Surface chemical reactivity and metal adsorptive properties of natural cyanobacterial mats from an alkaline hydrothermal spring, Yellowstone National Park

S.V. Lalonde\textsuperscript{a,*}, L.A. Amskold\textsuperscript{a}, L.A. Warren\textsuperscript{b}, K.O. Konhauser\textsuperscript{a}

\textsuperscript{a}Department of Earth and Atmospheric Sciences, 1-26 Earth Sciences Building, University of Alberta, Edmonton, AB, Canada T6G 2E3

\textsuperscript{b}School of Geography and Earth Sciences, 309 General Science Building, McMaster University, Hamilton, ON, Canada L8S 4K1

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Abstract

The propensity for microbes to adsorb dissolved metals onto their surfaces has been well documented, to the point where predictive surface complexation models can accurately account for these reactions under experimental conditions. However, critical surface chemical parameters, such as surface functional group concentrations and proton stability constants, have only been evaluated using laboratory cultures. Whether or not natural microbes are comparable in surface chemical reactivity to laboratory cultures, and whether they display variations across diverse populations, remains untested. To resolve this, we examined natural cyanobacterial mats of various morphologies (i.e., streamers, vertical spires, horizontally laminated structures), sampled from a single hydrothermal system in Yellowstone National Park, in terms of surface chemical parameters and acid-leachable metal contents. Potentiometric titration data of samples that were acid-washed to remove sorbed metals and reveal underlying organic surfaces indicated functional group concentrations of 0.98±0.28 to 2.84±0.41 mmol g\textsuperscript{-1} (dry weight) summed over a pKa range of 4 to 10, which is comparable to previously reported experimental values. In contrast, samples that were not acid-washed, but merely rinsed in titration electrolyte adjusted to stream pH, had functional group concentrations ranging from 6.12±1.39 to 19.23±3.14 mmol g\textsuperscript{-1}. They were also largely dominated by a single functional group of pKa $\sim$7 that may be explained by the presence of aqueous or solid phase metal carbonate species that are removed from the mats by acid-washing. Analysis of the acid-wash solutions indicate that different metals were concentrated to varying extents, and that metals with low metal-carbonate solubility products, such as Ba, Ca, Fe, Mg, Mn, Ni, Sr, and Zn, were preferentially concentrated by the mats, perhaps as the result of precipitation as, or complexation with, mat-hosted carbonate species. These results highlight the complexity of metal partitioning in natural microbial communities, where a variety of processes other than surface adsorption, such as metabolism, authigenic mineral precipitation, and the physical entrapment of detrital material, may contribute to metal sequestration.

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1. Introduction

Microbes are found in nearly every aqueous environment on Earth, where their highly reactive surfaces effectively adsorb metals to such an extent that
they exert significant control over elemental cycling on a variety of scales (see Konhauser, 2006). The adsorption of metals by microbial biomass has been well studied in a laboratory context, and predictive surface complexation models can now accurately account for microbial sorptive phenomena under a wide range of physiochemical conditions (e.g., Fowle and Fein, 1999; Borrok and Fein, 2005). However, the application of surface complexation modeling (SCM) towards natural microbial assemblages, in order to better understand their propensity to sequester metals, is complicated by several factors.

First, the determination of microbial surface chemical parameters, such as discrete surface functional group concentrations and their respective acidity constants, is required to enable SCM, yet is difficult to achieve for environmental samples. There exists no practical protocol for the culture-independent isolation of microbial biomass from most water-rock and soil systems, and indeed, surface chemical parameters have yet to be reported for natural microbial biomass. Second, mats contain a variety of microorganisms that are in different growth stages, and they produce extracellular organic materials (e.g., capsules, sheaths, S-layers) that each have their own unique chemical compositions. Third, the ability to identify and quantify metals adsorbed to microbial surfaces may be complicated by the presence of a variety of metal-bearing detrital and authigenic mineral phases that are inseparable from the microbial biomass. For instance, microbes that assemble as biofilm communities in the form of laminated mats and other macroscopic structures have been observed to trap detrital material and precipitate authigenic minerals to the point where they become fully mineralized (e.g., Konhauser and Urrutia, 1999). The extent to which organic surface adsorption reactions drive metal sequestration in such complex natural systems is unknown.

In order to better understand microbial metal sequestration processes as they occur in a natural context, we evaluated surface chemical parameters and acid-leachable metal contents of cyanobacterial mats collected from an alkaline hydrothermal spring system in Yellowstone National Park. Samples representing diverse mat morphologies (streamers, vertical spires, horizontally laminated mats) were collected from the hydrothermal system within a very small spatial area (~2.5 m²) and evaluated for diversity in their respective surface chemical properties and ability to sequester metals. Although we selected cyanobacterial mats that appeared to be wholly unmineralized, light microscopy revealed that all samples contained some mineral phases. Potentiometric titrations were performed on samples in two ways: (1) after washing in titration electrolyte that was approximately at spring pH and ionic strength, in order to best approximate the acid-base behavior of samples as they existed in-situ, and (2) after acid-washing, in order to remove minerals to the greatest possible extent, and thus reveal the inherent surface chemical properties of the organic components. This preliminary work is intended to serve as a starting point for better understanding the relative importance of the diverse chemical and physical processes that lead to metal sequestration in natural microbial assemblages.

2. Materials and methods

2.1. Site description and sample collection

Water and microbial mat samples were collected in July 2005 from a ~2.5 m² area approximately 8 m downstream from the vent of Mound Spring, an alkaline geothermal feature located in the Sentinel Meadows area of lower Geyser Basin, Yellowstone National Park (Fig. 1A). Spring temperature and pH were measured at the location of mat sampling using a Fisher digital thermometer, Beckman pH meter, and an Orion Ross electrode that was calibrated between 7 and 10 using commercial buffers heated to spring temperature. Source waters were collected in sterile 25 ml syringes, filtered through 0.45 μm in-line nylon syringe filters, acidified on-site by TraceMetal grade HNO₃, and cold-stored until analysis for dissolved and colloidal metals (described below). Additional spring water samples were collected for analysis by ion chromatography, but were not acidified. Total alkalinity was determined on-site by titrations with phenolphthalein and bromcresol green indicators, modified for the addition of reagents directly into a syringe containing the sample. Samples of microbial mat (0.55–3.37 g wet weight) were collected with sterile, stainless steel scalpels, placed in sterile 50 ml conical polypropylene centrifuge tubes, stored in spring water and ice-cooled during transport to the University of Alberta. All samples were stored in the dark at 4 °C until examination, and all titration and acid-wash experiments were completed within 4 days of sampling.

2.2. Sample preparation and acid-base titration

All glassware and plasticware were acid-washed in ~ 30% v/v concentrated HCl for at least 2 h and soaked in deionized water for 12 h prior to use. Microbial mat samples were divided by weight into two groups for separate preparation and titration. One half of sample
material was prepared by four alternating rinse (10 s agitation and 10 min soak) and harvest (centrifugation for 10 min at 10,000 \( \times g \)) cycles, using for each rinse \( \sim 35 \) ml 0.01 M KNO\(_3\) titration electrolyte that had been adjusted to spring pH (9.2) with 0.01 M NaOH in order to minimize the chemical disturbance of mat samples. These samples, prepared in and titrated from electrolyte adjusted to spring pH and ionic strength, are intended to best reflect the acid-base properties of the mats as they were found in-situ (i.e., retaining surface complexes and accessory minerals), and are thus referred to herein as “electrolyte-washed samples”. The second half was similarly prepared, however the 0.01 M KNO\(_3\) titration electrolyte was first adjusted to pH 3 with TraceMetal HNO\(_3\) in order to serve as an acid-wash, replacing surface complexed cations with protons, and dissolving any solid phase calcite. This acid-wash procedure is intended to reveal the proton-binding behavior of the

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Fig. 1. Site map (A) and photographs (B–F) showing sample location and mat morphologies. (B) All samples were obtained within an area \( \sim 2.5 \) m\(^2\) (highlighted by white box) that lay \( \sim 8 \) m from the spring source. The relative position of samples representing four mat morphologies are indicated on (C): (1) subaqueous samples with vertical spire morphology (shown enlarged in (D)) were collected from a small pooled section of stream, (2) samples with streamer morphology were collected from the pool outflow channel (upper part of photograph (E)), (3) yellow mat was collected from the left side of the same outflow channel (E), and (4) white mat were collected from the central portion of the large mat bounding the proximal edge of the stream pool (C). Upon return to the laboratory, thick (\( \sim 4 \) cm) samples of laminated mat morphology (3,4) were sectioned into upper and lower portions for individual analysis (F).
organic components comprising the biomass surface in a manner analogous to previous bacterial acid-base titrations (e.g., Fein et al., 2005 and references therein), and thus these samples are herein referred to as “acid-washed”. The rinsing solutions, 0.45 μm filtered final titration suspensions, and samples of stream water were subsequently analyzed for selected aqueous species (Al, As, Ba, Be, Ca, Cs, Fe, Ge, Li, Mg, Mn, Ni, Rb, Sb, Sr, Ti, Zn) using a Quadrupole ICP-MS (Perkin Elmer Elan 6000, University of Alberta).

For each titration, prepared sample (0.01 to 0.05 g dry weight) was suspended in ~50 ml of fresh titration electrolyte in a 125 ml titration flask, and fitted with a Ross-type pH electrode (Mandel Scientific, Guelph, Ontario), thermocouple, magnetic stir bar, titrant dispenser, and N2 purge line. Titrations were performed alkalimetrically to pH 11 with 0.01 M NaOH (Fisher Certified 0.01 M NaOH solution) and acidimetrically to pH 3 with 0.01 M HNO3 prepared from TraceMetal grade HNO3 and standardized against 0.01 M NaOH. A QC-Titrator autotitrator (Mandel Scientific) interfaced to a personal computer was set to variably dispense titrant for increments of 0.1 pH units with electrode stability criteria of <0.5 mV/s between additions. Equilibration time between additions averaged about 40 s, and each titration was completed in approximately 60 min. Suspensions were purged with N2 gas for 30 min prior to, and throughout, the titrations. Electrode calibrations using commercial buffers of pH 3.0, 4.0, 7.0, 10.0, and 12.0 were performed between every set of titration replicates (3–5) representing a single sample, and all pH measurements were automatically temperature-corrected. After the titrations, samples were filtered through pre-weighed 0.45 μm glass-fiber filters (GF/C #42, Whatman Inc, Florham park, NJ) and weighed after 7 days of air-drying. It should be noted that some cells may have lysed during storage, preparation, and titration, and that the heterogeneous nature of the mat biomass made it difficult to determine by light microscopy whether lysis occurred. As a proxy for cell integrity, pigment autofluorescence was examined for selected samples immediately pre-and post-titration, and no change in the frequency or intensity of cell autofluorescence was observed.

2.3. Discrete ligand modeling

Discrete ligand concentrations were fit to the titration data using a non-electrostatic linear-programming pKa spectrum approach described in detail by Smith and Kramer (1999) and previously applied in a variety of potentiometric titration studies of natural organic matter and microbial biomass (Brassard et al., 1990; Smith et al., 1999; Cox et al., 1999; Martinez and Ferris, 2001; Martinez et al., 2002; Phoenix et al., 2002; Fein et al., 2005). Fitting was performed using the software package Matlab (Mathworks, Natick, MA) with computer code kindly provided by D. Scott Smith (Wilfred Laurier University). Replicate blank titrations indicated negligible experimental error in system charge balance between pH ~3.5 and pH ~10.3, and data in the 4 to 10 pH range was used for modeling of ligand concentrations over a pKa range of 3 to 11 in 0.2 pH unit increments. Excess in the charge balance of the system results from the speciation of surface-associated proton-exchanging ligands and the influence of any proton-exchanging ligands in solution. Although the latter is generally assumed to be limited for titrations of vigorously washed biomass grown under laboratory conditions, this is likely not the case for the environmental samples titrated in this experiment (discussed below). Charge arising by proton-exchange with ligands is related to charge arising during titration by the following charge balance:

\[
\sum_{j=1}^{n} [L_j^-] - [\text{ANC}] = C_b - C_a + [H^+]/[OH^-],
\]

where, for the ith addition of titrant, \(C_b\) and \(C_a\) are the concentrations of base and acid, respectively, \([H^+]\) and \([OH^-]\) are measured by the pH electrode, \([L_j^-]\) is the concentration of deprotonated ligands for the jth mono-proptic ligand of n possible pK_a values set by the pK_a spectrum, and the acid neutralizing capacity (ANC) functions as a constant offset in charge excess. The ligand concentrations and ANC that provide the best fit to the measured charge excess (right hand side of Eq. (1)) is determined iteratively by minimizing the absolute error between measured and fitted charge excess (left hand side of Eq. (1)). The number of distinct-pK_a ligands that are required to describe the data is minimized as the linear-programming approach emphasizes zero as a possible solution. For each sample, between 3 and 5 replicate titrations were performed, and each titration was modeled independently.

2.4. Light microscopy

Small amounts of wet, refrigerated sample were dissected by sterile stainless steel scalpel, fixed to glass slides with several drops of low-fluorescence water-based fixative, air-dried for 15 min, and immediately examined. Photomicrographs were obtained using a Quorum Technologies 16 bit color QICAM and Zeiss
Axioskop mot 2 microscope operating in dark-field, differential interference contrast (DIC), bright-field, and phase contrast modes. Fluorescence images employed an excitation range of 530–585 nm. Samples were refrigerated before and after preparation, and all microscopy was performed within 7 days of sampling.

3. Results

3.1. Site and sample descriptions

At the time of collection in July 2005, Mound Spring had a source water temperature of 49 °C, declining to 46 °C over the ~8 m separating the source and 2.5 m² sampling area (Fig. 1B), and a pH of 9.2 at both source and sampling area. Four distinct cyanobacterial mat structures surrounding a pooled section of stream were investigated (Fig. 1C): submerged, “vertical spires” of biomass occupying calm waters of the pool (Fig. 1D), “streamers” found under flowing water at a pool outlet (Fig. 1E), and thick laminated structures forming on the pool walls, designated herein as “yellow mats” (Fig. 1E) and “white mats” (Fig. 1C). For both laminated mat samples, the surface was removed to a depth of ~5 mm for separate analysis (Fig. 1F).

Light microscopy showed that the vertical spires were dominated by long (>200 μm) filamentous Phormidium-like cyanobacteria that showed both random (Fig. 2A) and aligned orientation (Fig. 2B). Smaller rod-shaped cells were also ubiquitous in all samples, although their numbers appeared to be much lower and varied among samples. Almost all cells autofluoresced, indicating that the majority of cells in these mat structures were phototrophic (e.g., Fig. 2C). In the case of the streamer samples, autofluorescence micrographs indicated that the cell population consisted largely of filamentous, photosynthetic bacteria that were found almost equally in straight and an unusual, coiled form (Fig. 2C). Micrographs at high magnification indicate by cell size, as well as by apparent uncoiling phenomena, that the two forms represent different possible morphologies of a single cyanobacterial filament (images not shown). All of the biomass samples were encased to some extent in a matrix of extracellular...
polymeric substance (EPS); the abundant EPS enclosing the streamer structures was readily apparent in dark-field micrographs (Fig. 2D). The upper 5 mm of both laminated mats (yellow and white) were largely comprised of EPS (Fig. 3A) and filamentous bacteria lacking any alignment (Fig. 3B). Sections lower in the laminated mats (2–5 cm) displayed alternating regions of more or less dense EPS (Fig. 3C), the former being occupied by a mix of phototrophic rod-shaped and filamentous cells, and the latter by mostly rod-shaped cells, not all of which displayed autofluorescence (Fig. 3D).

Fig. 3. Dark-field and epifluorescent micrographs indicate that the upper 5 mm of both laminated mats consists of a dense EPS matrix (A) hosting randomly-aligned filamentous phototrophs (B). Lower sections of the mats show patches of more and less dense EPS (C), the former being occupied by a mix of phototrophic rod-shaped and filamentous cells, and the latter by mostly rod-shaped cells, not all of which displayed autofluorescence (D).

Fig. 4. Mineral grains of diverse size and morphology were observed in all samples. Crossed-polar light micrographs revealed strong birefringence for some mineral grains, and in consideration of the alkaline stream and mat chemistry, suggest potentially-authigenic carbonates. In the upper layers of the laminated mats, mineral grains larger than 20 μm were abundant (A), while in the lower layers of the laminated mats, as well as in the vertical spires and streamers (imaged in (B)), grains larger than 20 μm were absent, and smaller grains (<5 μm) dominated.
These rod-shaped non-phototrophic cells were found exclusively at lower depths in the laminated mats, and were visibly motile in wet-mount slide preparations. Collectively, the micrographs indicate that despite their spatial proximity in the stream, the four mat morphologies differ in the relative abundances of cell types, the alignment of filamentous bacteria (directional, coiled, or random), and in the nature or density of their EPS matrix. Vertical spire and streamer mat morphologies appeared to be dominated almost exclusively by photosynthetic bacteria of filamentous or rod shape, while laminated mat morphologies additionally contained non-photosynthetic, motile, rod-shaped bacteria at depth that may be heterotrophic. The consistency in cell size and cylindrical morphology of filamentous bacteria among samples suggests that similar bacteria contribute to all four mat morphologies, although it is impossible to confirm without further ecological study (that is beyond the focus of this work). This is also true for the photosynthetic bacteria of rod shape. In turn, the various mat morphologies would appear to arise largely as a function of differential EPS production and the varying relative abundances of the bacteria described above.

Interestingly, the diverse mat morphologies also differed with respect to their visible mineral content. In the upper 5 mm of the laminated mat samples, mineral grains were abundant and typically greater than 20 μm in their longest dimension (Fig. 4A), while in the lower sections, grains were distinctly smaller (typically less than 5 μm in longest dimension), with sizes ranging down to the limits of equipment resolution. By comparison, streamer and vertical spire structures not only possessed fewer grains, but they were also less than 5 μm in longest dimension (Fig. 4B). Analysis of the mineral grains under crossed-polars revealed the presence of calcite. Furthermore, acid washing for 30 min at pH 3 showed that a significant proportion of those

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**Fig. 5.** Acid-base data presented as excess charge in the titration system. Excess charge plots of electrolyte-washed and acid-washed mat preparations are as follows: (A) spire structures, (B) streamers, (C) upper 5 mm and (D) lower portions of yellow mat, and (E) upper 5 mm and (F) lower portions of white mat. Titration data is plotted as points, and lines demonstrate the excess charge fit by pK_a spectra models.
grains dissolved, as would be expected from calcium carbonate dissolution. However, some mineral grains remained, suggesting either that those grains were highly crystalline, or not carbonate in composition.

3.2. Acid-base titrations

In a natural mat, metals will be sequestered by a variety of submerged substrates, including the microbial biomass as well as any authigenic and detrital minerals that it hosts. To better understand the relative roles of these individual components, we have attempted to ascertain the acid-base behavior of samples containing both organic and mineral components, and the acid-base behavior of samples that we have acid-washed with the goal of removing mineral components. In order to achieve this, we have prepared the mat samples in two distinct ways. First, “electrolyte-washed” biomass was prepared by rinsing in titration electrolyte adjusted to stream pH (9.2), and then titrated down to pH 3 to approximate the excess charge behavior of the mat sample as it was found in-situ, i.e. with mineral components intact. Second, mat material was first acid-washed for 30 min in 0.001 M HNO₃ to strip off adsorbed metals, dissolve carbonate minerals, and protonate surface ligands. Samples were then similarly rinsed with titration electrolyte, and titrated from pH 3 to 11 to evaluate proton sorption by native organic surface components.

Excess charge plots of both “acid-washed” and “electrolyte-washed” mat preparations reveal that important changes in titration charging behavior are incurred by the acid-washing procedure (Fig. 5). Most notably, all suspensions of electrolyte-washed mat (top lines in Fig. 5A-F) exchanged more protons over the titration range than did their acid-washed counterparts. All samples also displayed an accumulation of excess charge at both extremes of the titration range (i.e., around pH 4 and 10). Although excess charge plots generated from titration data are ill-suited for

![Graphs showing excess charge plots for electrolyte-washed and acid-washed mats.](image)

**Fig. 6.** The concentrations and pKₐ values of ligands modeled from excess charge data of acid-washed samples are diverse: (A) spires, (B) streamers, (C) upper and (D) lower yellow mat, and (E) upper and (F) lower white mat. Ligand concentrations were summed over all replicates and divided by the total weight of all replicates in order to generate the composite plots presented above.
quantitative comparisons between samples, they do provide a visual assessment of proton sorption over the titration range, and thus are presented here for qualitative purposes.

3.3. Discrete functional group distributions

The excess charge data is more readily interpreted after being fitted with a distribution of discrete functional groups, in terms of functional group concentrations and acidity constants (henceforth presented as pKa, the negative log of the acidity constant; Fig. 6). This permits the concentrations of functional groups accounting for excess charge behavior to be compared between samples or preparations. Additionally, their pKa values can aid in the identification of reactive mat components, and the reported data as a whole can be related to past and future studies invoking a surface complexation model to explain charging behavior. Although a non-electrostatic model was employed here for the modeling of discrete functional groups, the results are likely comparable with other studies that employ electrostatic models, as surface capacitance values assumed for previous bacterial titration studies are sufficiently high (8.0 F/m²) as to be approximately equivalent to the non-electrostatic model (see Borrok et al., 2004 and references therein). Furthermore, in a recent comparison of discrete site modeling approaches, it was determined that for the gram-negative surface of two Pseudomonas species, surface electric field effects were negligible for proton adsorption, and a non-electrostatic model adequately described proton adsorption over a wide ionic strength range (0.001 to 0.6 M) (Borrok and Fein, 2005).

3.3.1. Acid-washed samples

The pKa spectra of acid-washed samples, assumed here to represent proton-active components of the native (organic) mat surface, reveal both conserved chemical features and diversity between samples. Samples of the spire-type mat indicated a pKa distribution of ligands that span the 5 to 10 range (Fig. 6A), while the ligand pKa distribution of streamer samples was dominated by low to neutral pKa values (Fig. 6B). The upper 5 mm of both the laminated yellow and white mats (Fig. 6C and D) showed reasonably comparable ligand densities near pKa 6 and 9, while lower sections of the mats indicated the presence of ligands closer to pKa 10 (Fig. 6E and F). In general, both upper and lower samples of laminated mat showed a paucity of ligands with pKa values below 5.5, and significant cluster of ligands between pKa ~5.5–7.5.

For purposes of comparison, ligands were grouped into 3 pKa classes (4–6, 6–8, and 8–10) (Table 1). The results indicate that the different mat structures vary with respect to total ligand concentration, as well as in the relative importance of individual ligand classes; however, the average standard error in most pair-wise comparisons of total ligand concentration was sufficiently high (55%) as to exclude appreciable confidence. Total ligand concentrations in the acid-washed preparations varied from 2.84±0.41 mmol g⁻¹ for the spire-type mat to 0.98±0.28 mmol g⁻¹ for the lower mat

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ligand concentration (mmol g⁻¹) per pKa class</th>
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<tbody>
<tr>
<td>Acid-washed preparations</td>
<td></td>
</tr>
<tr>
<td>Spires (n=5)</td>
<td>0.77±0.25, 0.53±0.19, 1.54±0.38, 2.84±0.41</td>
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<tr>
<td>Streamers (n=2)</td>
<td>0.40±0.40, 0.73±0.28, nd, 1.13±0.68</td>
</tr>
<tr>
<td>Upper yellow mat (n=3)</td>
<td>0.05±0.04, 0.88±0.35, 0.42±0.31, 1.36±0.59</td>
</tr>
<tr>
<td>Lower yellow mat (n=3)</td>
<td>0.32±0.18, 0.35±0.11, 0.32±0.32, 0.98±0.28</td>
</tr>
<tr>
<td>Upper white mat (n=5)</td>
<td>0.03±0.03, 0.55±0.18, 0.79±0.58, 1.36±0.76</td>
</tr>
<tr>
<td>Lower white mat (n=5)</td>
<td>0.25±0.14, 1.28±0.38, 0.29±0.29, 2.20±0.61</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Electrolyte-washed preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spires (n=5)</td>
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<tr>
<td>Streamers (n=2)</td>
</tr>
<tr>
<td>Upper yellow mat (n=5)</td>
</tr>
<tr>
<td>Lower yellow mat (n=5)</td>
</tr>
<tr>
<td>Upper white mat (n=4)</td>
</tr>
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<td>Lower white mat (n=5)</td>
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Uncertainty is expressed as one standard error.
section, similar in range to some previously reported values obtained for acid-washed suspensions of cultured Gram-negative (0.80–1.77 mmol g\(^{-1}\); Plette et al., 1995; Sokolov et al., 2001; Phoenix et al., 2002) and Gram-positive bacteria (0.22–2.29 mmol g\(^{-1}\); Fein et al., 1997; He and Tebo, 1998; Cox et al., 1999; Yee et al., 2004). However, the lack of sites with pK\(_a\) < 4 for the laminated mat samples differs from cultured representatives. These sites, often designated as carboxyl by their low pK\(_a\) values, have been implicated as primary sites for the adsorption of cations by bacteria (e.g., Beveridge and Murray, 1980). Their absence may explained by a retention of cations throughout the acid-wash and titration procedures, rather than an actual absence of carboxylic functional groups on the biomass surface, although additional (e.g., spectroscopic) evidence is required for any further inference.

### 3.3.2. Electrolyte-washed samples

The most significant observation of this study is that the electrolyte-washed samples contain significantly higher ligand concentrations than the acid-washed samples, with a single ligand typically dominating the pK\(_a\) spectra around 7 (Fig. 7, Table 1). In some instances, ligands with pK\(_a\) values near 4 and/or 10 were present, however they contributed no more than 48% of ligands for any electrolyte-washed sample, and on average constituted only 23% of the total ligands. The influence of an abundant ligand with pK\(_a\) ∼ 7 is evident in the plots of excess charge, where protons are strongly consumed in the pH 6–8 range (recall Fig. 5).

Unlike the acid-washed samples, some of the electrolyte-washed samples are statistically different in terms of total ligand concentration (Table 1). Total

![pK\(_a\) spectra of ligands modeled from excess charge data of electrolyte-washed samples reveal elevated concentrations and the dominance of a conserved ligand around pK\(_a\) 7: (A) spires, (B) streamers, (C) upper and (D) lower yellow mat, and (E) upper and (F) lower white mat. Ligand concentrations were summed over all replicates and divided by the total weight titrated of replicates in order to generate the composite plots presented above.](image)
3.4. Acid-leachable metal concentrations

Metal partitioning between the cyanobacterial mats and the spring waters was evaluated in terms of acid-leachable metal concentrations, which were obtained by analyzing the supernatant of each acid-wash solution for dissolved metals, and then normalizing the metal concentrations to the dry weight of acid-washed biomass. It should be noted that the metal concentrations reported here depend, to some degree, on the conditions of the acid-washing process and the presence of inorganic mat components; it is possible that the acid-wash procedure employed here failed to desorb some surface-complexed metals, and at the same time, released other metals by the dissolution of mineral components in the mat.

The concentrations of acid-leachable metals, in terms of μmol/Kg, are tabulated in Appendix 1. The ability of the mats to concentrate individual metals from the spring water may be better assessed by comparing the concentration of metals that were desorbed from the mats by acid-washing with their respective concentration in spring waters using the distribution coefficient, K_d (Fig. 8):

$$K_d = \frac{[\text{mat}](\mu\text{mol Kg}^{-1})}{[\text{spring}](\mu\text{mol L}^{-1})}$$

In the log K_d plot presented in Fig. 8, the metals are ordered by increasing aqueous concentration in spring water, and a range of distribution coefficients are apparent. Lower and upper values indicate that the concentration of metals leached from the mat ranged from 0.36 (Cs, Al) to almost 21,000 times higher (Mg) than the overlying water concentration. Distribution coefficients generally increased with decreasing spring water concentration, such that elements found at very low aqueous concentrations showed greater partitioning to the mats than did those elements found in the greatest aqueous concentration, although important exceptions are apparent (Ca, Zn). Another interesting observation is the degree of variation in elemental partitioning among samples. Some elements, such as Li, Ca, Ge, Sb, and Ti, were concentrated to similar extents by all mat samples, while others, such as Al, Rb, Cs, Be, Fe, and Mg, varied among samples in their concentration factors by up to two orders of magnitude. It further appears that the upper mat samples concentrated metals to the lowest extent, and streamer samples to the highest extent, yet ligand concentrations were lowest for the spires and streamers, but higher for the upper laminated mat sections, and highest for the bottom laminated mat sections.
once again, caution must be exercised as exceptions to this generalization are numerous.

4. Discussion

4.1. Identification of mat ligands

A variety of organic ligands have been invoked as chemical moieties that contribute to the chemical reactivity of the microbial surface. Their putative assignment to ligand clusters modeled from acid-base titration data is based on spectroscopic data (e.g., infrared (Jiang et al., 2004) and X-ray adsorption (Boyanov et al., 2002; Kelly et al., 2002) spectroscopy) and comparisons with model organic acids in terms of acid-base behavior (e.g., Fein et al., 1997). Cox et al. (1999) used organic acid pKa values from Martell and Smith (1989) to outline an approximate ligand classification scheme as follows: (1) carboxyl functional groups possess pKa values in the range of 2–6, phosphoryl functional groups in the range of 6–7, amino functional groups in the range 9–11, and hydroxyl functional groups in the range 8–13.

In this study, the ligands determined for acid-washed samples might include representatives from all of the above categories. The ligand classification scheme employed in Table 1, where ligands are grouped into pKa ranges of 4–6, 6–8, and 8–10, approximately correspond to the scheme of Cox et al., where the three classes represent carboxyl, phosphoryl, and hydroxyl/amin functional groups respectively. As spectroscopic evidence is required to confirm these identities, as well as distinguish between hydroxyl and amino functional groups whose pKa values may overlap, these assignments should be regarded as strictly operational.

For the electrolyte-washed samples, we believe that the dominant ligand at pKa ∼7 does not represent an organic functional group because of its loss upon acid-washing. Instead, we consider this behaviour to be consistent with mat-associated carbonate minerals and/or dissolved inorganic carbon (mostly as bicarbonate, HCO3−) that are at least partially dissolved or removed from the biomass by acid-washing, but are not removed by the rinsing procedure of electrolyte-washed samples. Indeed, as discussed above, calcite grains were observed within the EPS matrix of all samples, and their presence indicates local conditions of calcite supersaturation consistent with the microscopic observations indicating variably sized and located Ca-carbonate grains. A pKa value of ∼7 is in itself suggestive that the dissolution of calcite and/or production of H2CO3 from HCO3− is responsible for the dominant ligand, as the maximum buffer capacity for a closed system in equilibrium with calcite is around pH 7 (Langmuir, 1997). It is critical to note that the ligand concentrations of electrolyte-washed samples represent the combined contribution from both carbonate mineral grains and any mat-hosted aqueous carbonate species retained through the rinsing procedure, with the former dissolving to yield the latter over the course of titration. In other words, the ligand concentrations of electrolyte-washed samples are presumed to correspond to the total (aqueous + solid-phase) carbonate content of the mat.

For the electrolyte-washed samples, total ligand concentrations were lowest for the spires and streamers, higher for the upper laminated mat sections, and highest for the bottom laminated mat sections. This order is in general agreement with microscopy suggesting an abundance of calcite grains in thick laminated mat samples, with fewer grains contained in the spire and streamer structures. Such an observation is not unexpected given that the thick mat layers serve as diffusive barriers that prevent the rapid loss of soluble ions (e.g., HCO3−). This, in turn, maintains appropriate saturation states facilitating crystal growth. Conversely, soluble ions may be rapidly lost by diffusion and advection from the relatively thin spires and streamer structures.

Implicit in the above trends is the fact that the carbonate grains are authigenic in origin. Although we cannot be absolutely confident that some of the grains are not detrital, it is well known that cyanobacteria facilitate carbonate precipitation by the adsorption of Ca2+ cations to their cell surfaces and their metabolic production of HCO3− (Thompson and Ferris, 1990; Merz-Preiß, 2000). It is interesting to note that differences in organic surface functional group chemistry are small (total ligand concentrations ranging from 0.98±0.28 to 2.84±0.41 mmol g−1) relative to differences attributable to carbonate mineral content (i.e., electrolyte-washed total ligand concentrations ranging from 6.12±1.39 to 19.23±3.14 mmol g−1), suggesting that organic surface functional group chemistry was not a major determinant of the mat’s aqueous or mineral carbonate content.

4.2. Balance between available ligands and sequestered metals

As discussed above, acid-leachable metal concentrations in the microbial mats ranged from 0.36 to 21,000 times their respective stream water concentrations, and most metals were concentrated by a factor of 10 to 1000. This is not surprising for an alkaline spring system (pH
9.2 in this case), where most anionic functional groups (e.g., carboxyl and phosphoryl) attributable to the biomass should be in a deprotonated state and thus available as anionic sites for metal cation adsorption. However, mat-associated metal concentrations were approximately 3 orders of magnitude less than the number of available ligands determined for either acid-washed and electrolyte-washed samples. In other words, by a large majority, most ligands available within the microbial mats were unoccupied by metals. This fact, although counter-intuitive, is readily explained by the low concentrations of metals in the stream waters (see Appendix 1); at the stream pH of 9.2, the solubility of many of the metals examined in this study were very low. It is also worth noting that in this system, the microbial sequestration of metals is unlikely to affect aqueous metal concentrations, as the continuous supply of stream water approximates a flow-through system rather than a batch system, and the surfaces of interest should reach a steady-state equilibrium barring any chemical or biological change in the system. The end result is a system where metal adsorption is governed by equilibrium between metals in stream water and available ligands, the former of which is low, and the latter is high, as the result of elevated pH. Interestingly, the fact that the concentration of metals.

Nonetheless, it appears that there exist some relationships between biomass ligand availability and acid-leachable metal content (Fig. 9). As discussed above, and as readily apparent in Fig. 9, the electrolyte-washed samples have higher ligand concentrations that we consider, on the basis of their pKa values and effective removal by acid washing, to represent aqueous bicarbonate and/or mat-hosted carbonate mineral species. The total metal concentrations leached from the laminated mat samples (both upper and lower sections) correlated strongly with electrolyte-washed ligand concentrations (upper trend line), and to a lesser degree, with acid-washed ligand concentrations (lower trend line). In apparent discrepancy, the smaller mat structures (i.e., spires and streamers, individually labeled in Fig. 9) had much lower electrolyte-washed ligand concentrations, yet still possessed concentrations of metals that were comparable to the laminated mats. This discrepancy might be best explained by their response to the rinsing procedure; although electrolyte-washed samples were not exposed to low pH conditions, they were thoroughly rinsed in titration electrolyte. It is possible that the aqueous carbonate content of the smaller mat structures was removed to some extent during rinsing, as a consequence of their large surface area to volume ratios; the thicker laminated mat samples are likely to have better retained their aqueous carbonate contents during the rinse procedure simply because of their thickness. As a result, the spire and streamer structures provided low ligand concentrations during titration of electrolyte-washed samples, yet acid-washes of similar samples yielded metal concentrations that are comparable to the laminated structures. The relative roles in metal retention of mat-associated aqueous carbonate complexes and solid-phase carbonate minerals deserves further study; the apparent correlation between electrolyte-washed ligand concentrations and acid-leached metal concentrations does not distinguish metal carbonate minerals as the primary source of metals relative to aqueous metal carbonate complexes, and as discussed above, it is likely that the electrolyte-washed ligand concentrations represent the sum of both. In a retrospective and cautionary note, critical sample selection and careful preparatory techniques (i.e., sectioning mat biomass into parts of dimensions) may help avoid similar difficulties in the future study of metal sequestration by microbial mat structures.

In the case of the laminated mat samples, the apparent correlation of acid-leachable metal concentrations with both acid-washed and electrolyte-washed ligand concentrations is interesting, but unfortunately it provides little information regarding any definitive means of metal sequestration in these mats, whether by authigenic mineral precipitation, detrital entrapment, or surface adsorption. The correlations do indicate, however, that within a single hydrothermal system, and even over an area as small as the one detailed herein, variability in the chemical reactivity or metabolic activity of microbial

Fig. 9. Relationships between total mat metal concentrations and ligands determined for both acid-washed (●) and electrolyte-washed (▲) preparations. All of the laminated mat samples follow a correlation between ligand and metal concentrations, while the spire and streamer samples (labeled) are discrepant.
mats may be sufficient high as to be reflected in the concentrations of metals the mats sequester.

4.3. Trends in metal sequestration

Our work shows that various elements are concentrated to different extents by the microbial mats. Metals of low $K_d$ values ($0.5-1$), such as Al, As, Cs, Li, Rb, and Ti, are only slightly concentrated by the mats, in an apparently non-specific manner; this is consistent with laboratory experiments demonstrating general non-specificity during metal sorption by bacterial surfaces (e.g., Fein et al., 2001). However, those metals most concentrated by the mats (with $K_d$ values $>2$), such as Ca, Fe, Mn, Sr, Zn, Ba, and Mg, are preferentially sequestered in a manner that is inconsistent with non-specific adsorption to organic mat components. Importantly, these metals are primarily divalent cations with relatively low anhydrous metal carbonate solubility products ($K_{sp}$):

$$\text{MeCO}_3(s) \rightarrow \text{Me}^{2+} + \text{CO}_3^{2-}, K_{sp} = [\text{Me}^{2+}][\text{CO}_3^{2-}]$$

In other words, saturation for their respective metal carbonates may be achieved at low $[\text{Me}^{2+}]$ and $[\text{CO}_3^{2-}]$ values, and thus, these divalent metals are readily sequestered by co-precipitation with, or sorption to, calcium carbonate. Indeed, when their metal carbonate solubility products ($K_{sp}$ values from the WATEQ4F database, Ball and Nordstrom, 1991) are plotted against their respective biomass distribution coefficients, it becomes evident that their mat distribution coefficients increase as a function of increasing solubility, with the exception of Ca and Zn (Fig. 10). At first glance, this might indicate that these metals were sequestered in their metal carbonate form, and that the extent to which they were dissolved by the acid-leaching procedure depended on their respective mineral solubilities.

Stability constants for aqueous metal complexation by oxalate are also presented in Fig. 10, because it has been previously demonstrated that the adsorption of metals to bacterial surfaces are correlated to some extent with their degree of aqueous complexation by simple carboxylic acids such as oxalate (Fein et al., 2001). This apparent correlation is not surprising given the chemical homology between carboxylic acids on the bacterial surface and those of simple organic acids such as oxalate; in fact, the same principal of chemical homology explains why metal distribution coefficients in Fig. 10 correlate with both calcium carbonate solubility and oxalate complex stability. The stability constants of aqueous oxalate ($\text{CH}_3\text{O}_2$) and carbonate ($\text{CO}_3^{2-}$) metal complexes are known to correlate with each other as the result of their similarity in size, geometry, electron configurations and other bonding properties (Langmuir, 1997). The elements Ca and Zn, found at higher concentrations in the stream waters than the other carbonate-forming elements, poorly adhere to the above stability constant relationships. Zachara et al. (1991) previously investigated the sorption of divalent metals to calcite surfaces, and found that Zn deviates from other metals in the relationship between its calcite surface stability constant and its ionic radius relative to Ca, implying that additional factors related to the Zn atom’s electronic structure may additionally influence its sorption to calcite. However, in a plot similar to Fig. 10, they find that the sorption of Zn is equally related to its metal carbonate solubility product. According to the observed trends, Zn should have been found at higher surface concentration to correlate with its oxalate stability constant, and at lower surface concentrations to correlate with its metal carbonate solubility product. It is possible that this represents competition between organic and carbonate ligands for available Zn, however given the limited data, unknown factors may be equally responsible.

With respect to both apparent correlations, Calcium was consistently found at low concentrations. This trend may represent either incomplete dissolution of calcium carbonate grains or a failure for calcium to desorb from organic components during acid-washing. In the case of the former, time-lapsed dissolution experiments (unpublished)

![Fig. 10. A plot of anhydrous metal carbonate (MeCO_3(s)) solubility products (K_{sp}) vs. their average biomass distribution coefficients displays a strong linear relationship (R^2=0.97) for most of the carbonate-forming metals. Ca and Zn are treated as outliers and omitted from this regression (see text). However, a similarly linear relationship is observed with Me-oxalate stability constants (R^2=0.60, Ca omitted), highlighting the difficulty in relating metal sequestration to either mineral precipitation or biomass adsorption from a thermodynamic standpoint.](image-url)
indicated that the larger grains (as shown in Fig. 4A) were not completely removed within the 30 min acid-leach procedure. At the same time, the latter hypothesis may be equally valid, as carboxylic functional groups that were conspicuously absent from the acid-washed pKₐ spectra may have been ‘masked’ by cation occupancy even after the acid-washing procedure, as discussed above.

While interesting, the simultaneous correlation of metal distribution coefficients with metal carbonate K_{sp} values and metal-oxalate stability constants complicates any efforts to determine which metals may have been adsorbed to biomass vs. those that may have been hosted in carbonate minerals and subsequently released during acid-leaching. Overall, these results highlight the fact that metal distribution coefficients reflecting metal sequestration by natural microbial biomass may be highly ordered according to the thermodynamic properties of individual metals (e.g., ionic radius, ionic potential, electron configurations), yet vague in relation to actual sequestrative processes because of chemical homology between organic and mineral components in a natural microbial community.

It is clear that despite the analysis presented above, authigenic mineral precipitation, detrital entrapment, and surface adsorption all remain viable as mechanisms that may have lead to metal sequestration by the microbial mats. It is possible, if not probable, that all three processes occur simultaneously in this complex microbial community. At the very least, the results presented herein emphasize some of the difficulties inherent to the mechanistic understanding of metal sequestration in natural microbial systems. Perhaps more importantly, this study highlights the need for an encompassing view of metal sequestration by natural microbial assemblages that includes, but also looks beyond, organic surface complexation reactions. At least in this case, the partitioning of metals into a heterogeneous assemblage of cells, EPS, and diverse minerals must be considered in light of the fact that, despite their close proximity within in a single hydrothermal spring, such microbial communities may be highly varied with respect to their mineral content, organic surface reactivity, metabolic activity, and physical factors such as mat structure (e.g., streamer vs. spire vs. laminated mat) and permeability. It is clear from this preliminary work that future studies aiming to evaluate the microbial sequestration of metals may benefit from the use of natural samples for which laboratory analogues are poor or wholly unavailable.

5. Conclusions

Natural microbial mats grow under environmental conditions that are difficult, if not impossible to replicate, and the study of such systems may reveal complex geochemical processes that are not easily reproduced in a laboratory setting. Metal sequestration by microbial biomass is one such process, where simple laboratory analogues have been well characterized, but their relevance towards natural systems has remained untested. In order to better understand microbial metal sequestration as it occurs in natural settings, cyanobacterial mats of diverse morphology were collected from a small area within a single alkaline hydrothermal spring, Yellowstone National Park, and critically assessed for their ability to sequester metals from solution. Metal distribution coefficients indicate that some metals are non-specifically adsorbed, while other metals, primarily divalent cations characterized by low metal carbonate solubility products, were more strongly sequestered. While the latter might indicate sequestration by authigenic precipitation or detrital entrapment of carbonate mineral phases, a critical evaluation of pertinent thermodynamic parameters fails to distinguish whether these metals were sourced from mineral or organic mat components. Microscopy and acid-base titrations of electrolyte-washed samples demonstrate that the mats differ in their carbonate mineral content, and titrations of acid-washed samples imply that there exists some degree of variation between mats in the surface functional group chemistry of their organic components, despite the close proximity of mats within the hydrothermal feature. Total metal concentrations in the biomass correlated with both carbonate (electrolyte-washed) ligand concentrations and native organic (acid-washed) ligand concentrations, again failing to distinguish the exact mechanisms responsible for metal sequestration within the diverse mat structures. These results demonstrate that over small spatial areas, the extent of metal sequestration by diverse cyanobacterial mats may vary significantly, and that such variations may arise from the combined influences of metabolic activity, mineral phase entrapment and/or authigenic precipitation, and by the differences in surface functional group chemistry and physical organization of organic mat components. This study, being one of the first to evaluate metal sequestration by natural biomass in a framework relevant to surface adsorption models, highlights important considerations in the extrapolation of microbe-metal interaction from simple laboratory experiments to complex and heterogeneous environmental samples.

Acknowledgements

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Appendix 1

Concentrations of various elements in stream water and their corresponding mat concentrations as inferred by acid-washing

<table>
<thead>
<tr>
<th>Element</th>
<th>Stream concentration (μM)</th>
<th>Mat concentration (μmol/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spires</td>
<td>Streamers</td>
</tr>
<tr>
<td>Li</td>
<td>246.343</td>
<td>2726.467</td>
</tr>
<tr>
<td>Al</td>
<td>15.292</td>
<td>344.071</td>
</tr>
<tr>
<td>As</td>
<td>14.660</td>
<td>67.086</td>
</tr>
<tr>
<td>Ca</td>
<td>11.792</td>
<td>1216.319</td>
</tr>
<tr>
<td>Rb</td>
<td>1.384</td>
<td>129.767</td>
</tr>
<tr>
<td>Cs</td>
<td>0.688</td>
<td>79.847</td>
</tr>
<tr>
<td>Ge</td>
<td>0.658</td>
<td>26.181</td>
</tr>
<tr>
<td>Zn</td>
<td>0.399</td>
<td>822.112</td>
</tr>
<tr>
<td>Sb</td>
<td>0.386</td>
<td>4.258</td>
</tr>
<tr>
<td>Ti</td>
<td>0.157</td>
<td>1.305</td>
</tr>
<tr>
<td>Be</td>
<td>0.088</td>
<td>4.868</td>
</tr>
<tr>
<td>Ba</td>
<td>0.080</td>
<td>429.550</td>
</tr>
<tr>
<td>Mg</td>
<td>0.035</td>
<td>743.281</td>
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<tr>
<td>Mn</td>
<td>0.010</td>
<td>15.332</td>
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<tr>
<td>Sr</td>
<td>0.006</td>
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<tr>
<td>Ni</td>
<td>0.004</td>
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<tr>
<td>Sum</td>
<td>292.366</td>
<td>7723.898</td>
</tr>
</tbody>
</table>

Cl\(^{-}\) 223.914

\(\text{SO}_4^{2-}\) 15.941

Total alkalinity 5045.849

pH 9.2

Temperature (°C) 46.0

Ionic strength (M) 0.011

Elements are arranged by their stream water concentration. Also included for stream water are basic parameters as well as principle anion concentrations as determined by ion chromatography.

References


