In situ analysis of oxygen consumption and diffusive transport in 1 high-temperature acidic iron-oxide microbial mats

Authors: Hans C. Bernstein, Jacob P. Beam, Mark A. Kozubal, Ross P. Carlson, & William P. Inskeep

NOTICE: This is the peer reviewed version of the following article: Bernstein HC, Beam JP, Kozubal MA, Carlson RP, Inskeep WP, "In situ analysis of oxygen consumption and diffusive transport in 1 high-temperature acidic iron-oxide microbial mats," Environ Microbiol 2013 15(8):2360-2370, which has been published in final form at <http://dx.doi.org/10.1111/1462-2920.12109>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving

Bernstein HC, Beam JP, Kozubal MA, Carlson RP, Inskeep WP, "In situ analysis of oxygen consumption and diffusive transport in 1 high-temperature acidic iron-oxide microbial mats," Environ Microbiol 2013 15(8):2360-2370

***In situ* analysis of oxygen consumption and diffusive transport in high-temperature acidic iron-oxide microbial mats**

Hans C. Bernstein,^{1,3†} Jacob P. Beam,^{2,4†}
Mark A. Kozubal,^{2,4} Ross P. Carlson^{1,3,4} and
William P. Inskeep^{2,4*}

¹Department of Chemical and Biological Engineering,

²Department of Land Resources and Environmental Sciences, ³Center for Biofilm Engineering, and ⁴Thermal Biology Institute, Montana State University, Bozeman, MT 59717, USA.

Summary

The role of dissolved oxygen as a principal electron acceptor for microbial metabolism was investigated within Fe(III)-oxide microbial mats that form in acidic geothermal springs of Yellowstone National Park (USA). Specific goals of the study were to measure and model dissolved oxygen profile within high-temperature (65–75°C) acidic (pH = 2.7–3.8) Fe(III)-oxide microbial mats, and correlate the abundance of aerobic, iron-oxidizing *Metallosphaera yellowstonensis* organisms and mRNA gene expression levels to Fe(II)-oxidizing habitats shown to consume oxygen. *In situ* oxygen microprofile were obtained perpendicular to the direction of convective flow across the aqueous phase/Fe(III)-oxide microbial mat interface using oxygen microsensors. Dissolved oxygen concentrations dropped from ~50–60 µM in the bulk-fluid/mat surface to below detection (< 0.3 µM) at a depth of ~700 µm (~10% of the total mat depth). Net areal oxygen fluxes into the microbial mats were estimated to range from $1.4\text{--}1.6 \times 10^{-4} \mu\text{mol cm}^{-2} \text{ s}^{-1}$. Dimensionless parameters were used to model dissolved oxygen profile and establish that mass transfer rates limit the oxygen consumption. A zone of higher dissolved oxygen at the mat surface promotes Fe(III)-oxide biomineralization, which was supported using molecular analysis of *Metallosphaera yellowstonensis* 16S rRNA gene copy numbers and mRNA

expression of haem Cu oxidases (FoxA) associated with Fe(II)-oxidation.

Introduction

Yellowstone National Park (YNP), Wyoming, USA contains a diverse collection of pristine high-temperature environments containing stable thermophilic microbial communities. Acidic (pH ~3), amorphous Fe(III)-oxyhydroxide microbial mats located in Norris Geyser Basin (NGB) are of particular interest due to the interplay between geochemical cycling, nutrient transport, and unique microbial metabolic capabilities associated with dominant community members of the domain *Archaea* (Jackson *et al.*, 2001; Inskeep *et al.*, 2004; 2005; 2010; Macur *et al.*, 2004; Inskeep and McDermott, 2005; Kozubal *et al.*, 2008; 2011). Both autotrophic and heterotrophic Fe-metabolizing community members have been isolated and observed in metagenomes from Fe(III)-oxide mats in NGB (Kozubal *et al.*, 2008; 2012b; Inskeep *et al.*, 2010). Of these, Fe-oxidizing *Metallosphaera yellowstonensis*-like organisms have been shown to comprise near 20% of the total archaeal community in numerous Fe(III)-oxide microbial mats (Kozubal *et al.*, 2008). *M. yellowstonensis* is known to utilize dissolved O₂ as the principal terminal electron acceptor via a haem Cu oxidase (FoxA) associated with the Fox terminal oxidase complex (Kozubal *et al.*, 2008; 2011). An additional Fe-metabolizing community member (*Sulfolobales* strain MK5) has been characterized and found to be a heterotrophic Fe(II)-oxidizer and Fe(III)-reducer under aerobic and anaerobic conditions, respectively (Kozubal *et al.*, 2012b). Other dominant organisms in Fe-oxide mat communities include a deeply rooted archaeal lineage also thought to utilize O₂ based on analysis of respiratory pathways (Kozubal *et al.*, 2012a). The growth and development of Fe(III)-oxide microbial mats is likely influenced by the availability of electron donors and acceptors (and/or other key nutrients) present within the mat microenvironments. The mass transfer of dissolved O₂ to and within the Fe(III)-oxide microbial mat is of specific interest due to the O₂ requirement of microbially mediated Fe(II)-oxidation as well as aerobic oxidation of organic carbon (organotrophy) and arsenite (Hamamura *et al.*, 2009).

Iron-oxidizing microorganisms are also important in deep sea hydrothermal vents, acid mine drainages, seeps, and other geothermal springs (Emerson and Revsbech, 1994a; Emerson and Moyer, 2002; Baker and Banfield 2003; Inskeep *et al.*, 2004). Several neutrophilic Fe(II)-oxidizing microbial mat communities and structures have been characterized *in situ* using microsensors to evaluate gradients in oxygen (Emerson and Revsbech, 1994a,b; Sobolev and Roden, 2001; Druschel *et al.*, 2008). Typically, these studies have reported O₂ gradients extending from the oxic bulk-fluid interface towards anoxic regions. Microsensors (or microelectrodes) represent a reasonably non-invasive approach for measuring concentration profile of dissolved O₂ and other chemical gradients *in situ* (Revsbech and Jorgensen, 1986).

Amorphous Fe(III)-As(V) oxyhydroxide microbial mats from NGB have been shown to form as a result of microbial activity (Inskeep *et al.*, 2004). The rate of abiotic Fe(II)-oxidation is very slow at low pH (< 4.5) (Singer and Stumm, 1970; Konhauser, 1998). Moreover, *M. yellowstonensis* strain MK1 has been shown to be highly relevant in these habitats and oxidizes Fe(II) in pure culture (Kozubal *et al.*, 2008). Rapid microbial oxidation of As(III) to As(V) has also been observed in the outflow channels of these acidic hot springs, and which has been linked with the expression of arsenite oxidase genes (Langner *et al.*, 2001; Macur *et al.*, 2004; Hamamura *et al.*, 2009). These microbial mats are comprised of fairly porous Fe(III)-oxides that form on rhyolitic parent material and are essentially equivalent to biofilm structures (Hall-Stoodley *et al.*, 2004). In other aerobic biofilms it has been established that the transport of dissolved O₂ from the bulk fluid is influenced by the physical and structural properties of the biofilm matrix (de Beer *et al.*, 1994). Similarly, the consumption of dissolved O₂ and subsequent Fe(III)-oxide biomineralization rates in acidic geothermal systems are controlled by a combination of reaction kinetics (principally associated with microbial metabolism) and mass transport processes originating from the overlying gaseous oxygen ($p_{O_2} \approx 0.16$ atm at this elevation). Few studies have coupled *in situ* O₂ microprofile with the depth-wise distribution and transcriptional activity of a microbial community, especially in geothermal microbial mats.

Metallosphaera yellowstonensis is an Fe(II)-oxidizing *Sulfolobales* abundant in acidic, high-temperature Fe(III)-

oxide microbial mats in YNP, however, little is known regarding the distribution of these organisms with depth into the mat. It was previously shown that *M. yellowstonensis* is an obligate aerobe and actively transcribes its *foxA* gene within NGB geothermal springs (Kozubal *et al.*, 2008; 2011). The *foxA* gene encodes for subunit I (haem Cu oxidase, HCO) of a terminal oxidase complex that is specific for the oxidation of ferrous Fe (Bathe and Norris, 2007). Thus, this gene is essential as the final step of oxygen reduction in the electron transport chain of Fe(II) oxidation in *M. yellowstonensis* (Kozubal *et al.*, 2011). The *foxA* gene has also been identified in other iron-oxidizing *Sulfolobales* (Bathe and Norris, 2007; Auernik and Kelly, 2008).

The current study focuses on *in situ* O₂ analysis as a function of depth in high-temperature Fe(III)-oxide mats to evaluate the extent and possible mechanisms of microbial O₂ consumption in archaeal-dominated communities. The specific aims of this study were to (i) measure *in situ* dissolved oxygen profile within high-temperature acidic Fe(III)-oxide microbial mats, (ii) quantitatively compare O₂ mass transfer and consumption rates with rates of Fe(III)-oxide biomineralization, and (iii) link population abundance and mRNA gene expression levels of *Metallosphaera yellowstonensis* to Fe(II)-oxidizing habitats shown to consume O₂.

Results

Oxygen microprofile

The concentration of dissolved O₂ declined significantly as a function of depth in high-temperature Fe(III)-oxide mats (Fig. 1) from approximately 40–70 µM in the stream water to below detection (< 0.3 µM) at depths ranging from 400 to 750 µm (Fig. 2, Table 1). Oxygen was detected to depths of 250–1300 µm in *Beowulf* (BE_D) iron mats. An average depth of 730 ± 500 µm was obtained from three oxygen profiles located within a 2 cm radius. Depths of O₂ penetration were more similar among replicates measured at *One Hundred Springs Plain* (OSP_B), and averaged 800 ± 88 µm. Based on a measured total mat thickness (L_t) of ~ 7 mm, these results show that detectable levels of oxygen were observed in the top ~ 10% of the Fe(III)-oxide microbial mat.

Table 1. Select aqueous geochemistry at sampling positions (BE_D and OSP_B) within *Beowulf* and *OSP Springs*, Norris Geyser Basin (YNP).

Site	pH	pH (Mat)	Temperature (°C)	DS (µM)	O ₂ (aq), µM	CO ₂ (aq), µM	Fe (TS), µM
BE_D	3.0 (0.1)	2.7	68 (2)	6 (4)	60 (20)	800 (322)	39 (5)
OSP_B	3.4 (0.1)	3.75	75 (2)	4 (2)	51 (12)	340 (221)	32 (8)

Values represent multiple sampling times corresponding to the study period over two summer seasons (2010–2011).

Standard deviations are in parentheses ($n = 4$).

DS, dissolved sulfide; Fe(TS), total soluble Fe (essentially 98% ferrous-Fe).

Fig. 1. Photographs of sampling sites at (A) *Beowulf Spring* (BE) and (B) *One-Hundred Springs Plain* (OSP). Arrows represent approximate positions where aqueous geochemical and solid phases were sampled for molecular analysis and where oxygen microsensor measurements were obtained (scale bar = 20 cm).

Dissolved O_2 profile from both sites (*Beowulf* and *One-Hundred Springs Plain*) show the zones of most rapid concentration change near the surface of the mat (Fig. 2). The predominant concave curvature of all O_2 profile implies that there was no and/or negligible amounts of O_2 being produced and evolved from the mat. Although some O_2 profile exhibited minor inflection in curvature, these are likely due to flow-oriented laminations (i.e. thin layers exhibiting variable O_2 consumption rates) and structural heterogeneity (i.e. channels and voids) observed as a function of mat depth (Fig. 3). Dissolved O_2 measurements made at different water column depths above the Fe-oxide mat surface were fairly constant ($60 \pm 15 \mu M$) indicating that the stream water above the mat is well-mixed and below the measured O_2 saturation ($100 \mu M$; see *Experimental procedures*). Moreover, these measurements did not have the resolution to identify a diffusive boundary layer (DBL). The DBL thickness is primarily a function of velocity. Measured surface velocities over the

Fe mats were high ($5\text{--}20 \text{ cm s}^{-1}$), and mass transfer limitations external to the Fe mat were assumed negligible. The observed transition from a well-mixed bulk liquid to a depth-dependent O_2 concentration profile suggest that diffusion is the primary mechanism of mass transport within the Fe(III)-oxide mat.

Fick's law was used to calculate the net flux of dissolved O_2 into the near surface of the Fe(III)-oxide mat using measured oxygen concentration profile and the linear portions of the profile that span the aqueous-mat interface. Diffusion coefficients for dissolved O_2 in water were estimated to be 6.1×10^{-5} and $6.2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 68°C and 75°C (BE_D and OSP_B, respectively) using the Wilke-Chang correlation (Wilke and Chang, 1955). The calculated O_2 flux values were 1.64×10^{-4} and $1.41 \times 10^{-4} \mu\text{mol } O_2 \text{ cm}^{-2} \text{ s}^{-1}$ for BE_D and OSP_B, respectively. Assuming steady-state, these values represent a net areal rate of O_2 consumption by the Fe-oxide microbial mats.

Fig. 2. Dissolved O_2 microprofile measured at sampling locations (BE_D and OSP_B) within *Beowulf* and *OSP* geothermal springs. Each plot contains 3 individual profile taken at different positions within a $\sim 2 \text{ cm}$ radius (individual data points were measured in $50 \mu\text{m}$ step sizes; $x(\text{depth}) = 0$ corresponds with the mat surface).

Fig. 3. Stereo-micrographs of epoxy embedded thin sections of BE_D iron-oxide microbial mat cut perpendicular to the bulk stream flow. Orientated visual structural variations (e.g. laminations and voids) represent heterogeneity in the Fe(III)-oxide mat structure. A. Full-scaled image of an excised bulk-mat sample. B. Fine-scaled image of laminated portion of a second excised bulk-mat sample.

Reaction-diffusion analysis

Oxygen profiles were used to fit steady-state reaction-diffusion models assuming either first- or zero-order kinetics (Eqs 1 and 2) using profile data scaled to the unitless parameters u (dimensionless concentration) and ζ (dimensionless depth-position) (see *Experimental procedures*). A concentration-dependent (first-order) model fit the data from both sites (R^2 values ≥ 0.96) compared with poor fit using a zero-order model ($R^2 < 0.5$, data not shown). Consequently, the O_2 consumption rates are best described with concentration-dependent kinetics (Fig. 4). The first-order Thiele modulus ϕ_1 (see *Experimental procedures* and Appendix S1) is defined as a ratio of the reaction to diffusion time scales and was used as the single fitting parameter for modelling experimental dissolved O_2 profiles. Values of the fitted first-order Thiele moduli ranged from ~ 30 to 60 (OSP_B and BE_D profiles respectively), suggesting that under these conditions, the reaction time-scale is an order of magnitude

greater than the diffusion time-scale. Consequently, the rate of microbial oxygen consumption is likely controlled by the diffusion rate of dissolved oxygen in the mat.

Fe(III)-oxide accretion rates

The rate of Fe(III)-oxide deposition was determined *in situ* using glass slides ($75 \times 25 \times 1$ mm) (Fig. S1) introduced and analysed during the study period corresponding to O_2 profile measurements. The amount of Fe(III)-oxide accumulated on the slide surface increased linearly over 133 days (Fig. 5), resulting in measured Fe(III)-oxide accumulation rates of 0.4×10^{-5} and $3.0 \times 10^{-5} \mu\text{mol Fe cm}^{-2} \text{s}^{-1}$ for BE_D and OSP_B, respectively. Aqueous Fe(III) was also produced as a direct result of Fe(II) oxidation at these spring positions and was as much as 5–10% of the total aqueous Fe (Table 1) (Inskeep *et al.*, 2004; Macur *et al.*, 2004; Kozubal *et al.*, 2012b). Electron micrographs of material accumulated in OSP_B show cell attachment within 7 days of incubation, formation of amorphous

Fig. 4. Representative dissolved O_2 microprofile expressed in terms of dimensionless parameters u and ζ (also see *Experimental procedures*). Solid lines represent the fitted reaction diffusion models assuming a first-order rate equation (Eq. 1) and optimized values of the Thiele modulus (ϕ_1) to maximize R^2 values. The models are limited to spatial regions defined by $\zeta \geq 0$, corresponding to positions inside the Fe(III)-oxide mat matrix.

Fig. 5. Total iron accumulation on glass microscope slides incubated *in situ* for 133 days. Slopes of the linear accretion curves represent the net iron accumulation rates ($Rate_{BE_D}$ and $Rate_{OSP_B}$).

Fe(III)-oxide within 14 days and complete slide coverage within 30–45 days depending on site (Macur *et al.*, 2004; Beam *et al.*, 2011). The heterogeneous Fe(III)-oxide surfaces forming at the aqueous/mat interface are replete with Fe-encrusted cells resulting from the oxidation of Fe(II) (Fig. 6), and reveal the type of surface in which oxygen microsensors were introduced. Previous analysis of the Fe(III)-oxide mats in NGB have shown that the predominant Fe phases formed in these systems are amorphous Fe(III)-oxyhydroxides containing significant levels of sorbed arsenate ($As^V : Fe^{III}$ mole ratios ~ 0.65) (Inskeep *et al.*, 2004). Measured rates of Fe(III)-oxide accumulation represent the total rate of Fe(III) produced minus any aqueous Fe(III) and Fe(III)-oxides that are reduced or transported away in the stream channel (discussed further below). Accumulation of Fe(III)-oxide on the slides due to erosion from upstream sources is neg-

ligible, in part because there is a direct linkage between cell surfaces and Fe(III)-oxide mineralization. The measured net vertical growth (L_t = mat depth) of the Fe(III)-oxide microbial mat was ~ 7 mm (after 133 days of *in situ* incubation at the OSP_B site). Similarly, the measured L_t at BE_D ranges from ~ 5 –10 mm determined by direct measurement of the native mat and epoxy embedded thin sections (Fig. 3). Although the absolute mat depth varies across cm scales, localized microsensor (50 μ m diameter) measurements were made in mats with average thicknesses of ~ 7 mm at both sites.

Vertical distribution and activity of *M. yellowstonensis*

Metallosphaera yellowstonensis has been well established as an active and abundant Fe(II)-oxidizing *Sulfobacillus* in acidic, geothermal Fe(III)-oxide microbial mats in NGB (Kozubal *et al.*, 2008; 2011). Microbial mats subjected to O_2 profil measurements were sampled and dissected to remove top and bottom samples (see *Experimental procedures*), and to establish a direct linkage between the presence of this population and measured Fe(III)-oxide deposition. Significant numbers of *M. yellowstonensis* were observed in all Fe-mat samples, and quantitative estimates of 16S rRNA gene copy number were higher in the top 1 mm of mat (bulk-liquid/mat interface) at both sampling sites. This result was more evident at OSP_B where 16S rRNA gene copy numbers ranged from 2.3 ± 0.1 to $0.7 \pm 0.1 \times 10^5$ per gram in top and bottom mat samples respectively. At BE_D, gene copy numbers for *M. yellowstonensis* ranged from $5.5 \pm 1.2 \times 10^5$ to $4.9 \pm 0.6 \times 10^5$ per gram in top and bottom mat samples respectively (Fig. 7A).

Corresponding Fe(III)-oxide mat samples were also used to obtain semi-quantitative estimates of *M. yellowstonensis*-like *foxA* mRNA transcripts using

Fig. 6. SEM micrographs of glass slides incubated in the OSP_B site. A. Attached cells observed after 1 week. B. Mature amorphous Fe(III)-oxyhydroxide mat showing encrusted cells and ubiquity of biomineralized Fe(III)-oxides after 2 weeks.

Fig. 7. (A) Spatial distribution of *M. yellowstonensis* 16S rRNA gene copies and (B) relative expression of *foxA* mRNA to 16S rRNA gene copies in BE_D and OSP_B. The designations 'Top' refers to the first 1 mm of mat material at the mat/bulk-liquid interface; 'bottom mat' refers to the remainder of the mat. *P*-values: (A) = 0.46 and 9.6×10^{-5} for BE_D and OSP_B respectively, and (B) = 0.13 and 0.005 for BE_D and OSP_B respectively. Error bars represent ± 1 standard deviation from three technical replicates.

reverse transcriptase (RT)-PCR. *foxA* gene transcripts (mRNA) were observed in Fe(III)-oxide mats from both sites (semi-quantitative estimates are normalized to 16S rRNA gene copy number, Fig. 7B), and trends among the two sites and mat depths were similar to those observed using estimates of *M. yellowstonensis* 16S gene copy number. Specifically, *foxA* gene transcripts (mRNA) were also higher in top versus bottom mat samples from OSP_B (Fig. 7B). Normalized *foxA* transcript levels in BE_D were similar between top ($1.00 \pm 0 \times 10^{-2}$) and bottom ($0.94 \pm 0.05 \times 10^{-2}$) mat samples, suggesting that *M. yellowstonensis*-like organisms were still active in lower mat regions. These expression values agree with those found for *M. sedula* and *M. yellowstonensis* in pure culture grown on Fe(II) as the sole electron donor (Kappler *et al.*, 2005; Kozubal *et al.*, 2011). Among all Fe(III)-oxide samples evaluated, the top 1 mm of the OSP_B mat showed the highest localized O₂ concentrations (~50–70 μM), greater *M. yellowstonensis* 16S rRNA gene copy numbers, and higher amounts of *M. yellowstonensis* *foxA* mRNA.

Discussion

Contributions to biological oxygen consumption

High-temperature acidic Fe(III)-oxide microbial mats are reactive O₂ sinks. The net areal oxygen consumption rates estimated from the measured microprofile encompass the sum of all oxygen-consuming reactions occurring *in situ*. The microbial populations present in high-temperature acidic Fe(III)-oxide mats from Norris Geyser Basin have been studied in considerable detail (e.g. Inskeep *et al.*, 2010; Kozubal *et al.*, 2012a,b) and results from these efforts form a basis for interpreting O₂ consumption observed in the current study. The microbial processes that contribute to O₂ consumption in these systems include the aerobic oxidation of electron donors such as reduced-C, Fe(II), As(III), hydrogen and reduced sulfur (e.g. sulfid and/or elemental S). Of these possible electron donors, those likely to be responsible for the

majority of O₂ consumption include reduced-C, Fe(II), and As(III), based on the concentrations measured *in situ*. Organic carbon utilized by aerobic chemoorganotrophs (Table 2) likely originates from three sources: decaying biomass of autotrophic microorganisms (e.g. *M. yellowstonensis*), dissolved organic carbon from source waters (~50 μM C), and exogenous inputs from the local landscape (e.g. atmosphere, plant, animal, soil). Although highly exergonic, the concentrations of other electron donors (hydrogen, reduced S, methane) are too low to support significant O₂ consumption (Inskeep *et al.*, 2005).

The rates of Fe(III)-oxide deposition (e.g. 3×10^{-5} μmol Fe cm⁻² s⁻¹ at OSP_B) were used to estimate the O₂ flu required to account for this fraction of total Fe(II) oxidation. It is understood that the actual oxygen flu attributable to the total Fe(II) oxidation rate in these systems will be higher, because some Fe(II) oxidized is lost as aqueous Fe(III), eroded as Fe(III)-oxides in the stream channel, and/or lost due to reductive-dissolution. For OSP_B, the amount of oxygen consumed by Fe(II) oxidation, specifically resulting in Fe(III)-oxide solids accumulated, was estimated at 5.3% of the total measured O₂ flu [1 mol Fe(II) requires 0.25 mol O₂]. The estimates are an order of magnitude lower at BE_D (only 0.61% of the measured O₂ flu can be attributed to Fe(III)-oxide deposition). The lower values at BE_D are due in part to higher stream channel velocities in *Beowulf Spring* (~14–20 cm s⁻¹), which results in greater erosion of Fe(III)-oxide particles. The OSP site discharges on a lower slope than *Beowulf Spring* resulting in a shallower water column with lower channel velocities (~6–10 cm s⁻¹). Shear forces from bulk liquid flow coupled with photo-reduction and dissolution are both potential mechanisms for Fe(III)-oxide material loss, but the lower velocities in *OSP Spring* suggest that Fe(III)-oxide deposition rates measured at OSP_B are closer to an upper value of the contribution of Fe(II)-oxidation to O₂ flu (~5%). These estimates are reasonably consistent with the abundance of *M. yellowstonensis* like organisms in BE and OSP determined from prior studies (Kozubal *et al.*, 2008;

Table 2. Estimates of abundance of predominant taxa determined in iron oxide mat samples (BE_D and OSP_B) taken at similar locations to those used for oxygen microelectrode profiles

Site/population	Estimated abundance ^a	HCO ^b	Reference
OSP_B (T = 75 C, pH = 3.5)			
Novel Archaeal Group 1	50–60	Y	Kozubal <i>et al.</i> (2012a,b)
<i>M. yellowstonensis</i>	15–22	Y ^c	Kozubal <i>et al.</i> (2008)
<i>Vulcanisaeta</i> spp.	13–15	N	Jay <i>et al.</i> (2011)
<i>Acidilobus</i> spp.	5–10	N	Jay <i>et al.</i> (2011)
<i>Hydrogenobaculum</i> spp.	~ 2–3	Y	Kozubal <i>et al.</i> (2012b)
Other	< 2		
BE_D (T = 68 C, pH = 3.0)			
Novel Archaeal Group 2	35–40	Y	Kozubal <i>et al.</i> (2012b)
<i>M. yellowstonensis</i>	20–25	Y ^c	Kozubal <i>et al.</i> (2008)
<i>Sulfolobales</i> _MK5	5–10	Y ^c	Kozubal <i>et al.</i> (2012b)
<i>Thermoplasma</i> -like	5–10	Y	Inskeep <i>et al.</i> (2010)
Thaumarchaeota	5–10	Y	Beam <i>et al.</i> (2013)
Novel Archaea group 1	3–5	Y	Kozubal <i>et al.</i> , (2012a)
<i>Acidilobus</i> spp.	3–5	N	Inskeep <i>et al.</i> (2010)
<i>Hydrogenobaculum</i> spp.	3–5	Y	Inskeep <i>et al.</i> (2010)
Other	< 2		

The presence or absence of haem copper oxidases (HCO) used for the reduction of oxygen in aerobic respiration is summarized.

a. Estimated abundance based on phylogenetic classification of contigs (> 4000 bp) from assembled metagenome sequence (see references cited).

b. Yes/No is based on the presence or absence of a terminal oxidase complex containing a subunit I haem Cu oxidase (HCO).

c. Contains a Fox terminal oxidase complex specific for Fe(II)-oxidation in the *Sulfolobales* (Kozubal *et al.*, 2011; 2012a).

2012b; Inskeep *et al.*, 2010) to range from ~ 12–23% of the community using different molecular approaches (summarized in Table 2). Another aerobic Fe(II)-oxidizing organism from the BE site Fe(III)-oxide mat is the *Sulfolobales* strain MK5 confirmed to be an Fe(II)-oxidizing organism in culture and by the presence of a Fox terminal oxidase complex (Kozubal *et al.*, 2012b).

Correlation of oxygen consumption and Fe(III)-oxide deposition rates coupled with expression of Fe(II)-oxidizing haem Cu oxidases (*foxA*) from *M. yellowstonensis*-like organisms provides direct evidence for the linkage between the activity of this population and measured O₂ profile across a mat depth of approximately 700 µm. However, a significant percentage of the total O₂ consumption is due to other aerobic members present in these communities. For example, microbially mediated As(III) oxidation by *Hydrogenobaculum* sp. (order *Aquificales*) represents another O₂ consuming process (Table 2) that has been reported in other studies (Langner *et al.*, 2001; Macur *et al.*, 2004; Hamamura *et al.*, 2009). Estimated arsenite oxidation rates in these same Fe(III)-oxide mat systems are 0.26×10^{-5} and 1.95×10^{-5} µmol As(III) cm⁻² s⁻¹ for BE_D and OSP_B respectively [Fe(III) accretion rates scaled by As^V : Fe^{III} mole ratios ~ 0.65] (Inskeep *et al.*, 2004). Stoichiometrically, this corresponds to oxygen consumption rates of 0.13×10^{-5} and 0.98×10^{-5} µmol O₂ cm⁻² s⁻¹ for BE_D and OSP_B, which represents approximately 0.8 and 6.9% of the net areal O₂ consumption directly measured in BE_D and OSP_B respectively.

The dominant members of the OSP_B and BE_D Fe(III)-oxide microbial mats also include representatives from two novel lineages in the *Archaea* (Kozubal *et al.*, 2012a; 2012b) (Table 2). The 'novel archaeal group 1' (NAG1) population is in higher abundance at OSP_B, while the 'novel archaeal group 2' lineages are more abundant in BE_D. However, all of these community members appear to be aerobic chemoorganotrophs based on the presence of terminal oxidase complexes including the subunit I HCO (Inskeep *et al.*, 2010; Kozubal *et al.*, 2012a,b). Although *de novo* sequence assemblies of NAG1 show no evidence of Fe(II)-oxidation genes, the population appears capable of aerobic oxidation of complex carbon and CO as potential energy sources (Kozubal *et al.*, 2012a). The *Desulfurococcales* and *Thermoproteales*-like populations present in OSP_B and BE_D do not likely respire on O₂, based on the absence of haem Cu oxidases in *de novo* assemblies from these sites (Inskeep *et al.*, 2010; Jay *et al.*, 2011) as well as the fact that most cultured organisms from these orders are strict anaerobes that often require reduced forms of sulfur (Jay *et al.*, 2011).

Oxygen mass-transfer limitations

The rapid biotic consumption of O₂ in these Fe(II)-oxidizing biofilm is ultimately limited by the diffusive mass transfer rates through the Fe(III)-oxide matrix. Estimates of the first-order Thiele modulus (used as a single data fitting parameter) allowed quantification of time

scales for oxygen consumption rates relative to mass transfer (diffusion) rates, and suggested that the system is *mass transfer limited* with regards to dissolved O₂. The rapid rates of O₂ consumption are consistent with the importance of terminal oxidase complexes found in the organisms inhabiting these Fe mats (Inskeep *et al.*, 2010; Kozubal *et al.*, 2012a,b). However, the biomineralization and evolution of an Fe(III)-oxide biofilm (matrix) results in a *diffusion-limited* system that exhibits a pronounced gradient in dissolved O₂ from bulk-aqueous levels of ~50–60 μM to below detection (< 0.3 μM), all within approximately 700 μm. Given that the top of these mineralized structures contain the most oxygen, it is expected that Fe(II)-oxidation rates are greater near the aqueous-mat interface. Evidence for this hypothesis was obtained for OSP_B; however, *foxA* transcripts were nearly equally abundant throughout the entire depth of BE_D Fe(III)-oxide mats. Certainly, the Fe-oxide mats studied here also exhibit heterogeneity that could contribute to differential O₂ diffusion rates to lower mat depths (e.g. preferential versus restricted convective or diffusive transport, Fig. 3).

Biological iron-cycling

Acidic geothermal springs in NGB are anoxic at the source and Fe(II)-oxidizing microbial communities do not occur until the springs have sufficient dissolved O₂ from atmospheric mixing, after dissolved sulfide has degassed. Abiotic Fe(II)-oxidation in the springs is negligible in contrast to the microbially mediated process at these pH values (Singer and Stumm, 1970). Anaerobic Fe(II)-oxidation has not been observed *in situ*. In fact, the ΔG_{rxn} for the reduction of nitrate [$8\text{Fe(II)} + \text{NO}_3^- + 21\text{H}_2\text{O} = 8\text{Fe(OH)}_3(\text{s}) + 14\text{H}^+ + \text{NH}_4^+$] has been shown to be endergonic and would not be expected to be a dominant metabolism in these systems (Inskeep *et al.*, 2005). Therefore, Fe(II)-oxidation is a direct result of aerobic members of the orders *Sulfolobales* (e.g. *M. yellowstonensis* and *Sulfolobales* MK5-like organisms).

The reduction of Fe(III)-oxides has not been measured directly in these iron mats, however, this process cannot be ruled out. Prior work on these systems shows other community members that may contribute to the reduction of Fe(III)-oxides including *Sulfolobales* strain MK5 (Kozubal *et al.*, 2012b). The predominant geochemical process in these environments is Fe(II)-oxidation as evinced from several important observations that have preceded this work. First, depth-wise mineralogical characterization of these amorphous iron oxides revealed no transformation to other Fe oxide phases (e.g. magnetite) (Inskeep *et al.*, 2004; Kozubal *et al.*, 2012b), which is a likely indicator that Fe(III)-reduction does not contribute significantly to losses of Fe(III). Second, these mats

contain very few bacteria and prior 16S rRNA gene surveys show no evidence of known Fe(III)-reducing populations (Inskeep *et al.*, 2010; Kozubal *et al.*, 2012b). Additionally, no outer membrane cytochromes known to catalyze Fe(III)-reduction have been found in metagenomes from these sites (Inskeep *et al.*, 2010).

Conclusion

Results from this study highlight the importance of a potentially limiting substrate (dissolved oxygen) for microbial metabolism at high temperatures and the subsequent aerobic biomineralization of Fe(III)-oxide phases in acidic springs of YNP. These biotic oxygen-sinks are ultimately limited by the mass transfer of O₂ through the Fe(III)-oxide matrix. The consumption of O₂ is a multi-scale process spanning molecular, cellular and macroscopic (pore-scale) regimes. Measured O₂ depth-profile in two acidic springs of Norris Geyser Basin reveal a defined spatial zone where the majority of oxygen-consuming reactions are occurring. The spatial abundance of an established Fe(II)-oxidizing community member (*M. yellowstonensis*) was shown to correlate with the presence of dissolved oxygen and we expect the same will be true for other aerobic heterotrophs containing terminal oxidase complexes that require oxygen as a terminal electron acceptor (Table 2). The formation of microbial structures (e.g. mats, biofilms) containing Fe(III)-oxides via biomineralization is inextricably linked to the diffusive transport of oxygen, which also defines a pronounced gradient of dissolved oxygen in the top ~700 μm of Fe(III)-oxide microbial mat.

Experimental procedures

Site description and geochemistry

Two iron-oxide microbial mats from NGB were chosen for this study: *Beowulf Spring*, east discharge, position D (BE_D) and One Hundred Springs Plains, position B (OSP_B) (Fig. 1, Table 1). These sites were prime candidates for this study due to prior detailed geochemical, physical, and microbiological characterization (Inskeep *et al.*, 2004; 2005; 2010; Kozubal *et al.*, 2008; 2011). *Beowulf Spring* is a heavily terraced iron-oxide mat system flowing north on a relatively steep-grade, whereas *OSP Spring* is a slower (pulse-type) stream channel flowing southwest on a lower grade that yields a shallower, lower-velocity system. The coordinates of the study sites are: BE_D (44°43'53.224"N, 110°42'40.727"W) and OSP_B (44°43'58.8"N, 110°42'32.1"W). Temperature was measured with a digital thermometer equipped with a T-type thermocouple (Omega Engineering) with an error of ± 1°C. A portable Accumet® AP71 pH/mV/°C meter (Fisher Scientific) was used to measure pH and was calibrated in the field using pH standards of 4.01 and 7.0. Dissolved oxygen, aqueous CO₂, total sulfide and ferrous-ferric iron were measured from duplicate samples in the field as previously

described using gas chromatographic and colorimetric methods (Inskeep, *et al.*, 2005).

Oxygen profile

Dissolved O_2 profile were obtained in the direction normal to the bulk flow and surface of the mat (i.e. the aqueous-mat interface). All profile were taken with high-temperature tolerant 50 μm (tip diameter) Clark-type microsensors (custom equipment provided by Unisense A/S, Aarhus DK). The O_2 consumption rate by the microsensors was estimated at $8 \times 10^{-3} \mu mol year^{-1}$, which was considered negligible. Sensor positioning was controlled with a micromanipulator coupled to a motor controller. The micromanipulator was housed on a custom, adjustable tripod stand that was used in the field to perform profile measurements. Data collection and positioning controls were performed with Sensor Trace-Pro software (Unisense). Sensors were individually calibrated in the field using the anoxic calibration point from either 100 mM Na_2S (pH = 10) or by positioning the sensor inside the iron-oxide mat where the oxygen signal was less than 5% of the bulk liquid value (e.g. near detection). The oxic calibration point was obtained by positioning the sensor in the bulk liquid directly above the Fe-oxide mat; this value was also confirmed using independent measurements obtained by the Winkler method. The O_2 saturation values were also directly measured by the Winkler method by aerating 100 ml spring water at ~ 1 LPM inside a beaker (incubated at 75°C inside the spring) connected to an air pump and air stone. All profile were performed in triplicate (three positions within a ~ 2 cm radius of each other) for each sample site. Triplicate technical replicates were done for profile taken in the OSP_B site, however the brittle nature of the mat at BE_D only allowed for a single accurate profile per position. Locations corresponding to the surface of the mat were confirmed both visually and by initial changes in the profile slope.

Calculations and modelling

Classical reaction and diffusion theory was used to quantitatively analyse coupled reaction and mass transfer rates. Reaction and diffusion of substrates in microbial mats, biofilm and catalyst materials has been studied extensively (Thiele, 1939; Williamson and McCarty, 1976; Karel and Robertson, 1987; Stewart and Raquelas, 1995; Stewart, 2003). Briefly, by assuming a steady-state mass balance and one-dimensional slab geometry, Fick's law was used as a basis for modelling the experimental data and the empirical analysis of the bulk O_2 flux. The model was simplified by assuming constant effective diffusion constants and a singular reaction term which represents the sum of all O_2 consuming processes. The volumetric reaction rate was assumed to be bounded by either a first-order ($R = k_1 C_{O_2}$) or zero-order rate expression ($R = k_0$). Dimensionless parameters were defined as:

$$u = C_{O_2}/C_{O_2}', \quad \zeta = z/L_t, \quad \phi_0^2 = k_0 L_t / C_{O_2}' D_e, \quad \phi_1^2 = k_1 L_t^2 / D_e$$

where u is the ratio of the observed dissolved O_2 concentrations in the mat relative to the bulk liquid, ζ is the

dimensionless depth parameter, z is the depth-position within the mat, L_t is the total depth of the Fe(III)-oxide mat, ϕ is the Thiele modulus, a dimensionless rate constant defined using a ratio of the reaction rate coefficient to the effective diffusion coefficient (D_e) scaled to a characteristic area (L_t^2) (Thiele, 1939). Actual L_t values were obtained either by microscopy of excised mat sections (site BE_D) and by direct measurement of iron oxide mat accretion samples under steady-state conditions (site OSP_B) (see *Results and Discussion*). The effective diffusion coefficient of O_2 in the iron oxide microbial mat matrix is not known nor easily measured; consequently, the Thiele modulus is a useful parameter, which is inversely proportional to D_e .

Dimensionless analytical solutions (Eqs 1 and 2) to differential mass balances were obtained by imposing a zero-flux boundary condition at the bottom of the mat ($z = L_t$) and setting the observed concentration of dissolved O_2 to the constant bulk liquid concentration at the top of the mat ($z = 0$).

$$u = \cosh(\phi_1 \cdot \zeta) - \tanh(\phi_1) \sinh(\phi_1 \cdot \zeta) \quad (1)$$

$$u = (\phi_0^2 \cdot \zeta^2) / 2 - \phi_0^2 \cdot \zeta + 1 \quad (2)$$

Equations 1 and 2 are the analytical solutions for the first and zero-order reaction models, respectively. The analytical solutions were used to fit O_2 profile data (non-linear least squares regression) resulting in estimates of the Thiele moduli. Detailed and step-wise interpretations of the reaction-diffusion models and dimensionless number interpretations are contained in Appendix S1 of this manuscript.

Iron oxide accretion rates

Glass microscope slides (75 $\times$ 25 $\times$ 1 mm) were cleaned in a 2% hydrochloric acid bath for ~ 2 h to remove organic carbon, rinsed in 18 M Ω H_2O (free of organic carbon), and autoclaved for 15 min. The cleaned slides were placed in the primary outflow channel of OSP_B (73°C) and BE_D (68°C) such that the 75 $\times$ 25 mm surface was exposed to flow and visually flushed with the mat surface (see Fig. S1). Slides were carefully removed at 7, 15, 28 and 133 days, rinsed in bulk stream water to remove non-adhered material and placed in 0.175 M ammonium oxalate buffer (pH = 3) to dissolve Fe-oxides (Loeppert and Inskeep, 1996). Temperatures at these spring conditions remained relatively constant ($\pm 2^\circ C$) over the timescale of the experiment. Ferrous and total soluble iron were measured using the Ferrozine method at an absorbance of 562 nm (Stookey, 1970).

Microscopy

Images of thin sections from excised Fe(III)-oxide mat were taken with a stereo microscope (Nikon SMZ1500). Excised mat samples from BE_D were embedded with epoxy under a vacuum and thin sectioned. Stereoscope images were calibrated by direct measurement with a 1 mm stage micrometer. Scanning electron micrographs were obtained of slides sampled from field incubations with a field emission-scanning electron microscope set at low voltage (1 kV) after sputter-coating with Ir to minimize charging.

Quantitative PCR and spatial mRNA analysis

The relative spatial abundance of *Metallosphaera yellowstonensis* strain MK1 16S rRNA gene (1 copy per chromosome) was determined by slicing 1 mm from the top of an excised portion of mat. The remainder of the mat, known to contain lower levels of oxygen, was termed 'bottom-mat'. Community DNA was extracted from both top and bottom samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, OH). Quantitative PCR (qPCR) was performed using *M. yellowstonensis* specific 16S rRNA gene primer and the Sso Fast EvaGreen qPCR Kit and protocol in 50 µl reactions (Bio-Rad) on a RotorGene 3000 qPCR machine (Corbett Research). The specific primer and reaction conditions were as previously described (Kozubal *et al.*, 2008). Statistical analysis was performed using an independent, two-tailed Student's *t*-test.

Iron-oxide microbial mat squares (approximately 2 × 2 cm) were excised at the position where microsensor measurements were made for each sampling position. The mat sections were placed in sterile Petri dishes and sliced into 'tops' and 'bottoms' in same fashion as described in the qPCR section. All mat samples were stored in RNA Later in RNase free tubes. Total RNA was extracted (in triplicate) from approximately 500 mg of cut microbial mat using the FastRNA[®] Pro Soil Direct Kit (Qbiogene, CA) and FastPrep[®] Instrument (Qbiogene), for 40 s at a speed setting of 6.0. Total RNA was then treated with RNase free DNase I (New England Biolabs, MA) for 15 min and 37°C. The DNase I reaction was terminated by precipitation of total RNA with nuclease free lithium chloride (final concentration 1.6 M) and absolute ethanol overnight (Sambrook and Russell, 2001). RNA was resuspended in nuclease free water after centrifugation at 4°C for 15 min and washing once in ice cold 70% ethanol. The terminal haem copper oxidase subunit I (*foxA*) specific to the iron oxidation pathway of *M. yellowstonensis* was reverse transcribed using the AccessQuick RT-PCR System and protocol (Promega) with *M. yellowstonensis* specific *foxA* primers (Kozubal *et al.*, 2011). The cDNA *foxA* amplicons were purified with the GenElute[™] PCR CleanUp Kit (Sigma-Aldrich). The qPCR reaction was the same as for the 16S rRNA gene from *M. yellowstonensis* as described in the above section. Relative expression was determined by comparing the observed *C_T* values of *foxA* and *M. yellowstonensis* 16S rRNA gene (Kozubal *et al.*, 2011). Statistical analysis was performed as described in the above section.

Acknowledgements

The authors acknowledge funding from the National Science Foundation-Integrative Graduate and Education Training (IGERT) Program (DGE 0654336) for support to H.C.B. and J.P.B. and the Department of Energy-Genome Science Program, Microbial Interactions Foundational Science Focus Area (Pacific Northwest National Laboratory subcontract 112443 to M.S.U.). Additional support was provided by Unisense A/S (Aarhus, DK), the DOE-Joint Genome Institute Community Sequencing Program (CSP 787081), E. Booth for building customized field equipment, C. Hendrix and S. Gunther (YNP Center for Resources) for research permit

administration, and Drs. P. Stewart, M. Kühl and Mr. S. Davis for helpful discussions and field assistance.

References

- Auernik, K.S., and Kelly, R.M. (2008) Identification of components of electron transport chains in the extremely thermoacidophilic crenarchaeon *Metallosphaera sedula* through iron and sulfur compound oxidation transcripts. *Appl Environ Microbiol* **74**: 7723–7732.
- Baker, B.J., and Banfield J.F. (2003) Microbial communities in acid mine drainage. *FEMS Microbiol Ecol* **44**: 139–152.
- Bathe, S., and Norris, P.R. (2007) Ferrous iron- and sulfur-induced genes in *Sulfolobus metallicus*. *Appl Environ Microbiol* **73**: 2491–2497.
- Beam, J.P., Bernstein, H.C., Kozubal, M., Carlson, R.P., and Inskeep, W.P. (2011) Distribution and activity of iron-oxidizing microorganisms in acidic geothermal environments. Goldschmidt Conference, August 15–19, 2011, Prague, CR. *Mineral Mag* **75**: 503.
- Beam, J.P., Jay, Z., Kozubal, M., and Inskeep, W.P. (2013) Novel thermoacidophilic Thaumarchaeota inhabit iron oxide and elemental sulfur sediment habitats of Yellowstone National Park. *ISME J* (submitted).
- de Beer, D., Stoodley, P., Roe, F., and Lewandowski, Z. (1994) Effects of biofilm structures on oxygen distribution and mass-transport. *Biotechnol Bioeng* **43**: 1131–1138.
- Druschel, G.K., Emerson, D., Sutka, R., Suchecki, P., and Luther, G.W. (2008) Low-oxygen and chemical kinetic constraints on the geochemical niche of neutrophilic iron(II) oxidizing microorganisms. *Geochim Cosmochim Acta* **72**: 3358–3370.
- Emerson, D., and Moyer, C.L. (2002) Neutrophilic Fe-oxidizing bacteria are abundant at the Loihi Seamount hydrothermal vents and play a major role in Fe oxide deposition. *Appl Environ Microbiol* **68**: 3085–3093.
- Emerson, D., and Revsbech, N.P. (1994a) Investigation of an Iron-oxidizing microbial mat community located near Aarhus, Denmark: field studies. *Appl Environ Microbiol* **60**: 4022–4031.
- Emerson, D., and Revsbech, N.P. (1994b) Investigation of an iron-oxidizing microbial mat community located near Aarhus, Denmark: laboratory studies. *Appl Environ Microbiol* **60**: 4032–4038.
- Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004) Bacterial biofilms from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**: 95–108.
- Hamamura, N., Macur, R.E., Korf, S., Ackerman, G., Taylor, W.P., Kozubal, M., *et al.* (2009) Linking microbial oxidation of arsenic with detection and phylogenetic analysis of arsenite oxidase genes in diverse geothermal environments. *Environ Microbiol* **11**: 421–431.
- Inskeep, W.P., and McDermott, T.R. (2005) *Geomicrobiology of Acidsulfate-Chloride Springs in Yellowstone National Park*. Bozeman, MT, USA: Montana State University.
- Inskeep, W.P., Macur, R.E., Harrison, G., Bostick, B.C., and Fendorf, S. (2004) Biomineralization of As(V)-hydrous ferric oxyhydroxide in microbial mats of an acid-sulfate-chloride geothermal spring, Yellowstone National Park. *Geochim Cosmochim Acta* **68**: 3141–3155.
- Inskeep, W.P., Ackerman, G.G., Taylor, W.P., Kozubal, M., Korf, S., and Macur, R.E. (2005) On the energetics of

- chemolithotrophy in nonequilibrium systems: case studies of geothermal springs in Yellowstone National Park. *Geobiology* **3**: 297–317.
- Inskeep, W.P., Rusch, D.B., Jay, Z.J., Herrgard, M.J., Kozubal, M.A., Richardson, T.H., *et al.* (2010) Metagenomes from high-temperature chemotrophic systems reveal geochemical controls on microbial community structure and function. *PLoS ONE* **5**: e9773. doi:10.1371/journal.pone.0009773.
- Jackson, C.R., Langner, H.W., Donahoe-Christiansen, J., Inskeep, W.P., and McDermott, T.R. (2001) Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. *Environ Microbiol* **3**: 532–542.
- Jay, Z.J., Planer-Friedrich, B., Rusch, D.B., and Inskeep, W.P. (2011) Linking geochemistry to microbial community structure and function in sulfidic geothermal systems of Yellowstone National Park. *Goldschmidt Conference*, August 15–19, 2011, Prague, CR. *Mineral Mag* **75**: 1104.
- Kappler, U., Sly, L.I., and McEwan, A.G. (2005) Respiratory gene clusters of *Metallosphaera sedula* – differential expression and transcriptional organization. *Microbiology* **151**: 35–43.
- Karel, S.F., and Robertson, C.R. (1987) Reaction-rate calculations for cosubstrates diffusing into catalyst layer from opposite sides. *Biotechnol Bioeng* **30**: 427–438.
- Konhauser, K.O. (1998) Diversity of bacterial iron mineralization. *Earth Sci Rev* **43**: 91–121.
- Kozubal, M., Macur, R.E., Korf, S., Taylor, W.P., Ackerman, G.G., Nagy, A., and Inskeep, W.P. (2008) Isolation and distribution of a novel iron-oxidizing crenarchaeon from acidic geothermal springs in Yellowstone National Park. *Appl Environ Microbiol* **74**: 942–949.
- Kozubal, M.A., Romine, M., deM Jennings, R., Jay, Z.J., Tringe, S.G., Rusch, D.B., *et al.* (2012a) Geoarchaeota: a new candidate phylum in the Archaea from high-temperature acidic iron mats in Yellowstone National Park. *ISME J*. doi:10.1038/ismej.2012.132.
- Kozubal, M.A., Macur, R.E., Jay, Z.J., Beam, J.P., Malfatti, S.A., Tringe, S.G., *et al.* (2012b) Microbial iron cycling in acidic geothermal springs of Yellowstone National Park: integrating molecular surveys, geochemical processes, and isolation of novel Fe-active microorganisms. *Front Microbiol* **3**: 109.
- Kozubal, M.A., Dlakic, M., Macur, R.E., and Inskeep, W.P. (2011) Terminal oxidase diversity and function in ‘*Metallosphaera yellowstonensis*’: gene expression and protein modeling suggest mechanisms of Fe(II) oxidation in the *Sulfobacterales*. *Appl Environ Microbiol* **77**: 1844–1853.
- Langner, H.W., Jackson, C.R., McDermott, T.R., and Inskeep, W.P. (2001) Rapid oxidation of arsenite in a hot spring ecosystem, Yellowstone National Park. *Environ Sci Technol* **35**: 3302–3309.
- Loeppert, R.H., and Inskeep, W.P. (1996) *Methods of Soil Analysis: Part 3 – Chemical Methods*. Madison, WI, USA: Soil Science Society of America and American Society of Agronomy.
- Macur, R.E., Langner, H.W., Kocar, B.D., and Inskeep, W.P. (2004) Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulphate-chloride geothermal spring. *Geobiology* **2**: 163–177.
- Revsbech, N.P., and Jorgensen, B.B. (1986) Microelectrodes – their use in microbial ecology. *Adv Microbiol Ecol* **9**: 293–352.
- Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
- Singer, P.C., and Stumm, W. (1970) Acidic mine drainage: the rate-determining step. *Science* **167**: 1121–1123.
- Sobolev, D., and Roden, E.E. (2001) Suboxic deposition of ferric iron by bacteria in opposing gradients of Fe(II) and oxygen at circumneutral pH. *Appl Environ Microbiol* **67**: 1328–1334.
- Stewart, P.S. (2003) Diffusion in biofilms. *J Bacteriol* **185**: 1485–1491.
- Stewart, P.S., and Raquepas, J.B. (1995) Implications of reaction–diffusion theory for the disinfection of microbial biofilm by reactive antimicrobial agents. *Chem Eng Sci* **50**: 3099–3104.
- Stookey, L.L. (1970) Ferrozine – a new spectrophotometric reagent for iron. *Anal Chem* **42**: 779–781.
- Thiele, E.W. (1939) Relation between catalytic activity and size of particle. *Ind Eng Chem* **31**: 916–920.
- Wilke, C.R., and Chang, P. (1955) Correlation of diffusion coefficients in dilute solutions. *AIChE J* **1**: 264–270.
- Williamson, K., and McCarty, P.L. (1976) Model of substrate utilization by bacterial films. *J Water Pollut Control Fed* **48**: 9–24.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary modelling methodology.

Fig. S1. Photograph of *in situ* incubated glass microscope slides over 28 total days of biomineralization from OSP_B and BE_D. Scale bar = 2.5 cm.