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8 Corresponding Author: Ryan M. Nell

9 Corresponding Author's Institution: University of Notre Dame

10 First Author: Ryan M. Nell

11 Order of Authors: Ryan M. Nell; Jeremy B. Fein

12 **Influence of sulfhydryl sites on metal binding by bacteria**

13 Ryan M. Nell*, Jeremy B. Fein

14 Civil & Environmental Engineering & Earth Sciences,

15 University of Notre Dame, IN, 46556 USA

16 *Corresponding author: Ryan M. Nell

17 Tel.: (574) 631-4534

18 E-mail address: rnell@nd.edu (R. M. Nell)

19 Postal address:

20 156 Fitzpatrick Hall

21 Notre Dame, IN 46556

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23 *oneidensis* MR-1

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27 **Abstract**

28 The role of sulfhydryl sites within bacterial cell envelopes is still unknown,
29 but the sites may control the fate and bioavailability of metals. Organic sulfhydryl
30 compounds are important complexing ligands in aqueous systems and they can
31 influence metal speciation in natural waters. Though representing only
32 approximately 5-10% of the total available binding sites on bacterial surfaces,
33 sulfhydryl sites exhibit high binding affinities for some metals. Due to the potential
34 importance of bacterial sulfhydryl sites in natural systems, metal-bacterial
35 sulfhydryl site binding constants must be determined in order to construct accurate
36 models of the fate and distribution of metals in these systems. To date, only Cd-
37 sulfhydryl binding has been quantified.

38 In this study, the thermodynamic stabilities of Mn-, Co-, Ni-, Zn-, Sr- and Pb-
39 sulfhydryl bacterial cell envelope complexes were determined for the bacterial
40 species *Shewanella oneidensis* MR-1. Metal adsorption experiments were conducted
41 as a function of both pH, ranging from 5.0 to 7.0, and metal loading, from 0.5 to 40.0
42 μmol/g (wet weight) bacteria, in batch experiments in order to determine if metal-
43 sulfhydryl binding occurs. Initially, the data were used to calculate the value of the
44 stability constants for the important metal-sulfhydryl bacterial complexes for each
45 metal-loading condition studied, assuming a single binding reaction for the
46 dominant metal-binding site type under the pH conditions of the experiments. For
47 most of the metals that we studied, these calculated stability constant values
48 increased significantly with decreasing metal loading, strongly suggesting that our
49 initial assumption was not valid and that more than one type of binding occurs at

the assumed binding site. We then modeled each dataset with two distinct site types with identical acidity constants: one site with a high metal-site stability constant value, which we take to represent metal-sulfhydryl binding and which dominates under low metal loading conditions, and another more abundant site that we term non-sulfhydryl sites that becomes important at high metal loadings. The resulting calculated stability constants do not vary significantly as a function of metal loading and yield reasonable fits to the observed adsorption behaviors as a function of both pH and metal loading. We use the results to calculate the speciation of metals bound by the bacterial envelope in realistic bacteria-bearing, heavy metal contaminated systems in order to demonstrate the potential importance of metal-sulfhydryl binding in the budget of bacterially-adsorbed metals under low metal-loading conditions.

1. INTRODUCTION

Bacteria are ubiquitous in subsurface and surface water environments, affecting metal contaminant speciation, distribution, and mobility in these aqueous systems (e.g., Beveridge and Murray, 1976; Beveridge, 1989; Mullen et al., 1992). A wide range of metals adsorb onto bacterial cell envelopes due to reactions with abundant cell envelope binding sites (e.g., Churchill et al., 1995; Fein et al., 1997; Ledin et al., 1997; Yee and Fein, 2001). Furthermore, adsorption of metals onto the bacteria can promote biomineralization (e.g. Konhauser et al., 1993; Warren and Ferris, 1998; Dunham-Cheatham et al., 2011), and can control metabolic processes through metal bioavailability (Barkay et al., 1997; Borrok et al., 2005; Sheng et al., 2011; Flynn et al., 2014). Studying important metal-bacterial cell envelope binding

73 mechanisms, therefore, is critical for understanding global cycling of metals and
74 metal mobilities in natural systems.

75 A plethora of techniques have been used in studying metal adsorption onto
76 bacteria including: calorimetry (Gorman-Lewis et al., 2006; Gorman-Lewis, 2009;
77 Harrold and Gorman-Lewis, 2013), bulk adsorption experiments (Churchill et al.,
78 1995; Fein et al., 1997; Ledin et al., 1997; Yee and Fein, 2001), X-ray photo-electron
79 spectroscopy (XPS) (Kushwaha et al., 2012; Chubar et al., 2013), Fourier transform
80 infrared spectroscopy (FTIR) (Jiang et al., 2004; Yee et al., 2004; Fang et al., 2011),
81 molecular dynamics simulations (Johnson et al., 2006; Lins et al., 2008; Barkleit et
82 al., 2009), and X-ray absorption spectroscopy (XAS) (Kelly et al., 2001; Boonfueng et
83 al., 2009; Dunham-Cheatham et al., 2011). The first studies of the binding sites of
84 metals on bacterial cell walls quantified adsorption under high metal loading
85 conditions and identified carboxyl, phosphoryl, and amino moieties as the dominant
86 groups responsible for metal binding (Beveridge and Murray, 1976, 1980; Guiné et
87 al., 2006; González et al., 2014). However, recent spectroscopy studies document the
88 importance of sulfhydryl sites as the primary bacterial cell envelope binding sites
89 for Cd^{2+} , Hg^{2+} , and Zn^{2+} (and likely other chalcophilic elements as well) under low
90 metal loading conditions (Guiné et al., 2006; Mishra et al., 2009; 2010; 2011;
91 Dunham-Cheatham et al., 2015). Organic sulfhydryl compounds are important
92 complexing ligands for soft Lewis acids (or chalcophiles), and the concentration of
93 sulfhydryl sites within dissolved organic matter is known to influence the speciation
94 of these metals in natural waters (e.g., Hering and Morel, 1988). The role of
95 sulfhydryl sites within bacterial cell envelopes is unknown, but they likely control

96 the fate and bioavailability of metals by: 1) providing high-affinity binding sites that
97 are important under the low metal loading conditions typical of even contaminated
98 environments, 2) mediating redox transformations, and 3) controlling precipitation
99 and colloidal formation of soft metals such as Hg and Zn.

100 Thermodynamic modeling of metal adsorption onto bacteria has become a
101 powerful tool in determining metal speciation and distributions in bacteria-bearing
102 geologic systems. A wide range of metals and bacteria have been examined, and
103 stability constants have been determined for the dominant metal-bacteria
104 complexes under experimental conditions (e.g., Churchill et al., 1995; Yee and Fein,
105 2001; Burnett et al., 2007; Ginn et al., 2010; Ngwenya et al. 2010). Virtually all of
106 these experimental studies were performed at relatively high metal loading
107 conditions. Sulfhydryl sites concentrations and acidity constants have been
108 determined for a number of bacterial species (Joe-Wong et al., 2012; Kenney et al.,
109 2012; Yu et al., 2014) and typically represent only approximately 5-10% of the total
110 binding sites. Under the high metal loading conditions of most previous studies,
111 non-sulfhydryl sites dominate relative to metal-sulfhydryl binding. The derived
112 'site-specific' equilibrium constant values from these studies likely represent
113 abundance-normalized averages for metal-sulfhydryl and metal-non-sulfhydryl
114 binding constants. Under high metal loading conditions, non-sulfhydryl binding
115 dominates, thus overshadowing any sulfhydryl binding contribution. EXAFS results
116 indicate that metals bind preferentially to sulfhydryl sites under low metal loading
117 conditions (Guiné et al., 2006; Mishra et al., 2010; Kenney et al., 2012). Therefore,
118 determinations of metal-sulfhydryl binding constant values are needed in order to

119 construct accurate models of metal speciation and bacterial adsorption under low
120 metal loading conditions. The only study to report explicit metal-sulfhydryl binding
121 constant values has been a study of Cd binding onto bacteria, conducted over a wide
122 range of metal loading conditions, 0.5 to 40 $\mu\text{mol Cd/g}$ (wet weight) bacteria (Yu
123 and Fein, 2015). Yu and Fein (2015) demonstrate that the stability constant for the
124 Cd-sulfhydryl complex is approximately three orders of magnitude greater than that
125 for the Cd-non-sulfhydryl bacterial complex.

126 The calculation of metal speciation and distribution in realistic complex
127 bacteria-bearing systems requires stability constants for each metal-bacterial
128 complex that can form in those systems. Typical environmental settings have
129 relatively low aqueous metal concentrations and thus low metal loading conditions.
130 Therefore, it is imperative to determine stability constants for the metal-sulfhydryl
131 bacterial complexes that should dominate under these conditions. The objective of
132 this study is to extend the approach taken by Yu and Fein (2015) in order to
133 quantify the thermodynamic stability of a range of metal-sulfhydryl bacterial cell
134 envelope complexes, and to derive a relationship that enables estimation of stability
135 constants for metals that have not been included in this study. We measured the
136 adsorption of Mn, Co, Ni, Zn, Sr, and Pb onto *Shewanella oneidensis* MR-1 under a
137 range of metal loading conditions in batch adsorption experiments. We use the
138 results to determine if metal-sulfhydryl binding occurs, and to constrain the value of
139 the stability constants for the important metal-sulfhydryl bacterial complexes.

140 **2. MATERIALS AND METHODS**

141 **2.1 Bacterial Preparation**

142 *Shewanella oneidensis* MR-1 is a Gram-negative bacterium capable of growth
143 in both aerobic and anaerobic conditions and having the ability to reduce a variety
144 of metals. In this study, cells were grown aerobically in an incubating shaker to
145 maintain constant mixing, initially in 5 mL of trypticase soy broth amended with
146 0.5% yeast extract at 32 °C for 24 h, and then transferred to 2 L of the same growth
147 medium for an additional 24 h at 32 °C to achieve early stationary phase. Bacterial
148 cells were then harvested via centrifugation at 8,100 *g* for 5 min in pre-weighed
149 centrifugation tubes. The resulting bacterial cell pellets were washed five times with
150 0.1 M NaCl, with centrifugation at 8,100 *g* for 5 min and re-suspended in fresh 0.1 M
151 NaCl performed between rinses. After the final rinse, the supernatant was removed
152 and two additional 30 min centrifugations were conducted at 8,100 *g*, with the
153 supernatant discarded after each centrifugation step. No specific EPS removal
154 procedure was performed, though prior studies indicate that *S. oneidensis* does not
155 have a significant EPS-associated sulfhydryl site concentration (Yu et al., 2015). The
156 final bacterial wet weight was measured, and all bacterial concentrations in this
157 study are reported as the wet weight of bacteria per g of solution. The wet to dry
158 weight conversion factor for *S. oneidensis* MR-1 was previously determined to be
159 4.75 (Yu and Fein, 2015). Cells were washed, weighed, and used for experiments all
160 on the same day.

161 **2.2 Adsorption Experiments**

162 Metal adsorption experiments were conducted at metal:biomass ratios
163 ranging from approximately 0.5 to 40 μmol metal/g bacteria as a function of pH for

Mn, Co, Ni, Zn, Sr, and Pb systems. Additional metal loadings of 23.0, 26.0, 33.0, and 36.0 $\mu\text{mol/g}$ were conducted for the Pb-bacteria system in order to better evaluate changes in Pb adsorption as a function of metal loading. These metal loading values refer to the initial metal loading for the adsorption experiments. For each metal studied, a parent solution was made by dilution of a 1000 ppm commercial aqueous metal standard solution into 0.1 M NaCl to the desired concentration for a given metal:biomass ratio. Each parent solution was adjusted to $\text{pH } 6.5 \pm 0.1$ using aliquots of NaOH ranging in concentration from 0.1 to 6 M. Metal concentrations in the parent solutions were chosen such that concentrations were measureable via inductively coupled plasma optical emission spectroscopy (ICP-OES) and all experimental solutions were under-saturated with respect to any potential mineral precipitates in the pH range of interest. Pre-weighed and washed bacterial cell pellets, at concentrations that would yield the desired metal:bacterial ratios, were suspended using a vortex shaker in the metal parent solutions. Bacteria were metabolically inactive, and because no nutrients or electron donors were included in the experimental solutions, the bacteria remained inactive throughout the course of the experiments. The well-mixed metal-biomass suspensions were divided into 5 mL portions in Teflon test tubes with the pH of each suspension adjusted using small aliquots of HNO_3 or NaOH to the desired pH for each experiment such that the pH range covered was between 5.0 and 7.0. Significant concentrations of Mg and Ca can be released from bacteria at pH values less than 5.0 (Yu and Fein, 2015), and metal (hydr)oxide precipitation can occur above pH 7 at the metal concentrations used here.

187 The tubes were capped and rotated end over end for 2 h at room
188 temperature to ensure that equilibrium was reached (Fowle and Fein, 2000; Borrok
189 and Fein, 2005). The final pH, which is the reported pH value for each experiment,
190 was measured before samples were centrifuged for 5 min at 8,100 *g* and filtered
191 using 0.45 µm nylon filters to separate the cells from the aqueous supernatant. Each
192 sample was acidified using 18 µL of 15.8 M HNO₃ per 10 mL of sample. The final
193 aqueous phase metal concentrations were determined using ICP-OES, and the extent
194 of adsorption was determined by difference between the known initial metal
195 concentration and the final measured metal concentration for each experiment. No
196 reversibility experiments were conducted, but previous experiments have
197 demonstrated that metal cation adsorption onto bacterial cells is rapid and fully
198 reversible on the time scale of our experiments (e.g., Fowl and Fein, 2000; Yu and
199 Fein, 2015).

200 **2.3 Analysis of Metals**

201 The metal concentrations in each aqueous supernatant sample was
202 measured by ICP-OES. Matrix-matched standards were prepared using 1000 ppm
203 commercial aqueous metal standards, diluted to the appropriate concentration
204 range using 0.1 M NaCl. The wavelengths used for analysis of Mn, Co, Ni, Zn, Sr, and
205 Pb were 257.610, 228.616, 221.648, 213.857, 232.235, and 217.000 nm,
206 respectively. Blanks and standards were analyzed before and after every 16
207 experimental samples in order to monitor instrument drift. Analytical uncertainty,

determined by repeat analyses of the standards, was $< \pm 5\%$ for all metals studied with detection limits of approximately 0.02 ppm for all metals studied.

3. RESULTS

3.1 Metal Adsorption Experiments

All of the metal systems studied exhibited typical metal adsorption behavior as a function of pH (Figure 1). For simplicity of discussion, we classify the metal loadings that we studied into three categories: low (less than 2.0 $\mu\text{mol/g}$), medium (2.0-10.0 $\mu\text{mol/g}$), and high (greater than 10.0 $\mu\text{mol/g}$). Two general trends were observed in each of the metal systems studied: the percentage of total metal in each system that was removed from solution due to adsorption increased with increasing pH and with decreasing metal loading on the bacteria. In the Pb experiments, the low and medium metal loading conditions led to almost complete adsorption of the Pb onto the bacterial cells, and only the highest Pb loading conditions exhibited a decrease in the percentage of Pb adsorbed (Figure 1E). Additional Pb-bacteria experiments were conducted under high metal loading conditions (23.0, 26.0, 33.0, and 36.0 $\mu\text{mol/g}$) in order to better constrain the important Pb-bacterial surface binding constants (Figure 1E). In all cases, the percentage of total Pb removed in each system decreased as a function of metal loading. The maximum observed extents of metal removal under the low metal loading conditions occurs according to the following order: $\text{Pb} > \text{Zn} > \text{Mn} > \text{Ni} > \text{Co} > \text{Sr}$, with nearly complete removal of the Pb and only approximately $\sim 58\%$ removal of the Sr. Under the highest metal loading conditions studied, the maximum observed extent of metal adsorption ranged from

230 83% adsorption in the Pb experiments to only 10% adsorption in the Mn
231 experiments, with a trend of $\text{Pb} > \text{Zn} > \text{Ni} > \text{Co} > \text{Sr} > \text{Mn}$.

Figure 1: Measured extents of A) Co, B) Zn, C) Ni, D) Mn, E) Pb, and F) Sr adsorption onto *S. oneidensis* cells at metal loadings of 0.5, 1.3, 2.5, 5.0, 14.0, and 40.0 $\mu\text{mol/g}$ (wet weight) bacteria. Additional metal loading concentrations were conducted for Pb at 23, 26, 33, and 39 $\mu\text{mol/g}$ (wet weight) bacteria. The legend in “A” applies to each graph, and the values represent approximations of the actual metal loadings in each system, with precise values given in Table S1. The percent metal removed is calculated using the initial parent solution concentration.

4. DISCUSSION

4.1 Calculating Stability Constants for Bacterial Metal-Sulfhydryl Complexes

We use a thermodynamic approach to model aqueous metal speciation and metal adsorption onto bacterial cell envelopes as a function of both pH and metal loading conditions. As was done by Yu and Fein (2015), we first model the adsorption behavior as a function of pH and metal loading with a single metal-bacteria adsorption reaction and we solve for the associated equilibrium constant, or K , value. If a single adsorption reaction mechanism is responsible for the adsorption observed under all metal loading conditions, our calculated K values would not change as a function of metal loading. However, if, as Yu and Fein (2015) found for Cd-bacterial systems, the calculated K values decrease systematically with metal loading, then we can use the data to constrain metal binding onto an additional high affinity reactive site that we refer to as 'sulfhydryl', based on EXAFS results which indicate that metal binding is dominated by sulfhydryl groups under low metal loading conditions (Guiné et al., 2006; Mishra et al., 2010; Kenney et al., 2012). We define 'non-sulfhydryl' as the remaining portion of bacterial reactive sites that are responsible for metal adsorption, with this 'non-sulfhydryl' component likely composed of a combination of carboxyl and/or phosphoryl groups (Boyanov et al., 2003; and Kelly et al., 2001; and Mishra et al., 2010).

In our modeling approach, the *S. oneidensis* cell envelope buffering capacity is described using a non-electrostatic four proton-active site model (Yu et al., 2014), having site concentration values of 105.0 ± 9.5 , 86.3 ± 15.1 , 44.0 ± 2.9 and 113.7 ± 8.7 $\mu\text{mol/g}$ (wet weight) (hereafter referred to as Sites 1-4), with corresponding

268 pKa values of 3.9 ± 0.2 , 5.2 ± 0.2 , 7.0 ± 0.1 , and 9.4 ± 0.1 . Proton buffering for Sites 1-
 269 4 are described using the following reaction:

271 where R represents the macromolecule within the cell envelope to which each site,
 272 L_n , is attached. The mass action equation for Reaction (1) is:

$$273 \quad K_a = \frac{[R-L_n^-] a_{H^+}}{[R-L_n-H^0]} \quad (2)$$

274 where $[R-L_n^-]$ and $[R-L_n-H^0]$ are the concentrations of the deprotonated and
 275 protonated form of site type L_n , respectively, and a_{H^+} represents the activity of H^+ in
 276 solution. Our metal adsorption experiments ranged in pH from 5.0 to 7.0, thus Sites
 277 2 and 3 are the ones most likely to be responsible for metal adsorption. We use a 1:1
 278 metal:site stoichiometry to account for the metal adsorption:

280 where $R - L_n - M^{(m-1)}$ represents the metal-bacterial surface complex. Reaction
 281 (3) has the corresponding mass action equation:

$$282 \quad K_{Metal-L_n} = \frac{[R-L_n-M^{(m-1)}]}{[R-L_n^-] a_{M^{m+}}} \quad (4)$$

283 Note that in our initial modeling treatment, binding onto sulfhydryl and 'non-
 284 sulfhydryl' sites both with pKa values of 7.0 is all represented by a single $K_{Metal-L_n}$
 285 value for Reaction 4. Therefore, our initial test determines if this site truly is a single
 286 type of functional group, or if the site represents a mixture of groups that change in

importance as a function of metal loading. The model calculations were performed using FITEQL 2.0 (Westall, 1982) with inclusion of relevant aqueous metal hydrolysis reactions, aqueous metal-chloride complexation reactions, bacterial envelope reactive site concentrations and corresponding acidity constants (see TABLE S1 for a list of all included reactions, equilibrium constants, and sources). We tested one-site models involving Site 2 and Site 3 separately, and for all metals studied, the one-site models using deprotonated Site 3 to account for metal adsorption produced better fits as a function of both pH and metal loading than the one-site models using deprotonated Site 2 (illustrated for the Zn data in Figure 2).

The Reaction 3 $\text{Log } K_{\text{Metal-Site } 3}$ values calculated from these one-site models for the Pb- and Sr-bacteria systems remain constant at 7.1 ± 0.1 and 3.9 ± 0.1 (Figure 3), respectively, over the metal loading range studied here (model fits not shown). Conversely, Reaction 3 $\text{Log } K_{\text{Site } 3}$ values calculated from these one-site models for the other metals studied decrease significantly with increasing metal loading (Figure 4). For example, the calculated one-site Reaction 3 $\text{Log } K_{\text{Zn-Site } 3}$ values for Zn decrease from 5.5 to 4.3 as metal loading increases from 0.5 to 40 $\mu\text{mol Zn/g}$ bacteria, with similar decreasing $\text{Log } K_{\text{Metal-Site } 3}$ values as a function of metal loading for the Co, Ni, and Mn systems (Figure 4A, C, E, and G). These results strongly suggest that the binding mechanism changes as a function of metal loading for these metals. A similar decrease in thermodynamic equilibrium constant values as a function of Cd/bacteria loading was determined by Yu and Fein (2015), who invoked Cd^{2+} binding onto sulfhydryl and ‘non-sulfhydryl’ sites, each with the same pKa values, but with different site concentrations, in order to successfully model the

310 pH and Cd loading dependence to the observed adsorption behavior. It is important
311 to remember that each site type, L_n , invoked in this modeling approach is not
312 necessarily a single type of functional group, but may represent a mixture of group
313 types with similar pKa values. The evidence from this study, although
314 circumstantial, strongly suggests that sulfhydryl sites dominate at low metal loading
315 for most of the metals studied here, except for Sr. These sites have a high affinity for
316 metal adsorption, so metals preferentially bind to the sulfhydryl sites when there is
317 availability of all site types. The 'non-sulfhydryl' sites dominate under high metal
318 loading conditions because the abundance of sulfhydryl sites is limited (Yu et al.,
319 2014) and represents only approximately 5-10% of the total sites available for
320 metal binding. Although metals still bind to sulfhydryl sites under high metal
321 loading conditions, these sites saturate and metals also bind to the more abundant
322 | 'non-sulfhydryl' sites, which can dominate the bound metal budgets.

Figure 2: Modeling results using one-site models (dashed for models involving Site 2 only; solid for models involving Site 3 only) of Zn adsorption onto *S. oneidensis* cells under metal loadings of: A) 0.5, B) 1.3, C) 2.6, D) 5.0, E) 13.4, and F) 40.0 $\mu\text{mol Zn/g}$ (wet mass) bacteria.

Figure 3: Calculated stability constants from the one-site model consisting of Reaction (3): A) Log $K_{Pb-Site\ 3}$ values as a function of metal loading; B) Log $K_{Sr-Site\ 3}$ values as a function of metal loading.

Following the approach taken by Yu and Fein (2015), we attempt to account for the metal-loading dependence of the adsorption behaviors by invoking a two-site model for each metal dataset, and testing these models by determining the metal-loading dependence to the calculated metal-sulfhydryl site stability constant. That is, we construct models involving a high-affinity sulfhydryl site, $R - Site\ 3_{Sulf}^-$, and a lower affinity 'non-sulfhydryl' site, $R - Site\ 3_{non-Sulf}^-$. Both of these sites are assumed to have the same pK_a value of 7.0. Yu et al. (2014) determined a total Site 3 concentration for *S. oneidensis* of $44.0 \pm 2.9\ \mu\text{mol/g}$ bacteria. The potentiometric titrations conducted by Yu et al. (2014) are not sensitive to site type, so 'Site 3' refers to all sites with similar pK_a values of approximately 7.0, and consists of both sulfhydryl and non-sulfhydryl sites. However, Yu et al. (2014) also used a sulfhydryl-blocking technique to determine the Site 3 sulfhydryl portion concentration of $3.4 \pm 1.6\ \mu\text{mol/g}$ bacteria. The two governing metal adsorption reactions are described as:

where $R - Site\ 3_{Sulf} - M^{(m-1)}$ and $R - Site\ 3_{non-Sulf} - M^{(m-1)}$ represent metal complexes with high- and low-affinity sites, respectively. The resulting stability constants are expressed as:

$$K_{Metal-Site\ 3_{Sulf}} = \frac{[R-Site\ 3_{Sulf}-M^{(m-1)}]}{[R-Site\ 3_{Sulf}^-] a_{M^{m+}}} \quad (7)$$

$$K_{Metal-Site\ 3_{non-Sulf}} = \frac{[R-Site\ 3_{non-Sulf}-M^{(m-1)}]}{[R-Site\ 3_{non-Sulf}^-] a_{M^{m+}}} \quad (8)$$

Stability constants for two or more metal-bacterial complexes can be determined from adsorption measurements only when the variable parameter in the experiments (pH, metal loading, etc.) changes the distribution of the adsorbed metal between those complexes. In our experiments, changes in pH do not change the distribution of adsorbed metal between sulfhydryl and non-sulfhydryl sites, so a single dataset conducted at a fixed metal loading can not be used to calculate K values for both Reactions 7 and 8 simultaneously. However, we make use of the fact that metal-sulfhydryl binding under the highest metal loading conditions studied is dominated by ‘non-sulfhydryl’ binding sites. EXAFS studies indicate that non-sulfhydryl sites dominate under high metal loading conditions (Guiné et al., 2006; ; Mishra et al., 2010; Kenney et al, 2012), suggesting that the calculated Log $K_{Metal-Site\ 3}$ from the highest metal loading experiments in our study can be assumed to represent the equilibrium constant for metal adsorption onto the ‘non-sulfhydryl’ sites. Therefore, for the Zn, Co, Ni, and Mn systems, we fix the value of

369 $\log K_{Metal-Site\ 3_{non-Sulf}}$ and use the measured adsorption data to solve for a value of
370 $K_{Metal-Site\ 3_{Sulf}}$ from a particular metal loading dataset. We only use the low and
371 medium metal loading datasets to solve for the metal-sulfhydryl binding constant,
372 as these are the ones where metal-sulfhydryl binding is likely to be most significant,
373 so provide the best constraints on the stability constant value.

374 The calculated metal-sulfhydryl binding constants for the Zn-, Mn-, Ni-, and
375 Co-bacteria systems do not change systematically as a function of metal loading
376 (Figure 4). The calculated binding constants for each metal-bacteria system are
377 compiled in Table 2, and the values are 1.2 (Mn-bacterial system) to 2.4 (Zn-
378 bacterial system) orders of magnitude greater than the stability constants for the
379 corresponding complexes between the metals and the 'non-sulfhydryl' site (Table
380 2). Although we do not have direct spectroscopic evidence for the identity of what
381 we refer to as 'sulfhydryl' sites for the systems studied here, EXAFS results from Cd-
382 and Hg-bacteria systems, and the much higher binding affinity exhibited by the low
383 abundance sites in our model, strongly suggest that sulfhydryl binding is
384 responsible for metal loading under low-metal loading conditions for each of the Zn,
385 Mn, Ni, and Co systems.

Figure 4: Calculated stability constants as a function of metal loading for the one-site models consisting of Reaction (3): A) $\text{Log } K_{\text{Zn-Site } 3}$; C) $\text{Log } K_{\text{Co-Site } 3}$; E) $\text{Log } K_{\text{Ni-Site } 3}$; G) $\text{Log } K_{\text{Mn-Site } 3}$. Calculated stability constants as a function of metal loading with fixed Reaction (6) K values, for two-site models consisting of Reactions (3.5) and (3.6) : B) $\text{Log } K_{\text{Zn-Site } 3, \text{Sulf}}$; D) $\text{Log } K_{\text{Co-Site } 3, \text{Sulf}}$; F) $\text{Log } K_{\text{Ni-Site } 3, \text{Sulf}}$; H) $\text{Log } K_{\text{Mn-Site } 3, \text{Sulf}}$.

Table 2: Calculated best-fitting model Log stability constant values

Metal	^a Log $K_{Metal-Site\ 3_{non-Sulf}}$	^b Log $K_{Metal-Site\ 3_{Sulf}}$
Mn ²⁺	4.8 ± 0.2	6.0 ± 0.2
Co ²⁺	4.0 ± 0.3	5.5 ± 0.2
Ni ²⁺	3.9 ± 0.2	5.8 ± 0.2
Zn ²⁺	4.3 ± 0.2	6.7 ± 0.1
Sr ²⁺	3.9 ± 0.1	N/A
Pb ²⁺	7.1 ± 0.1	N/A
Cd ²⁺	5.2 ^c	8.3 ± 0.3 ^c

^aUncertainties for the non-sulfhydryl site K values represent the envelope of K values that account for ~95% of a dataset under the highest conducted metal loading condition for a given system.

^bMetal-Sulfhydryl binding constant uncertainties represent the standard deviation of calculated Log $K_{Metal-Site\ 3_{Sulf}}$ values.

^cYu and Fein (2015)

The two calculated stability constants for each metal determined in this study ($K_{Metal-Site\ 3_{Sulf}}$ and $K_{Metal-Site\ 3_{non-Sulf}}$) can be used to calculate the adsorbed metal distribution between cell envelope sulfhydryl and ‘non-sulfhydryl’ sites under each of the conditions in this study. For example, our two-site model provides a good fit to the observed extent of aqueous Zn adsorption as a function of both pH and Zn:bacteria ratio, covering almost two orders of magnitude in metal-loading conditions (Figure 5). Under the low and medium metal-loading systems (Figures 5A-D), Zn-sulfhydryl binding represents virtually the entire budget of adsorbed Zn across the pH range studied. More specifically, at 0.5 µmol/g, the Zn-sulfhydryl complex represents 95 and 94% of the total adsorbed Zn at pH 5 and 7, respectively. At 5.0 µmol/g, these percentages decrease only slightly to 92 and 70% of the total adsorbed Zn at pH 5.0 and 7.0, respectively. At the highest metal loadings studied (Figures 5E and F), ‘non-sulfhydryl’ sites become more important. At 13.4 µmol/g, Zn bound to sulfhydryl sites dominates below approximately pH 6.0, with Zn bound to ‘non-sulfhydryls’ dominating above pH 6.0. At 40.0 µmol/g, Zn bound to

the ‘non-sulfhydryl’ sites represents virtually all of the adsorbed Zn across the pH range studied, justifying our use of the 40.0 $\mu\text{mol/g}$ adsorption data to constrain the value of $K_{\text{Zn-Site } 3_{\text{non-Sulf}}}$. Our calculated bacterial cell envelope speciation results are in general consistent with EXAFS studies of Cd and Hg adsorption onto similar bacterial species (Mishra et al., 2010; Dunham-Cheatham et al., 2014) showing the relative importance of sulfhydryl sites increasing with decreasing metal-loading conditions.

Figure 5: Calculated extent and speciation of Zn adsorption using our two-site model with $\log K_{\text{Zn-Site } 3_{\text{Sulf}}}$ and $\log K_{\text{Zn-Site } 3_{\text{non-Sulf}}}$ values of 6.7 and 4.3, respectively, at metal loadings of: A) 0.5, B) 1.3, C) 2.5, D) 5.0, E) 13.4, and F) 40.0 $\mu\text{mol Zn/g}$ (wet weight) bacteria. Open circles indicate experimental data; the solid curve represents the calculated total percent of Zn that is adsorbed; the dashed curve represents the contribution of Zn bound to sulfhydryl sites, and the dotted curve represents the contribution of Zn bound to non-sulfhydryl sites.

4.2 Linear Free-Energy Relationships

We can use our results to estimate the stability constants of metal-bacterial cell envelope complexes that have not been determined here, using linear free-energy relationships (LFERs) which relate the calculated K values from this study to

stability constants of corresponding metal-ligand aqueous complexes. These LFERs are most effective when the ligands in each type of complex being compared are similar. Here, we relate metal-*S. oneidensis* Site 3 (sulfhydryl and 'non-sulfhydryl') binding constants to those for metal-cysteine and metal-acetate aqueous complexes, respectively. We use cysteine, which is a simple amino acid capable of metal binding via a thiol side chain, as an analogue to the sulfhydryl reactive site on the bacterial cell envelope. We use aqueous metal-acetate binding as an analogue for metal adsorption onto the 'non-sulfhydryl' sites, similar to the approach taken by Fein et al. (2001) and Ginn et al. (2010). All values are referenced to zero ionic strength, using the activity coefficient calculations conducted within FITEQL. We use metal-cysteine binding constant values compiled by Berthon (1995), with subsequent correction to zero ionic strength using the extended Debye-Hückel equation for each metal studied.

Figure 6 illustrates that a reasonable correlation exists for both relationships tested, with linear correlations described by the following equations:

$$\text{Log } K_{\text{Metal-Site 3, non-Sulf}} = 2.15 * \text{Log } K_{\text{Metal - Acetate}} + 1.17 \quad (9)$$

$$\text{Log } K_{\text{Metal-Site 3, Sulf}} = 0.28 * \text{Log } K_{\text{Metal - Cysteine}} + 3.70 \quad (10)$$

Although the scatter in the graphs in Figure 6 leads to fairly high uncertainties associated with predicted stability constants from each relationship (the average misfit between the predicted value for each metal and the actual value is equal to ± 0.2 log units and ± 0.4 log units for Figures A and B, respectively), the relationships derived from our data do enable for the first time estimation of stability constants

for metal-sulphydryl cell envelope complexes for metals that have not been studied here.

Figure 6: Linear free energy relationships between the calculated values for $\text{Log } K_{\text{Metal-Site 3, non-Sulf}}$ from this study and the corresponding stability constants for aqueous metal-acetate complexes (A); and the relationship between $\text{Log } K_{\text{Metal-Site 3, Sulf}}$ and corresponding stability constants for aqueous metal-cysteine complexes (B). The plotted Cd-bacteria complex values are from Yu and Fein (2015) and the solid lines represent linear fits to the data.

4.3 Environmental Applications

In the absence of direct measurements of metal speciation in bacteria-bearing surface water systems, we can use our calculated metal-bacterial stability

constants to estimate the importance of metal-sulphydryl binding in these systems. In order to explore the potential influence of bacterial sulphydryl site binding on aqueous metal distributions, we apply our high- and low-affinity binding site model to two hypothetical heavy metal contaminated systems, ranging in degree of metal contamination and hence metal loading on the bacteria. The total metal concentrations that we assume for these systems were chosen to represent low- and high-metal loading conditions, and we use these total values (see Figure 7 caption) in conjunction with our results to estimate the distribution of metals adsorbed onto bacterial sulphydryl sites (Figure 7). These calculations use a bacterial concentration of 3.7×10^{12} cells/L, which is a bacterial concentration that has been measured in the upper sediment layers of a fairly typical lowland river system (Fischer et al., 2002). Using a dry weight conversion of 0.4×10^{-12} g bacteria (dry weight)/L (Bakken and Olsen, 1983), and a wet:dry weight ratio of 4.75 (Yu et al., 2014), yields a river sediment pore water bacterial concentration of approximately 7.0 g (wet weight)/L. We calculate the distributions of Zn, Cd, and Ni in each system, using the stability constants for $K_{Metal-Site\ 3,\ Sulf}$ and $K_{Metal-Site\ 3,\ non-Sulf}$ that were determined in this study (Table 2). In this simplified calculation, we only consider the speciation of metals adsorbed onto bacteria. Metal adsorption onto the plentiful other surfaces in complex environmental systems such as mineral surfaces and solid and/or dissolved organic matter would reduce the concentration of adsorbed metal, and hence could augment the importance of metal-sulphydryl binding by further lowering the bacterial metal loading conditions.

499 Our calculations suggest that bacterial sulfhydryl sites can control the
500 distribution of metal bound onto the bacterial envelope under low metal loading
501 conditions (Figure 7). Under the high metal-loading conditions that we considered,
502 sulfhydryl binding of Zn, Cd, and Ni was relatively unimportant, with only 10, 36,
503 and 3 percent of the adsorbed metal present bound to sulfhydryl sites, respectively.
504 However, under lower metal-loading conditions, sulfhydryl sites dominate the
505 adsorbed metal budgets, with 95, 99, and 87 percent of the adsorbed Zn, Cd, and Ni
506 present bound to sulfhydryl sites, respectively. These calculations demonstrate the
507 range of potential importance for bacterial sulfhydryl binding of heavy metals in
508 environmental systems. In high metal loading systems, sulfhydryl sites are likely to
509 represent a small portion of the total adsorbed metal budget, swamped by the more
510 plentiful lower-affinity non-sulfhydryl sites within bacterial cell envelopes.
511 However, in the low metal loading systems that are perhaps more typical of most
512 environments, sulfhydryl sites are not saturated and can dominate the speciation of
513 bacterially-adsorbed heavy metals. These calculations also demonstrate the utility
514 and flexibility of our approach to quantify the speciation of bacterially adsorbed
515 metals in these systems.

516

517

518 Figure 7: Calculated distribution of metal adsorbed onto bacterial sulfhydryl sites.
519 Gray bars are the results for the low-metal loading system; black bars are for the
520 high-metal loading system. The following concentrations were used in the
521 speciation calculations: 1) For the low metal loading conditions: 0.011, 0.002, 0.007
522 mg/L of Zn, Cd, and Ni, respectively. 2) For high metal loading conditions: 41, 0.19,
523 and 0.110 mg/L of Zn, Cd, and Ni, respectively. Both systems included 4.3 mg/L of
524 total chloride and 1300 mg/L of total dissolved sulfate. The calculations were
525 conducted for a hypothetical aerobic zone within a sediment, and therefore neglect
526 aqueous sulfide complexation and precipitation.

527

528 5. CONCLUSIONS

529 In this study, we demonstrate the importance of metal loading on metal
530 binding mechanisms for a range of metal-*S. oneidensis* systems. Using a surface
531 complexation modeling approach first described by Yu and Fein (2015), we
532 deconvolve metal binding onto high- and low-affinity binding sites likely associated
533 with sulfhydryl and non-sulfhydryl reactive sites, respectively, within the cell
534 envelope. Yu and Fein (2015) applied this approach to Cd adsorption, finding that
535 although sulfhydryl sites are far less abundant within bacterial cell envelopes, the
536 stability constant for the Cd-sulfhydryl complex is over three orders of magnitude
537 higher than that for Cd binding onto non-sulfhydryl sites. We have extended this

approach to demonstrate similar findings for Mn, Co, Zn, and Ni binding. Further work must be done to verify binding coordination changes from metal-sulfhydryl to metal-non-sulfhydryl groups. In our Pb experiments, virtually all of the Pb was adsorbed onto the cell envelope sites, so equilibrium between adsorbed and un-adsorbed Pb could not be constrained. The near complete adsorption of Pb that was exhibited under low and medium metal loading conditions masks any potential preferential binding between sulfhydryl and non-sulfhydryl sites seen in the other metal systems. Sr, perhaps not surprisingly, does not appear to interact with sulfhydryl sites significantly and all Sr adsorption data could be modeled with one stability constant for Sr-non-sulfhydryl binding.

The sets of metal-sulfhydryl and metal-non-sulfhydryl stability constants that we determine for the other metals in this study yield excellent fits to the adsorption data as a function of both pH and metal loading, with metal loading conditions ranging over approximately two orders of magnitude. Our study suggests that sulfhydryl binding is most likely important for chalcophilic metals, playing potential roles in bioavailability of these elements in bacterial metabolic processes. Low metal loadings are typical of most natural systems, and our sample calculations of the importance of bacterial metal-sulfhydryl binding demonstrate the importance of bacterial sulfhydryl site binding, especially for low metal-loading environments. For accurate predictions, metal-sulfhydryl site binding constants must be determined in order to model the speciation and distribution of metals in complex realistic systems. Our results provide evidence for the importance of metal-sulfhydryl binding under these conditions, and suggest that a site-specific approach

like the one described here offers a means for quantifying the effect of metal loading and sulfhydryl binding on metal adsorption in bacteria-bearing systems.

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