despite low concentrations of soluble Fe in the near-neutral geothermal springs (JC3 and NSG-PS), Fe solid phases are important in both systems. In NGB-PS, FeIII-oxides form as hard coatings on SiO2 phases surrounding the source pool, and as soft microbial mats in the outflow channel from temperatures of 85–65 °C (Fig. 1A). Although there is a significant amount of soluble AsV present in NGB-PS, the Fe-oxide phases do not contain significant quantities of As. This is likely due to the pH dependence and resultant competitive complexation of silicate and arsenate binding for surface Fe-O sites (Meng et al., 2000; Davis et al., 2001). Conversely, pyrite (FeS2) is the dominant Fe phase formed in the source pool of JC3 (Fig. 1B), and no evidence of significant Fe oxidation is exhibited throughout the outflow channel. This may be due in part to the slower rate of H2S degassing and O2 ingassing in JC3 (Fig. 4). Energetic analyses of the source waters of these two springs indicated that both the oxidation of FeII and the reduction of FeIII was exergonic, but that reduction of FeIII via H2 was favoured in JC3 relative to NGB-PS.

In summary, gas exchange rates and spring water velocity define zones of H2, CH4, H2S and CO2 degassing as well as O2 ingassing. Although air–water gas exchange rates are controlled by abiotic processes, they have a substantial impact on the solid phase geochemistry and the types of microbial communities that develop throughout the outflow channels. The solid phases formed in these microbial mats may often reveal clues regarding important microbial metabolisms operative in situ. The deposition of elemental S, for example, appears to only occur when total dissolved sulfide concentrations exceed ∼5 µM (at least at these spring velocities), and this zone is in large part defined by the degassing of H2S(aq). It is also clear that Fe-oxide mats develop upon significant oxygenation due to ingassing and are not likely to form in the presence of dissolved sulfide >2–3 µM. Conversely in JC3, the presence of dissolved sulfide and the resultant slower ingassing of O2 (Fig. 4) encourages the formation of pyrite (FeS2).

### Microbial population distribution

The distribution of observed 16S rRNA gene sequences in these geothermal springs is summarized in Table 4, where the predominant sequences with close cultivated relatives are presented as a function of distance within the outflow channel. Although not all sampling sites are listed for each spring, this description summarizes several of the major changes in phylogeny that occur in the solid phases and microbial mats found near the source waters or down gradient in the outflow channels. The possible metabolisms of organisms represented by these sequences are listed to suggest potential roles for these organisms in mediating geochemical processes observed in situ. It is important to note that in all springs, additional...
bacterial and archaeal 16S rRNA gene sequences were observed, but these were not closely related to known cultured relatives and are not presented here (W. P. Inskeep, unpublished data).

Several similar 16S rDNA sequences were observed in the acidic springs as a function of distance from geothermal discharge. For example, *Stygiolobus*-like sequences have been detected in the near source water environments where dissolved O$_2$ levels are at or below detection. The reported metabolism of this organism suggests that it oxidizes H$_2$ using elemental S as an electron acceptor (Segerer *et al.*, 1991). If so, this organism is actually establishing a small S cycle.

### Table 4 Summary of 16S rDNA sequence types* identified in the prominent zones of YNP geothermal springs indicated by the closest cultivated relatives, phylogenetic affiliation, GenBank Accession numbers and possible metabolisms at specific spring locations based on geochemical conditions and known physiologies of cultivated relatives

<table>
<thead>
<tr>
<th>Spring</th>
<th>Spring location</th>
<th>Genus of nearest cultivated relative</th>
<th>Phylogenetic group</th>
<th>Accession number</th>
<th>Donors</th>
<th>Acceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidd</td>
<td>H$_2$S, S$_0$ zone</td>
<td><em>Stygiolobus</em> (98)</td>
<td>Crenarchaeota</td>
<td>AY191883</td>
<td>H$_2$</td>
<td>S$_0$</td>
</tr>
<tr>
<td>Spring</td>
<td>75–84 °C</td>
<td><em>Caldococcus</em> (97)</td>
<td>Crenarchaeota</td>
<td>AY191881</td>
<td>OC**</td>
<td>S$_0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Thermoclaudium</em> (98)</td>
<td>Crenarchaeota</td>
<td>AY191886</td>
<td>OC</td>
<td>S$_0$</td>
</tr>
<tr>
<td>NGB-B</td>
<td></td>
<td><em>Caldisphaera</em> (96)</td>
<td>Crenarchaeota</td>
<td>AY191890</td>
<td>OC</td>
<td>S$_0$</td>
</tr>
<tr>
<td>NGB-S</td>
<td>H$_2$S, S$_0$ zone</td>
<td><em>Hydrogenobaculum</em> (99)</td>
<td>Aquificales</td>
<td>AY191873</td>
<td>H$_2$, S$_0$, As$^{III}$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>60–75 °C</td>
<td><em>Desulfurella</em> (96)</td>
<td>6–Proteobacteria</td>
<td>AF325180</td>
<td>OC</td>
<td>S$_0$, SO$_4$</td>
</tr>
<tr>
<td></td>
<td>50–65 °C</td>
<td><em>Metallophaea</em> (98)</td>
<td>Crenarchaeota</td>
<td>AY191874</td>
<td>Fe$^{II}$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>55–70 °C</td>
<td><em>Sulfobacillus</em> (98)</td>
<td>Firmicutes</td>
<td>DQ350778</td>
<td>Fe$^{II}$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>50–65 °C</td>
<td><em>Thiomonas</em> (98)</td>
<td>β-Proteobacteria</td>
<td>AY191879</td>
<td>Fe$^{II}$, S$_0$, As$^{III}$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>55–70 °C</td>
<td><em>Acidimicrobium</em> (98)</td>
<td>Actinobacteria</td>
<td>AY191899</td>
<td>OC, Fe$^{II}$</td>
<td>O$_2$, Fe$^{III}$</td>
</tr>
<tr>
<td></td>
<td>50–65 °C</td>
<td>*Acetobacteraceae Y008 (99)</td>
<td>α-Proteobacteria</td>
<td>AY191880</td>
<td>OC</td>
<td>O$_2$, Fe$^{III}$</td>
</tr>
<tr>
<td>Acidd</td>
<td>H$_2$S, S$_0$ zone</td>
<td><em>Stygiolobus</em> (99)</td>
<td>Crenarchaeota</td>
<td>AY181882</td>
<td>H$_2$</td>
<td>S$_0$</td>
</tr>
<tr>
<td>Spring</td>
<td>70–75 °C</td>
<td><em>Hydrogenobaculum</em> (99)</td>
<td>Aquificales</td>
<td>AY882797</td>
<td>H$_2$, S$_0$, As$^{III}$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Metallophaea</em> (96)</td>
<td>Crenarchaeota</td>
<td>AY882800</td>
<td>S$_0$, Fe$^{II}$</td>
<td>O$_2$</td>
</tr>
<tr>
<td>RS2</td>
<td>Fe(OH)$_3$, jarosite</td>
<td><em>Hydrogenobaculum</em> (98)</td>
<td>Aquificales</td>
<td>AY882804</td>
<td>H$_2$, S$_0$, As$^{III}$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>55–70 °C</td>
<td><em>Metallophaea</em> (96)</td>
<td>Crenarchaeota</td>
<td>AY882806</td>
<td>Fe$^{II}$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>55–70 °C</td>
<td><em>Acidianus</em> (93)</td>
<td>Crenarchaeota</td>
<td>AY882801</td>
<td>Fe$^{II}$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>50–65 °C</td>
<td><em>Metallophaea</em> (95)</td>
<td>Crenarchaeota</td>
<td>AY882814</td>
<td>Fe$^{II}$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>50–65 °C</td>
<td><em>Sulfobacillus</em> (93)</td>
<td>Firmicutes</td>
<td>AY882815</td>
<td>Fe$^{II}$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>50–65 °C</td>
<td>*Acetobacteraceae Y008 (98)</td>
<td>α-Proteobacteria</td>
<td>AY882809</td>
<td>OC</td>
<td>O$_2$, Fe$^{III}$</td>
</tr>
<tr>
<td>Neutral</td>
<td>Fe$<em>{S_2}$, S$</em>{bS}$, jarosite</td>
<td><em>Thermocraubacterium</em> (99)</td>
<td>Thermodesulfobacteria</td>
<td>AY882717</td>
<td>H$_2$</td>
<td>Fe$^{III}$</td>
</tr>
<tr>
<td>Spring</td>
<td>75–90 °C</td>
<td><em>Vulcanisaeta</em> (97)</td>
<td>Crenarchaeota</td>
<td>AY882719</td>
<td>OC</td>
<td>S$_2$O$_3$, S$_0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Caldococcus</em> (97)</td>
<td>Crenarchaeota</td>
<td>AY882714</td>
<td>OC</td>
<td>S$_0$</td>
</tr>
<tr>
<td>JC3</td>
<td>SiO$_2$, S$_0$</td>
<td><em>Caldococcus</em> (96)</td>
<td>Crenarchaeota</td>
<td>AY882764</td>
<td>OC</td>
<td>S$_0$</td>
</tr>
<tr>
<td></td>
<td>50–75 °C</td>
<td><em>Caldisphaera</em> (97)</td>
<td>Crenarchaeota</td>
<td>DQ333311</td>
<td>OC</td>
<td>S$_0$</td>
</tr>
<tr>
<td></td>
<td>50–75 °C</td>
<td><em>Desulfurella</em> (98)</td>
<td>6–Proteobacteria</td>
<td>DQ333312</td>
<td>OC</td>
<td>S$_0$, SO$_4$</td>
</tr>
<tr>
<td></td>
<td>50–75 °C</td>
<td><em>Sulfurhydrogenibium</em> (98)</td>
<td>Aquificales</td>
<td>AY882757</td>
<td>H$_2$, S$_2$O$_3$, S$_0$, NO$_3$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>50–75 °C</td>
<td><em>Thermocraubacterium</em> (98)</td>
<td>Aquificales</td>
<td>AY882760</td>
<td>H$_2$, S$_2$O$_3$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td></td>
<td>50–75 °C</td>
<td><em>Thermus</em> (99)</td>
<td>Deinococcus–Thermus</td>
<td>AY882763</td>
<td>OC, As$^{III}$ ?</td>
<td>O$_2$</td>
</tr>
<tr>
<td>Neutral</td>
<td>SiO$_2$, jarosite</td>
<td><em>Thermocrinis</em> (99)</td>
<td>Aquificales</td>
<td>DQ324878</td>
<td>H$_2$, S$_2$O$_3$, S$_0$</td>
<td>O$_2$</td>
</tr>
<tr>
<td>Spring</td>
<td>80–85 °C</td>
<td><em>Thermus</em> (99)</td>
<td>Deinococcus–Thermus</td>
<td>DQ324868</td>
<td>OC</td>
<td>O$_2$</td>
</tr>
</tbody>
</table>

*16S rRNA sequences from ASC springs in NGB were compiled from Jackson *et al.*, 2001, Inskeep *et al.*, 2004 and Macur *et al.*, 2004.

1Major geochemical (e.g. dominant solid phase) and temperature zones at and down gradient of geothermal source.

2Values in parentheses are the percentage similarity to nearest cultivated relatives in GenBank (Benson *et al.*, 2005). Sequences whose closest cultivated relatives are <93% are not discussed here.

3The exact stoichiometry of the stibnite-like, Sb-S phase is under study.

4Primary studies are cited in text.

*OC = organic carbon; As$^{III}$ ? = close relatives have been shown to oxidize As$^{III}$ but not necessarily for energy gain.*
[between S(0) and S(-II)] after the formation of elemental S on the spring floor, regenerating dissolved sulfide. *Hydrogenobaculum*-like sequences are also common to both the acid-sulfate-chloride springs of Norris Basin and the acid-sulfate Rainbow Spring (RS2). In fact, numerous *Hydrogenobaculum*-like sequences are detected throughout the outflow channels in the acidic spring types (Macur *et al*., 2004a; Table 4). In culture, these Aquificales are generally aerobic to microaerobic and may oxidize H$_2$, S$^0$ and or As$^{III}$ using O$_2$ as an electron acceptor (Donahoe-Christiansen *et al*., 2004; Reysenbach *et al*., 2005). Consequently, these organisms will depend on variable fluxes of O$_2$ supplied via ingassing, and if oxidizing H$_2$ or S$^0$, would likely be in their optimum habitat throughout the S$^0$ depositional zones.

As the acidic outflow channels become oxygenated and H$_2$S levels decline to below 1–2 µM, Fe-oxide phases become the dominant solid phase. Much of the Fe biomineralization occurs as encrustations and sheaths around microbial filaments (Inskeep *et al*., 2004; Macur *et al*., 2004a). Pronounced changes in phylogeny occur in both acidic spring types including appearance of archaeal and bacterial sequences whose close relatives have all been implicated in the oxidation of Fe$^{III}$. The presence of *Metallospirillum*, *Sulfobacillus*, *Acidimicrobium* and *Thiobacillus*-like sequences in the Fe-oxide mats is generally consistent with possible chemolithotrophy on Fe$^{II}$ using O$_2$ as an electron acceptor (Peeples & Kelly, 1995; Norris *et al*., 1996; Bruneel *et al*., 2003; Johnson *et al*., 2003). There is also evidence of heterotrophic populations that may reduce Fe$^{III}$ such as sequences related to *Acetobacteraceae* Y008, an isolate obtained from Norris Basin (Johnson *et al*., 2003).

Comparison of 16S rDNA sequences obtained from the near-neutral springs reveals several important clues about geochemical factors controlling the types of microorganisms found in these geothermal ecosystems. The presence of significant dissolved sulfide at higher pH and the subsequent slower disappearance rates of sulfide down gradient contribute to slower oxygenation. Within the source pool and adjacent sediments, predominant sequence types observed were highly related to *Geothermobacterium ferrireducens*, an organism capable of oxidizing H$_2$ with Fe$^{III}$ as an electron acceptor (Kashefi *et al*., 2002), and a suite of chemoorganotrophic organisms that are likely reducing elemental S using organic C (OC) as an electron donor (e.g. *Vulcaniseta*, *Caldisphaera*, *Thermofilum*; Kjems *et al*., 1990; Aoshima *et al*., 1996; Itoh *et al*., 2002). Below temperatures of 70 °C, *Thermocrinum* and *Sulfurihydrogenibium*-like sequences become important. In culture, these Aquificales require H$_2$, S$^0$, S$_2$O$_3$ or Fe$^{III}$ for growth under aerobic to microaerobic conditions (Huber *et al*., 1998; Aguiar *et al*., 2004). Consequently, although O$_2$ ingassing is considerably slower in JC3, these organisms require some flux of O$_2$ and are not observed in the source pool sediments. Interestingly, the sequence data from JC3 suggest that microaerobic to aerobic Aquificales and anaerobic S$^0$-reducing organisms such as *Desulfiurella* and *Caldisphaera* (Miroshnichenko *et al*., 1998; Itoh *et al*., 2003) are both important groups of organisms down gradient; this is consistent with the minimal oxygenation occurring throughout the outflow channel of JC3.

Conversely, the near-neutral NGB-PS contains very little dissolved sulfide and the source waters appear partially oxygenated upon discharge (e.g. As$^{V}$/As$^{III}$ ratios are ~0.38 at source water discharge as compared to ~0.05–0.1 in the other systems; Fig. 4). *Thermocrinum* and *Thermus*-like sequences are the most dominant clones observed in NGB-PS (Table 4). Organisms represented by the *Thermocrinum*-like sequences may be oxidizing H$_2$ with O$_2$ as an electron acceptor (Huber *et al*., 1998). Unlike the other springs, there were no sequences observed in NGB-PS that could be inferred through close relatives to be engaged in any kind of elemental S reduction, consistent with the direct observation that low dissolved sulfide values of <2–3 µM are not sufficient to deposit elemental S, and support subsequent S reducers. Interestingly, no obvious sequences were detected that could be used to support the assumption that Fe$^{III}$ chemotrophy is occurring in this system. The biotic rates of Fe$^{III}$ oxidation to form Fe$^{III}$ oxides increase dramatically above pH values of 4.5 (Nordstrom & Southam, 1997); consequently, abiotic Fe oxidation may make a greater contribution to Fe$^{III}$-oxide deposition than in the acidic springs where phylogenetic evidence strongly supports the presence of Fe$^{III}$-oxidizing chemotrophs. The free-energy values for Fe oxidation are $-65$ to $-75$ kJ mol$^{-1}$ e$^{-}$ (e.g. Reactions 24, 25) in NGB-PS compared to only $-25$ to $-30$ kJ mol$^{-1}$ e$^{-}$ in the acidic systems. Consequently, reliance on the magnitude of free-energy values for Fe oxidation to infer population distribution is not entirely consistent with observed phylogeny. As temperatures in the outflow channel of NGB-PS cool to $-65$ C, *Thermomicrobium*-like sequences have been recovered. Cultivated *Thermomicrobium* sp. are aerobic heterotrophs of the Chloroflexi (Jackson *et al*., 1973), some members of which can potentially oxidize As$^{III}$ via aox-like genes (LeBrun *et al*., 2003).

**Variation in free-energy values within outflow channels**

Calculated free-energy values for several representative redox reactions are each poised within a fairly narrow energy window as a function of distance within the outflow channels (Fig. 5). This behaviour is essentially mirrored in the estimated electron activity values from various half-cell reactions (Fig. 3) down gradient in each spring and is controlled by the respective standard state free-energy values and a corresponding range of possible ratios of oxidized to reduced species. For example, there is no significant change in free-energy values (per mol electron) for H$_2$ oxidation, H$_2$S oxidation or Fe$^{II}$ oxidation as a function of distance within the outflow channels that would assist in demarcating termination of S$^0$ deposition followed by deposition of Fe$^{III}$-oxide like phases observed in the pH 2.5–3 springs (e.g. see Fig. 1; Table 3), or the striking As$^{III}$
oxidation patterns observed in all springs that occur at well-defined positions based in part on the different amounts of dissolved oxygen and dissolved sulfide (Fig. 4). Although significant changes in geochemistry and phylogeny occur within outflow channels (Fig. 4, Table 4), the calculated free-energy values are not particularly sensitive to these changes and offer poor resolution for understanding geochemical and temperature gradients occurring down stream of source discharge. Moreover, although the variation in observed microbial populations down gradient (Table 4) appear to reflect changes in geochemistry and temperature, the free-energy values do not reveal significant changes with distance that would correlate with the distribution of micro-organisms.

**DISCUSSION**

**Sensitivity analysis of free-energy calculations**

Redox couples involving H$_2$, H$_2$S, S$^0$, Fe$^{2+}$, H$_3$AsO$_3$, CH$_4$ and NH$_4$ as electron donors with O$_2$ as the oxidant are all highly exergonic in the source waters of the springs studied. As O$_2$ concentrations increase down gradient due to air–water gas exchange, reactions with O$_2$ generally become slightly more exergonic, but in some cases the activities of electron donor are declining simultaneously, such as with the degassing of H$_2$S(aq), H$_2$(aq) and CH$_4$(aq). The value of $\Delta G_{\text{rxn}}$ for each couple is fairly insensitive to changes in the activity of O$_2$(aq), within the range of detectable O$_2$(aq) to saturation at 0.16 atm O$_2$(g) (e.g. 0.08–140 µM, total pressure at YNP elevation is approximately 0.77 atm). Consequently, in the case of highly exergonic reactions with O$_2$, it is obvious that detailed analytical measurements of individual sites will not change the conclusion that these reactions may be important for chemolithotrophic micro-organisms. As pointed out in Fig. 6 (Reaction 1), whether one assumes $10^{-3.7}$ M O$_2$ (e.g. saturation) or $10^{-20}$ M O$_2$ (e.g. 10 orders of magnitude lower than a reasonable detection limit) will have little impact on the conclusion that the knall-gas reaction (Reaction 1) is highly exergonic. Interpretations regarding the importance of H$_2$ as an electron donor for microbial metabolism in geothermal systems cannot be based solely on energetic calculations, because it is clear that the reaction will always be exergonic, provided O$_2$(aq) and H$_2$(aq) are greater than $\sim 10^{-25}$ M (Fig. 6, Reaction 1). Consequently, the fact that this reaction is highly exergonic regardless of position within the outflow channel should not be over interpreted as evidence that H$_2$
Fig. 6 Sensitivity analysis of the dependence of free-energy values on reactant concentrations for several redox reactions (Reactions 1, 8, 4, 20, 28, 33; Table 2) that may be important for chemolithotrophic metabolism in geothermal springs. Circles represent estimated regions of the range in free-energy values corresponding to reactant detection limits or realistic upper concentration limits. Sensitivity analyses (lines) investigate ranges in pH, dissolved O$_2$ and dissolved H$_2$S ($p = \ln$).

Results show that for most conditions experienced in natural systems, electron donor reactions with O$_2$(aq) are highly exergonic and detailed chemical analyses of specific sites will not change this conclusion, given current detection limits.
oxidation is the dominant metabolism for all thermophiles across these geothermal gradients. Numerous thermophilic organisms (especially members of the Aquificales) may utilize the knall-gas reaction (Eder & Huber, 2002; Reysenbach et al., 2005; Spear et al., 2005a,b), but are likely confined to zones of higher H$_2$(aq) as defined by degassing rates (e.g. Figure 4). In fact, we have observed several 16S rRNA gene sequences within the higher H$_2$ zones whose close relatives are known H$_2$ oxidizers. These include Hydrogenobaculum-like sequences in ASC (NGB-B, NGB-S, NGB-D) and AS (RS2) springs (Shima & Suzuki, 1993) and Sulfurilyhydrogenibium and Thermocronius-like sequences in near-neutral springs of JC3 and NGB-PS, respectively (Huber et al., 1998; Aguilar et al., 2004; Table 4). Many of the inferred H$_2$-oxidizing populations observed in the subject geothermal springs are microaerobic or aerobic organisms; consequently, some flux of O$_2$ is necessary to support these populations in situ. The rates of O$_2$ ingassing and aqueous O$_2$ mass transfer are likely critical parameters for understanding the actual distribution of thermophiles that capitalize on the free energy available from O$_2$ electron acceptor reactions. Phylogenetic evidence supports the assumption that H$_2$ is an important electron donor, but not necessarily always with O$_2$ as an electron acceptor, and not across all positions within the outflow channels (Table 4).

**Sulfur oxidation**

Reactions involving the oxidation of reduced S species by O$_2$(aq) are all highly exergonic in the systems considered (e.g. Reactions 8 [H$_2$S/SO$_4$], 9 [H$_2$S/SO$_4$] and 14 [SO$_4$/ SO$_4$]; Fig. 2). Again, this is not particularly surprising considering the standard state $\Delta G_{\text{rxn}}$ values for these electron transfer reactions. Figure 6(B) identifies the plausible range of calculated $\Delta G_{\text{rxn}}$ values for the oxidation of H$_2$S via O$_2$ (Reaction 8) and indicates that irrespective of the amounts of H$_2$S or O$_2$ measured (within detection), this reaction will be highly exergonic. Indeed, $\Delta G_{\text{rxn}}$ values for each spring fall within the detection-saturation window shown in Fig. 6(B). It is important to question the utility of this result realizing that all systems will yield similar exergonic values for this redox couple. Several close relatives of 16S rDNA sequences obtained from within the H$_2$S degassing zone (Fig. 4) of the acidic springs (e.g. NGB-S and RS2) are known H$_2$S and S$^0$ oxidizers (Table 4). For example, Hydrogenobaculum-like populations are commonly observed, while Sulfurilyhydrogenibium-like populations appear in the higher pH spring JC3. Both genera are represented by cultured organisms capable of oxidizing reduced S species (Shima & Suzuki, 1993; Reysenbach et al., 2005). Given that several members of the Aquificales have been shown to be capable of oxidizing reduced S and or H$_2$, it is difficult to use energetic favourability as an argument for which metabolism is most important in the near source water environments of sulfidic geothermal springs.

**Sulfur reduction**

The oxidation of H$_2$ with S$^0$ as the electron acceptor (Reaction 4) is an interesting redox couple to consider further, especially given that it represents one of the most primitive possible chemolithotrophic processes in the absence of O$_2$. Energetic analyses (Fig. 6C) show that this reaction is significantly less exergonic than many other possible reactions with O$_2$ as an electron acceptor, yielding values of only $\sim$10 to $\sim$20 kJ mol$^{-1}$ electron, near the potential limits of minimum energy requirements to support cellular metabolism (Hoehler et al., 2001; Jackson & McInerney, 2002). The plausible window of $\Delta G_{\text{rxn}}$ values shows that this reaction will generally be exergonic, although only down to H$_2$(aq) activities of $10^{-10}$ to $10^{-12}$ M at high H$_2$S (e.g. mm levels). Do the lower values of $\Delta G_{\text{rxn}}$ suggest that this reaction is less important for chemolithotrophs and that this will not be a preferred metabolism in these systems? Phylogenetic analyses of the springs studied indicate that Stygiolobus, Caldococcus, Vulcainiaeta and Caldisphaera-like sequences (Table 4) are important populations observed in the S$^0$ depositional zones of all springs studied with the exception of NGB-PS, which contains dissolved sulfide values <3 $\mu$m. Cultivated relatives of these sequences are known to utilize S$^0$ as an electron acceptor via either H$_2$ (Styglibus azorius; Segerer et al., 1991) or organic C (Vulcainiaeta, Caldococcus, Caldisphaera sp.; Aoshima et al., 1996; Itoh et al., 2002; Itoh et al., 2003) as electron donors. Consequently, despite the lower $\Delta G_{\text{rxn}}$ values for the reduction of elemental S$^0$ via H$_2$ (Reaction 4), this reaction may be quite important in supporting anaerobic primary producers. Free-energy values for organotrophic reduction of elemental S are also estimated to be low ($\sim$25 to $\sim$35 kJ mol$^{-1}$ e$^{-}$; data not shown) relative to other highly exergonic reactions. In all springs studied, where present, these populations were confined to the near-source water zones prior to significant O$_2$ ingassing, and where S$^0$ was ubiquitous along the channel floor. Consequently, although steep gradients in dissolved H$_2$S and H$_2$ are important in defining specific niches within the outflow channel, the importance of anaerobic S reducers would have been difficult to predict based solely on the magnitude of calculated free-energy values (e.g. Figures 2 and 6).

**Arsenic oxidation**

The oxidation of H$_3$AsO$_4^-$ with O$_2$ is also highly exergonic ($\sim$50 to $\sim$80 kJ mol$^{-1}$ electron), independent of dissolved O$_2$ concentrations ranging from detection to saturation (Fig. 6D). Rapid oxidation rates of As$^{III}$ are observed in all springs studied (Fig. 4), and this has been shown to be mediated by micro-organisms in several of the ASC springs (Langner et al., 2001; Macur et al., 2004a), and in other geothermal watersheds (Wilkie & Her ing, 1998; Gihring et al., 2001). However, the actual mechanisms and dynamics of microbial As$^{III}$ oxidation are not well understood and may
or may not be related to chemolithotrophic metabolism (e.g. see reviews by Silver & Phung, 2005; Cervantes et al., 1994; Mukhopadhyay et al., 2002; Oremland & Stolz, 2003). Definitive chemolithotrophic metabolism on AsIII has only been reported for several micro-organisms (Santini et al., 2000, 2002; Oremland et al., 2002; Bruneel, 2004), yet detoxification strategies involving anaer-like genes may also contribute to observed AsIII oxidation (LeBrun et al., 2003; Muller et al., 2003; Macur et al., 2004b; Silver & Phung, 2005). Dissolved sulfide (H2S(aq)) has been shown to inhibit AsIII oxidation in Hydrogenobaculum-like populations (Donahoe-Christiansen et al., 2004) and this observation is consistent with As speciation profiles in ASC springs (e.g. NGB-B; Fig. 4) where AsIII oxidation commences after dissolved sulfide concentrations drop below ~2 µM. However, in the near-neutral JC3 thermal spring, AsIII oxidation occurs despite the presence of total dissolved sulfide ranging from 15 to 30 µM within the outflow channel. At this time, it is not known whether organisms within these springs are oxidizing AsIII for energy gain, but it is clear that the dynamics of As speciation within the geothermal outflow channels are controlled by a combination of biotic and abiotic factors that cannot be resolved through general examination of free-energy values. Microbial detoxification pathways, which often have a significant impact on trace element cycling in natural systems, generally require energy (Rosen, 2002); consequently, the energetic favourability of a particular inorganic couple may not be of immediate value for understanding microbial-geochemical linkages.

The oxidation of CH4 to CO2 (methanotrophy) and NH4+ to NO3− (nitrification) are also considerably exergonic under all plausible conditions (Fig. 6E,F). The free-energy values are poised from approximately ~80 to ~100 kJ mol−1 electron for the oxidation of CH4 via O2, and from ~20 to ~40 kJ mol−1 electron for the oxidation of NH4+ via O2 (Fig. 6E,F). The acidic geothermal springs (NGB-B, S and RS2) and the near-neutral JC3 spring contain significant quantities of CH4(aq) in the source waters (Table 1). However, it is not clear from phylogenetic analyses whether there are organisms within these springs that may be utilizing CH4(aq) as an electron donor. To date, none of the 16S rDNA sequences identified in these springs have obvious cultivated relatives known to oxidize CH4 (Jackson et al., 2001; Macur et al., 2004a; S. Korf, unpublished data). Certainly, it is possible that micro-organisms represented by 16S rDNA sequences with poor matches to cultivated relatives may be involved in CH4 oxidation. However, methane oxidation may be inhibited in highly acidic conditions (Hanson & Hanson, 1996). Consequently, until we understand the physiology and activity of relevant microbial populations in these systems, it is not possible to conclude that CH4 is an important electron donor, despite the fact that it is one of the most exergonic processes possible. Similarly, the oxidation of NH4+ to NO3− is highly exergonic; however, this reaction may not be important to chemolithotrophs in acidic geothermal systems (Kowalchuk & Stephen, 2001), despite the fact that concentrations of NH4+ are 1.7 and 5.5 mM in RS2 and JC3, respectively. No consumption of NH4+ or production of NO3− (or NO2−) has been observed throughout the outflow channels of the springs studied (data not shown), suggesting little microbial oxidation of NH4+ is occurring despite the fact that it is highly exergonic.

Reactions with iron

Redox reactions involving FeII as an electron donor or FeIII as an electron acceptor are particularly important in YNP geothermal springs (e.g. Reactions 3, 12, 22, 24–27, Table 3), and of all the redox reactions considered, free-energy values of reactions involving Fe are the most variable across sites. Moreover, due to the nonequilibrium nature of these systems, both the oxidation of FeII and the reduction of FeIII are exergonic in all the geothermal systems considered here (e.g. reactions 24 and 25 and 3, 12, 17 and 18 in Table 2). Given these divergent thermodynamic possibilities, factors other than the calculated thermodynamic favourability play an important role in defining the fate of Fe in these systems and explain the biomineralization of FeIII oxides observed in NGB-B, NGB-S and NGB-PS compared to the formation of FeS2 in JC3. In the current study, we examined two general situations: (i) low pH geothermal springs containing high aqueous FeII ranging from 0.05 to 0.1 mM (NGB-B, NGB-S, RS2), or (ii) near-neutral pH springs (NGB-PS, JC3) containing less than 2 µM aqueous Fe. In the acidic springs, FeII is the dominant valence state at the source (>98% FeII) and no consistent losses of dissolved Fe are observed down gradient, indicating that the flux of total soluble Fe is high relative to the accumulation rate of FeIII solid phases within the outflow channel (Langner et al., 2001). The amounts of FeIII and FeII in JC3 and NGB-PS are more difficult to assess at less than 2 µM total Fe, but we have consistently observed a mixture of FeII and FeIII in the source waters of these springs. The corresponding energetic analyses for all four geothermal springs show that oxidation reactions of FeII with O2 are exergonic (range of ~20 to ~70 kJ mol−1 electron) either written as a reaction to form soluble Fe3+ (Reaction 24) or to form FeIII-oxides such as ferricydrate (Fe(OH)3) (Reaction 25). Simultaneously, the reduction of FeII with H2(aq) (Reaction 3) is favourable in these geothermal springs, and free-energy values also range from ~25 to ~50 kJ mol−1 electron (Fig. 7C). Consequently, without additional knowledge of the processes actually occurring and the microbial populations actually inhabiting these sites, energetic approaches are of limited use for predicting dominant microbial processes involving Fe in these habitats.

When the actual processes and microbial populations are examined, it is clear that FeII oxidation is very important in the acidic springs, where copious amounts of Fe(OH)3 phases (and jarosite in RS2) are formed due to microbial processes (Inskeep et al., 2004; Macur et al., 2004a). Several of the microbial populations identified in the Fe mats of NGB-B,
NGB-S and RS2 via 16S rDNA sequence analysis are closely related to known Fe II-oxidizing organisms including 
\textit{Metallosphaera}, \textit{Acidmicrobium}, \textit{Sulfobacillus} and \textit{Thiomonas} (Table 4) (Fuchs \textit{et al}, 1995; Peeples & Kelly, 1995; Norris \textit{et al}, 1996; Bruneel \textit{et al}, 2003; Johnson \textit{et al}, 2003). The higher pH springs (JC3 and NGB-PS) clearly show higher free-energy values for Fe$^{2+}$ oxidation due to significant decreases in H$^+$ activity (Reaction 25), or the lower calculated Fe$^{3+}$ activities (Reaction 24) (Fig. 7A,B). The oxidation of Fe$^{II}$ in NGB-PS has not yet been linked to potential Fe$^{II}$ oxidizing populations, and may represent a greater contribution from the more rapid chemical rate of Fe$^{II}$ oxidation above pH 4.5 (Nordstrom & Southam, 1997). Conversely, no Fe$^{III}$ oxidation is observed throughout the outflow channel of JC3. In fact, pyrite (FeS$_2$) is a dominant solid phase formed in the source pool and is also found distributed down gradient. Phylogenetic analyses (16S rDNA) of sediments taken from JC3 indicate that \textit{G. ferrireducens}-like organisms are likely the predominant bacteria inhabiting the source pool sediments (Table 4). The metabolism reported for the anaerobic \textit{G. ferrireducens} involves H$_2$ oxidation via Fe$^{3+}$ as an electron acceptor (Kashefi \textit{et al}, 2002), and this is consistent with the formation of FeS$_2$ in this particular spring.

Although the energetic analyses show that both Fe$^{II}$ oxidation and Fe$^{III}$ reduction are exergonic in all springs studied, the dominant processes result in either the formation of Fe$^{III}$ oxide solid phases or the Fe$^{II}$-sulfide solid phases. The major factor that appears to control the fate of Fe is the rate of dissolved sulfide degassing and the inversely correlated rate of O$_2$ ingassing (Fig. 4). In the acidic springs, degassing of dissolved sulfide is encouraged due to the fact that H$_2$S(aq) is the dominant species of dissolved sulfide at low pH (<5), and Fe$^{II}$ oxidation commences as soon as dissolved sulfide levels drop to below 1–2 $\mu$M. Conversely, the rate of sulfide degassing is much slower in JC3 due to the fact that HS$^-$ is a more important soluble species of dissolved sulfide. The oxidation of Fe$^{II}$ is likely limited by insufficient O$_2$ mass transfer, and the slower degassing rates of dissolved sulfide encourages the formation of Fe$^{II}$-sulfide solid phases (Luther \textit{et al}, 2003). Consequently, the processes that appear to be most important in defining the geochemical and microbial community trajectory are related to rates of air–water gas exchange.

\textbf{Energy fluxes and kinetics in dynamic environments}

How might we improve the utility of energetic analyses for understanding the role of biota in geochemical cycling? One possibility would be to express the energy available to microorganisms as an energy flux in units of energy per unit area per unit time (e.g. kJ m$^{-2}$ min$^{-1}$). Although the velocity (m min$^{-1}$) at any single point within a watershed or geothermal outflow channel will be a constant scalar for all reactions considered, the actual concentrations of various electron donors will vary and modify the energy flux. Consequently, rather than considering the energy available per mole of electron, the energy flux provides an additional and perhaps more meaningful estimate of the energy available to microorganisms at a given location in the flowing channel. Energy fluxes can be calculated based on concentrations of either the
electron donors or the electron acceptor at specific points within the outflow channels. For example, energy fluxes were calculated for source-water conditions in NGB-B based on concentrations of electron donors including H2, H2S, AsIII, NH4 and CH4 and a measured velocity of approximately 5 m min−1. Resultant energy fluxes range from 0.007 to 0.02 kJ m−2 min−1 for reactions where H2 is an electron donor to values of 30–500 kJ m−2 min−1 for reactions with H2S, 5–15 kJ m−2 min−1 for reactions with AsIII, 0.4–7 kJ m−2 min−1 for reactions with FeII, and 0.7–3 kJ m−2 min−1 for reactions with CH4. These calculations show that although H2 oxidation is highly exergonic per mole electron transferred, the potential energy flux based on the actual concentration of H2 is fairly low compared to the energy flux from other electron donors. The reactions with H2S as an electron donor yield the greatest energy fluxes due to the higher concentration of H2S relative to other potential electron donors, and the energy fluxes for reactions where CH4, FeII and AsIII serve as electron donors are one to two orders of magnitude greater than reactions with H2. Despite the lower energy flux for reactions with H2, phylogenetic analyses of the ASC springs (Jackson et al., 2001; Macur et al., 2004a) suggest that H2 is likely an important electron donor, especially near the point of spring discharge, prior to significant degassing. Phylogenetic analyses and subsequent physiological inference of other near-neutral geothermal systems in YNP also suggest that H2 is an important electron donor driving primary productivity (Shock et al., 2005; Spear et al., 2005a,b). However, it is incorrect to assume that H2 is the only important electron donor in the source pools and especially the outflow channels of geothermal springs.

Although energy fluxes may be an additional approach for understanding the distribution of microbial populations in geothermal systems, they too will be limited in their ability to predict the distribution of microbial populations, in part due to the steep gradients in concentrations of dissolved gases and in part due to the physiological realities of adapted microbial populations. A complete understanding of geochemical–microbial interactions in geothermal systems will require integration of abiotic and biotic processes in a dynamic framework that includes relevant reaction rates coupled with the activities of relevant microbial populations (Inskipp & McDermott, 2005). In this regard, future studies will continue to identify the dominant community members in model geothermal systems and characterize their physiological attributes. Moreover, knowledge of the expression of particular functional genes in these chemotrophic environments would be extremely beneficial for testing hypotheses regarding the primary electron donors and acceptors responsible for primary productivity (Newman & Banfield, 2002).

It is clear that the actual distribution of adapted microbial populations and their respective activities are correlated with geochemical conditions, but it is not necessarily clear which abiotic geochemical processes may be the most important in actually controlling the distribution and activity of a specific micro-organism. Certainly, temperature and pH are important parameters that may directly impact microbial community structure. Indirectly, pH can have an enormous impact on other geochemical processes that may have a global impact on geochemical conditions and resultant phylogeny, such as the slower degassing rates of H2S in JC3.

Air–water exchange reactions are responsible for significant chemical mass transfer across the air–water interface over very short distances (cm to m). Consequently, steep gradients in dissolved O2 and other gases play an important role in defining the distribution of microbial populations and changes in aqueous and solid phase geochemistry occurring down gradient of thermal discharge. Physical factors such as velocity, air–water surface area and turbulence link directly to rates of air–water gas exchange, and these same processes control rates of temperature decline in geothermal outflow channels. Further phylogenetic analyses coupled with functional gene analysis and measurements of gene expression (Croal et al., 2004) may clarify the importance of gas exchange rates in defining the distribution of microbial populations in geothermal systems. An integration of abiotic and biotic reaction rates controlling geochemical processes could lead to the development of more comprehensive kinetic models (e.g. Wang & van Cappellen, 1996; Mayer et al., 2002) that recognize the importance of key abiotic reactions as well as microbial activities of specific adapted populations. Although energetic analyses can tell us what is possible, it is clear that our understanding of biogeochemical cycling and geobiology is predicated on the paths and actual rates of specific processes. Moreover, interpretations regarding the comparison of numerous free-energy values for redox couples across different sites must be tempered with the reality that the standard state free-energy value for a chosen redox reaction poises the reaction within a relatively narrow range of free-energy values, especially when activities of both oxidized and reduced species are above analytical detection limits.

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