INTRODUCTION

Metal sequestration of bacteria plays an important role in the biogeochemical cycling of metals in the environment. Consequently, there is an interest to understand how the bacterial surfaces interact with metals in solution and how this affects the bacterial surface. In this work we have used a surface-sensitive analysis technique, cryogenic X-ray photoelectron spectroscopy (cryo-XPS), to monitor the surface of *Bacillus subtilis* cells as a function of pH and Zn$^{2+}$ content in saline solution. The objective of the study was twofold: (1) to investigate the agreement between two data treatment methods for XPS, as well as to investigate to what extent sample pretreatment may influence XPS data of bacterial samples, and (2) to characterize how the surface chemistry of bacterial cells is influenced by different external conditions. (1) It was found that the two data treatment methods gave rise to comparable results. However, identical samples analyzed fast-frozen or dry exhibited larger differences in surface chemistry, indicating that sample pretreatment can to large extents influence the obtained surface composition of bacterial samples. (2) The bacterial cell wall (in fast-frozen samples) undergoes dramatic compositional changes with pH and with Zn$^{2+}$ exposure. The compositional changes are interpreted as an adaptive metal resistance response changing the biochemical composition of the bacterial cell wall. These results have implications for how adsorption processes at the surface of bacterial cells are analyzed, understood, modeled, and predicted.

Cell Wall Composition of *Bacillus subtilis* Changes as a Function of pH and Zn$^{2+}$ Exposure: Insights from Cryo-XPS Measurements

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Supporting Information

ABSTRACT: Bacteria play an important role in the biogeochemical cycling of metals in the environment. Consequently, there is an interest to understand how the bacterial surfaces interact with metals in solution and how this affects the bacterial surface. In this work we have used a surface-sensitive analysis technique, cryogenic X-ray photoelectron spectroscopy (cryo-XPS), to monitor the surface of *Bacillus subtilis* cells as a function of pH and Zn$^{2+}$ content in saline solution. The objective of the study was twofold: (1) to investigate the agreement between two data treatment methods for XPS, as well as to investigate to what extent sample pretreatment may influence XPS data of bacterial samples, and (2) to characterize how the surface chemistry of bacterial cells is influenced by different external conditions. (1) It was found that the two data treatment methods gave rise to comparable results. However, identical samples analyzed fast-frozen or dry exhibited larger differences in surface chemistry, indicating that sample pretreatment can to large extents influence the obtained surface composition of bacterial samples. (2) The bacterial cell wall (in fast-frozen samples) undergoes dramatic compositional changes with pH and with Zn$^{2+}$ exposure. The compositional changes are interpreted as an adaptive metal resistance response changing the biochemical composition of the bacterial cell wall. These results have implications for how adsorption processes at the surface of bacterial cells are analyzed, understood, modeled, and predicted.
The authors declare no competing financial interest.

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Zinc isotope fractionation during surface adsorption and intracellular incorporation by bacteria

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Abstract

Zinc (Zn) isotopes are fractionated during biogeochemical processing by microorganisms. Uncertainties remain, however, regarding the roles of cell surface adsorption and speculation of aqueous Zn on the extents of isotopic fractionation. In this study, we conducted bacterial surface adsorption and intracellular incorporation experiments using Zn and representative Gram-positive (Bacillus subtilis) and Gram-negative (Pseudomonas mendocina, Escherichia coli) bacterial species, as well as a natural bacterial consortium derived from soil. Under conditions of high Zn:bacteria ratio, surface complexes preferentially incorporated the heavier isotopes of Zn, resulting in an average Δ66Znadsorbed-solution of +0.46‰ (αadsorbed-solution ≈ 1.00046). Adsorption experiments conducted under conditions of low Zn:bacteria ratio appear to have been complicated by the presence of dissolved organic exudates that competed with surface functional group sites for Zn. We were able to empirically model this process to show that very small amounts of Zn-organic complexes with fractionation factors in the range of α = 1.002 to 1.003 could account for the observed δ66Zn of the experimental solutions. For the intracellular incorporation experiments, the presence of 0.2 and 2 mg/L of Zn (as Zn-citrate) resulted in a Δ66Znincorporated-solution ranging from −0.2% to +0.5%, depending upon the bacterial species and the growth phase. The addition of 0.2 and 2 mg/L Zn to the growth medium appeared to create a metal stress response (or at least a change in metal processing) in P. mendocina that resulted in a positive Δ66Znincorporated-solution of up to +2.04‰. Our study suggests that Zn isotopes have the potential to be used to elucidate metal-binding pathways associated with microorganisms in natural systems, but that the interpretation of these effects is likely complicated by factors such as competing surface interactions and differences in bacterial species and metal speciation.

1. Introduction

Zinc (Zn) is a critical element for biological functioning (Olhaberry et al., 1983; Shankar and Prasad, 1998; Hambridge, 2000; Andreini et al., 2006a,b; Maret, 2009), and the stable isotopes of Zn are substantially fractionated during biogeochemical processing (e.g., John et al., 2007). For these reasons, a number of investigations have examined Zn isotopic fractionation during plant uptake (Weiss et al., 2004; Viers et al. 2007; Moynier et al., 2009; Arnold et al., 2010; Caldelas et al., 2011; Jouvin et al., 2012; Tang et al., 2012) and interaction with yeast (Zhu et al., 2011) have shown that Zn isotopes of particulate matter and diatom frustules, respectively, can serve as proxies for the biochemical cycling of Zn.

Despite the increasing interest in understanding how Zn isotopes fractionate during biological cycling, there are still substantial uncertainties. In plant systems, for example, variable concentrations of Zn, as well as differences in biochemical pathways among different plant species, can lead to substantially different Zn isotopic signatures among plants (Caldelas et al., 2011; Jouvin et al., 2012; Tang et al., 2012). In microorganism systems, the relative importance of surface complexation versus intracellular incorporation is uncertain. For example, Gélart et al. (2006) showed that during sorption and uptake, freshwater and seawater diatoms were preferentially associated with heavier Zn isotopes (Δ66Zncell-solution of +0.35‰ and +0.27‰, respectively), while in a separate study John et al. (2007) found that seawater diatoms incorporated the lighter Zn isotopes (Δ66Zncell-solution ranged from −0.20‰ to −0.80‰, depending on the Zn homeostasis pathway), but adsorbed the heavier Zn isotopes. In addition, there is still uncertainty regarding the magnitude of Zn isotopic fractionation among different bacterial species and under differing conditions of Zn stress.

In order to try and address some of these uncertainties, we investigated the isotopic fractionation of Zn during surface adsorption and intracellular incorporation by representative Gram-positive (Bacillus subtilis) and Gram-negative (Pseudomonas mendocina and Escherichia coli) bacterial species and a natural bacterial consortium isolated from...
soil. The laboratory bacteria strains were chosen because of their different surface chemical properties. Moreover, Zn homeostasis mechanisms for each of these bacterial species have been described previously (e.g., Beard et al., 2000; Outten and O’Halloran, 2001; Cánovas et al., 2003; Moore and Helmann, 2005). We conducted separate batch adsorption experiments and bacterial growth experiments under a variety of chemical conditions. Samples of the associated fluids and/or bacterial cells (collected as a function of pH, time, or growth phase) were prepared and analyzed for their concentrations and isotopic compositions of Zn. Here we present the results of these experiments and discuss their implications for understanding Zn isotope variations in natural systems.

2. Methods

All volumetric flasks, sample bottles, test tubes, and pipette tips used for experiments were acid-washed in sub-boiling 10% HCl and rinsed 3 times with ultra-pure (18.2 MΩ) water. Chemical reagents and growth media were prepared using ultra-pure water. Ultra-pure acids and bases were used for experiments and sample digestion and preparation.

2.1. Bacteria collection, growth, and harvesting

*B. subtilis* (Gram-positive), *P. mendocina*, and *E. coli* (both Gram-negative) were chosen as representative laboratory strains of bacteria and were acquired through the Fein lab at the University of Notre Dame. The cellular surface of Gram-positive bacteria consists principally of a thick and rigid peptidoglycan layer (25 to 30 nm) bound together with secondary polymers such as teichoic acids (Konhauser, 2006). The surface of Gram-negative bacteria consists of a flexible outer membrane of lipopolysaccharides covering a thin (3 nm) peptidoglycan layer (Beveridge and Koval, 1981; Konhauser, 2006). *B. subtilis* was grown by inoculating (via sterile loop) an autoclaved medium of 30 g glycerol, 0.5 g K2HPO4, 1.0 g NH4Cl, 0.2 g MgSO4·7H2O, 0.2 g CaCl2·2H2O, 8.33 g succinate disodium salt, 30 mM Fe-EDTA, 4.77 g HEPES buffer, 1 g of glucose (for *E. coli* only), and 0.125 mL of trace elements solution (5 mg MnSO4·H2O, 6.5 mg CoSO4·7H2O, 3.3 mg ZnSO4 and 2.4 mg MoO3 per 100 mL of water; Hersman et al., 2001). Zn is an essential trace nutrient and the bacteria cannot grow effectively without it. It was eliminated from the growth experiments but replaced with another form of Zn (Zn-citrate or Zn+2). The deprotonated sites form surface complexes with Zn and other metals, impacting the transport and fate of metals in natural systems (e.g., Yee and Fein, 2001; Ngwenya et al., 2003; Ginn and Fein, 2008). These surface interactions are also a first step in intracellular uptake of metal (e.g., Borrok et al., 2005a). Batch adsorption experiments were conducted as a function of pH in experimental solutions with constant Zn/bacteria ratios using live cells of *B. subtilis* or the natural consortium and using live and dead cells of *P. mendocina*. Experimental component concentrations are summarized in Table 1.

2.2. Variable pH surface adsorption experiments

Batch adsorption experiments were designed to isolate Zn complexation reactions associated with bacterial surfaces. Bacterial surfaces contain a variety of organic–acid functional group sites (phosphoryl, carboxyl, hydroxyl, and some sulfhydryl, etc., depending on the species and Gram-type) that deprotonate as a function of pH (e.g., Beveridge, 1989; Kelly et al., 2002; McChure et al., 2003; Guiné et al., 2006; Mishra et al., 2010b). The deprotonated sites form surface complexes with Zn and other metals, impacting the transport and fate of metals in natural systems (e.g., Beard et al., 2000; Outten and O’Halloran, 2001; Cánovas et al., 2003; Moore and Helmann, 2005). Batch adsorption experiments were performed in a stock solution with a 0.01 M NaClO4 electrolyte. A measured volume of stock solution was amended with Zn (from an in-house Zn wire standard) and a pre-weighed bacterial pellet was suspended in the mixture. A control experiment where no bacterial pellet was added to the stock solution was also conducted to confirm that Zn did not interact with the experimental apparatus. The control experiment was subjected to the same procedures, including filtration and sample processing, that were part of the other experiments.

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Although the live cells were not thought to be actively metabolizing in the nutrient-free experimental solutions, the dead cell experiment was done to further test this possibility. In this case, *P. mendocina* cells were suspended in the experimental stock solution and treated for 12 h with UV radiation prior to the addition of Zn. We tested the effectiveness of the UV treatment by trying to culture the affected cells and no growth was observed. All batch adsorption experiments were performed in a stock solution with a 0.01 M NaClO4 electrolyte. A measured volume of stock solution was amended with Zn (from an in-house Zn wire standard) and a pre-weighed bacterial pellet was suspended in the mixture. A control experiment where no bacterial pellet was added to the stock solution was also conducted to confirm that Zn did not interact with the experimental apparatus. The control experiment was subjected to the same procedures, including filtration and sample processing, that were part of the other experiments.

The final Zn and bacteria concentrations for each experiment are provided in Table 1. Thirty milliliters of the homogenized experimental stock solution were added to each of eight 50 mL reaction vessels. The pH of each vessel was individually adjusted between pH 2.5 and 7 using 0.2 mL aliquots of dilute HNO3 or dilute NaOH (this small volume addition had a negligible impact on Zn concentrations). The pH of the experimental solutions was measured using a Thermo Scientific Orion Star Log™ pH Meter that was calibrated prior to each experiment. The reaction vessels were placed on a shaker table for 2 h after which time the final (equilibrium) pH of each vessel was recorded. Previous work has shown that a period of 2 h is enough time for bacteria–metal surface complexes to reach apparent equilibrium (e.g., Fowle and Fein, 2000). The reaction vessels were then centrifuged and the supernatant filtered through a pre-cleaned (with 2% nitric acid) 0.45 μm nylon filter. The supernatant was preserved for later analyses by adding 0.2 mL of concentrated HNO3. The solutions were later analyzed for their Zn concentrations using an ICP-OES and were further prepared for isotopic analysis (see below).

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Enthalpies and Entropies of Cd and Zn Adsorption onto Bacillus licheniformis and Enthalpies and Entropies of Zn Adsorption onto Bacillus subtilis from Isothermal Titration Calorimetry and Surface Complexation Modeling

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Enthalpies and Entropies of Cd and Zn Adsorption onto Bacillus licheniformis and Enthalpies and Entropies of Zn Adsorption onto Bacillus subtilis from Isothermal Titration Calorimetry and Surface Complexation Modeling

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Determining the thermodynamic driving force of metal-bacteria surface complexation is important for understanding why, from a thermodynamic perspective, these spontaneous reactions occur. We therefore determined the Gibbs energies, enthalpies, and entropies of Cd and Zn complexation onto Bacillus licheniformis and of Zn complexation onto Bacillus subtilis using surface complexation modeling and isothermal titration calorimetry. Our results indicated that Cd and Zn complexation onto Bacillus licheniformis is entropically driven at low pH and enthalpically driven at circumneutral pH. Zn complexation onto Bacillus subtilis is entropically driven, which suggests that Bacillus licheniformis has different donor ligands dominating reactivity around circumneutral pH.

Keywords: bioavailability, biogeochemical cycling, bioremediation

Introduction

Bacterial cell surfaces are known to complex a variety of metal ions. Much of the previous research on metal-bacterial surface reactions has focused on quantifying reactions for a better understanding of their implications on the movement of dissolved ions in environmental and on the exploitation of their potential for bioremediation of metal contamination. Less work has focused on understanding the thermodynamic driving force of these reactions, which ultimately explains why, from a thermodynamic perspective, these spontaneous reactions occur.

The thermodynamic driving force of surface reactions is a major influence in determining the selectivity of metal ions to adsorb to surfaces and the strength of the bonds formed. Determining the thermodynamic driving force of metal ion-bacterial surface adsorptions reactions will yield a better understanding of why these reactions occur, which will aid our understanding of microbial influences on the movements of dissolved ions in the environment and might also advance our understanding of the importance of adsorptions reaction for cell functions (Beveridge 1989; Beveridge and Murray 1980; Ferris and Beveridge 1985; Hoyle and Beveridge 1983, 1984; Pabst et al. 2010; Young 2006).

Metal-bacteria surface reactions have been studied using many techniques. Empirical models of metal-bacterial surface uptake can quantify adsorption under the conditions studied without extensive surface characterization, but might fail in predicting adsorption in systems that significantly differ from the studied conditions (Gorman-Lewis et al. 2006; Klimmek et al. 2001; Lin et al. 2012; Puranik and Paknikar 1999; Sulaymon et al. 2013). Surface complexation modeling frames surface adsorption reactions based on balanced chemical equations using thermodynamics, and is typically more robust in predicting adsorption under conditions not directly studied. Surface complexation modeling does, however, require more extensive characterization of the reactive surface than empirical models (Burnett et al. 2007; Fein et al. 1997; Gorman-Lewis et al. 2006; Ngwenya et al. 2003; Plette et al. 1995; Tourney et al. 2008).

Spectroscopic investigation offers the ability to more directly identify the ligands involved in binding metal to bacterial surfaces, and is complementary to surface complexation modeling because it provides a means of further understanding the coordination that surface complexation modeling attempts to capture (Boyavov et al. 2003; Guine et al. 2006; Kelly et al. 2002; Mishra et al. 2009, 2010; Song et al. 2012; Tourney et al. 2008, 2009; Yee et al. 2004). Although these types of investigations are critically important to our understanding of metal-bacteria surface reactions, they do not provide all the information necessary to determine the thermodynamic driving force (e.g., bond formation or dehydration) of a reaction.
under high Cd-to-biomass conditions (Boyanov et al. 2003). Our results for Cd complexing with \textit{B. licheniformis} site L2 are similar to results for Cd complexing onto \textit{B. subtilis}, which suggests a similar coordination environment. However, our results for Cd complexation onto site L3 are not consistent with results for \textit{B. subtilis}, which suggests that the coordinating ligand that deprotonates in the circumneutral pH range is different than the phosphonate found on the \textit{B. subtilis} surface.

Similar to Cd complexation with anionic oxygen ligands, Zn complexation with anionic oxygen ligands produces enthalpic to slightly exothermic enthalpies (Ashcroft and Mortimer 1970; Martell et al. 1998; Pettit and Powell 2005). Zn complexation on \textit{B. licheniformis} also produced thermodynamic parameters that are consistent with a site L2 and a site L3 of carboxyl and thiol ligands, respectively. Complexation onto site L2 produced an enthalpy and entropy of $+0.2 \pm 0.2\, \text{kJ/mol}$ and $+45 \pm 1\, \text{J/molK}$, respectively, and a $-T\Delta S$ value of $-13.4\, \text{J/molK}$. These parameters indicate the reaction is entropically driven, which is consistent with anionic oxygen complexation.

Complexation onto site L3 produced an enthalpy and entropy of $-15.5 \pm 0.2\, \text{kJ/mol}$ and $+28 \pm 1\, \text{J/molK}$, respectively, and a $-T\Delta S$ value of $-8.3\, \text{J/molK}$. These parameters indicate the reaction is enthalpically driven, which is consistent with a “borderline” metal complexed with a ligand that possesses vacant $p$ orbitals (i.e., deprotonated thiols), which act as acceptors for $d$ electrons of metal ions (Ahrland et al. 1958; Beck and Nagypal 1990; Martell et al. 1998; Martell and Hancock 1996; Pearson 1968a, 1968b; Pettit and Powell 2005).

Zn complexation on \textit{B. subtilis} produced only enthalpic enthalpies of complexation. This result is consistent with spectroscopic identification of carboxyl and phosphonate ligands on the \textit{B. subtilis} surface (Boyanov et al. 2003; Kelly et al. 2002). Enthalpies of Zn complexation onto \textit{B. subtilis} are approximately $+8\, \text{kJ/mol}$ to $+13\, \text{kJ/mol}$. Entropies of Zn complexation are approximately $+80\, \text{J/molK}$ for sites L1 and L2, and $+125\, \text{J/molK}$ for site L3. The $-T\Delta S$ values for Zn complexation on sites L1, L2, and L3 are $-23.8\, \text{kJ/mol}$, $-23.5\, \text{kJ/mol}$, and $-37.3\, \text{kJ/mol}$, respectively. The enthalpic enthalpies, along with the $T\Delta S$ values, indicate the Zn complexation reaction is enthalpically driven, which is the common mode of metal complexation with anionic oxygen ligands.

The interpretation of the results of Cd and Zn complexation on \textit{B. licheniformis} and \textit{B. subtilis} differs chiefly in the binding with surface sites that deprotonate around circumneutral pH. Coordination environments indicated by the enthalpies and entropies of complexation are distinct for anionic oxygens versus “soft” ligand donors like thiols. This work points to different ligands dominating Cd and Zn binding in the circumneutral pH range, with thiols present on \textit{B. licheniformis} and with anionic oxygen ligands present on \textit{B. subtilis}.

Detecting the participation of thiol functional groups in complexation reactions on bacterial surfaces is relatively new (Mishra et al. 2010, 2009; Song et al. 2012). Mishra et al. (2010), using EXAFS, found the presence of thiol sites on the \textit{B. subtilis} surface under low Cd-to-biomass loading. The sulphydryl signal, however, diminished under intermediate and high Cd-to-biomass loading, with carboxyl and phosphoryl functional groups taking over complexation. The authors postulated that the sulphydryl signal became masked by the carboxyl and phosphoryl binding with increased ratio of Cd-to-biomass. We performed our Zn-\textit{B. subtilis} ITC experiments under intermediate to high Zn concentrations, and our results did not suggest the presence of Zn-thiol binding on \textit{B. subtilis}.

Considering the work of Mishra et al. (2010), this suggests that Zn might follow the same complexation pattern as Cd, and that under intermediate to high Zn-to-biomass loading, carboxyl and phosphoryl surface sites are chiefly responsible for complexation. These reactions might mask any Zn-sulhydryl interactions, rendering them undetectable by ITC under these conditions. Further work under low metal-to-biomass ratios is needed to determine if high-affinity thiol sites are detectable on the \textit{B. subtilis} surface with ITC.

Decomposing Gibbs energy into its enthalpic and entropic contribution allows one to investigate subtle differences in energetics of reactions. Comparing Cd and Zn complexation onto \textit{B. licheniformis} shows that the more negative Gibbs energy of Cd complexation is due to the entropic contribution. Cd complexation onto \textit{B. licheniformis} sites L2 and L3 is more favorable than Zn complexation by approximately 4 kJ/mol. The enthalpies of Cd and Zn complexation on \textit{B. licheniformis} are within error of each other for both sites L2 and L3; however, there is a statistically significant increase in the $-T\Delta S$ values of Cd complexation of approximately 3 kJ/mol to 5 kJ/mol (we determined all statistically significant differences using an unpaired t test that produced $p$ values less than 0.05). This differs with Cd and Zn complexation onto \textit{B. subtilis} site L2 because Cd complexation is nearly thermoneutral, while Zn complexation is significantly more endothermic. The more negative Gibbs energy of Cd complexation is from the enthalpic contribution of complexation, because the entropy of Zn complexation is larger than that of Cd. These subtle differences in complexation would not be detectable with other methods and are not necessarily predicted based on simply knowing the identity of donor ligands and Gibbs energy of reaction. Because there are few full thermodynamic characterizations of metal complexation on bacterial surfaces, it is impossible to compare these differences to results with other metals or other bacterial species. More work is needed to decompose the Gibbs energy of metal complexation of other metals and bacterial species to determine if variation in contributions to Gibbs energy is predictable based on the identity of the donor ligand and metal ion.

**Conclusions**

By using a combined approach of surface complexation modeling and ITC to investigate Zn adsorption onto \textit{B. licheniformis} and \textit{B. subtilis} and Cd adsorption onto \textit{B. licheniformis}, we characterized these interactions with the full suite of thermodynamic parameters ($AG$, $\Delta H$, and $\Delta S$) that describe the complexation reactions. Enthalpies and entropies of Cd and Zn complexation on \textit{B. licheniformis} were consistent with the previously postulated existence of carboxyl and thiol surface...
sites. Enthalpies of Zn complexation on \textit{B. subtilis} were consistent with the presence of anionic oxygen ligands on the surface corresponding to carboxyl and phosphoryl functional groups. This thermodynamic data makes is possible to examine the driving force for these reactions and to interrogate the identity of the surface sites involved in complexation.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

**References**


Metal adsorption on mosses: Toward a universal adsorption model

A.G. González, O.S. Pokrovsky

Abstract

This study quantifies the adsorption of heavy metals on 4 typical moss species used for environmental monitoring in the moss bag technique. The adsorption of Cu²⁺, Cd²⁺, Ni²⁺, Pb²⁺ and Zn²⁺ onto Hypnum sp., Sphagnum sp., Pseudoscleropodium purum and Brachythecium rutabulum has been investigated using a batch reactor in a wide range of pH (1.3–11.0) and metal concentrations in solution (1.6 µM–3.8 mM). A Linear Programming Model (LPM) was applied for the experimental data to derive equilibrium constants and the number of surface binding sites. The surface acid–base titration performed for 4 mosses at a pH range of 3–10 in 0.1 M NaNO₃ demonstrated that Sphagnum sp. is the most efficient adsorbent as it has the maximal number of proton-binding sites on the surface (0.65 mmol g⁻¹). The pKᵢ computed for all the moss species suggested the presence of 5 major functional groups: phosphodiester, carboxyl, phosphoryl, amine and polyphenols. The results of pH-edge experiments demonstrated that B. rutabulum exhibits the highest percentage of metal adsorption and has the highest number of available sites for most of the metals studied. However, according to the results of the constant pH "Langmuirian" isotherm, Sphagnum sp. can be considered as the strongest adsorbent, although the relative difference from other mosses is within 20%. The LPM was found to satisfactorily fit the experimental data in the full range of the studied solution parameters. The results of this study demonstrate a rather similar pattern of five metal adsorptions on mosses, both as a function of pH and as a metal concentration, which is further corroborated by similar values of adsorption constants. Therefore, despite the species and geographic differences between the mosses, a universal adsorption edge and constant pH adsorption isotherm can be recommended for 4 studied mosses. The quantitative comparison of metal adsorption with other common natural organic and inorganic materials demonstrates that mosses are among the most efficient natural adsorbents of heavy metals.

1. Introduction

Atmospheric pollution constitutes one of the most important environmental problems of human health [1–3]. This is especially true for heavy metal pollutants that enter the food chain via plant uptake and subsequent amplification [4]. To assess the degree of atmospheric contamination by metals, bioindicators have been widely used in both urban and industrial areas. Among various bioindicators, mosses were among the first ones for tracing pollution indicators, mosses were among the first ones for tracing pollution.

Moss is a phylum of small, soft plants with around 12000 species classified as Bryophyta [24] which inhabit most of the earth. Mosses are unique in the sense that they (1) are able to store water up to 16–26 times dry weight and (2) the phenolic compounds embedded in the mosses' cell walls readily avoid moss decay [25]. Peat moss can also acidify its surroundings by taking up cations such as Ca²⁺ and Mg²⁺, and releasing H⁺. These characteristics determine the very important role of moss as the interface be-
Table 3
Experimental conditions and LPM parameters for metal adsorption on moss as a function of metal concentration in solution (Langmuir-isotherm) in 0.01 M NaNO₃ with biomass of 1.0 g dry L⁻¹. Kₘ corresponds with the equilibrium constant for the reaction between metal in solution and the available sites as a function metal aqueous concentration in solution.

<table>
<thead>
<tr>
<th>Species</th>
<th>Metal studied</th>
<th>pH-range</th>
<th>[Me⁺] M</th>
<th>pKₘ</th>
<th>Binding sites mmol g⁻¹</th>
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</thead>
<tbody>
<tr>
<td>Hypnum sp.</td>
<td>Copper</td>
<td>5.50 ± 0.04</td>
<td>5.19 10⁻⁵–3.65 10⁻⁴</td>
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<td>0.430</td>
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<td>Sphagnum sp.</td>
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<td>Pseudoscleropodium purum</td>
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<td>1.57 10⁻⁵–3.78 10⁻⁴</td>
<td>0.75</td>
<td>0.104</td>
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<td>Brachytecium rutabulum</td>
<td>5.53 ± 0.04</td>
<td>1.93 10⁻⁵–2.85 10⁻⁴</td>
<td>1.45</td>
<td>2.20</td>
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<tr>
<td>Hypnum sp.</td>
<td>Cadmium</td>
<td>5.62 ± 0.04</td>
<td>2.31 10⁻⁶–1.41 10⁻⁴</td>
<td>2.55</td>
<td>0.172</td>
</tr>
<tr>
<td>Sphagnum sp.</td>
<td>Zinc</td>
<td>6.52 ± 0.05</td>
<td>2.31 10⁻⁶–1.34 10⁻⁴</td>
<td>1.45</td>
<td>0.029</td>
</tr>
<tr>
<td>Pseudoscleropodium purum</td>
<td>6.52 ± 0.05</td>
<td>2.31 10⁻⁶–1.47 10⁻⁴</td>
<td>3.35</td>
<td>0.591</td>
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<tr>
<td>Brachytecium rutabulum</td>
<td>6.62 ± 0.08</td>
<td>3.11 10⁻⁶–2.13 10⁻³</td>
<td>2.45</td>
<td>0.114</td>
<td></td>
</tr>
<tr>
<td>Hypnum sp.</td>
<td>Nickel</td>
<td>5.63 ± 0.03</td>
<td>9.37 10⁻²–2.89 10⁻³</td>
<td>1.25</td>
<td>0.073</td>
</tr>
<tr>
<td>Sphagnum sp.</td>
<td>Nickel</td>
<td>5.65 ± 0.05</td>
<td>9.88 10⁻³–3.17 10⁻³</td>
<td>1.25</td>
<td>0.081</td>
</tr>
<tr>
<td>Pseudoscleropodium purum</td>
<td>5.67 ± 0.03</td>
<td>9.08 10⁻²–2.41 10⁻³</td>
<td>1.35</td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>Brachytecium rutabulum</td>
<td>5.58 ± 0.04</td>
<td>9.37 10⁻²–2.91 10⁻³</td>
<td>5.70</td>
<td>7.669</td>
<td></td>
</tr>
<tr>
<td>Hypnum sp.</td>
<td>Lead</td>
<td>6.55 ± 0.07</td>
<td>1.93 10⁻⁵–9.93 10⁻⁴</td>
<td>2.95</td>
<td>0.799</td>
</tr>
<tr>
<td>Sphagnum sp.</td>
<td>Lead</td>
<td>6.52 ± 0.05</td>
<td>2.03 10⁻⁵–1.04 10⁻³</td>
<td>2.05</td>
<td>0.137</td>
</tr>
<tr>
<td>Pseudoscleropodium purum</td>
<td>6.55 ± 0.06</td>
<td>1.98 10⁻¹–1.03 10⁻³</td>
<td>2.80</td>
<td>0.334</td>
<td></td>
</tr>
<tr>
<td>Brachytecium rutabulum</td>
<td>6.53 ± 0.05</td>
<td>2.03 10⁻⁶–6.97 10⁻⁴</td>
<td>3.10</td>
<td>1.117</td>
<td></td>
</tr>
<tr>
<td>Hypnum sp.</td>
<td>Zinc</td>
<td>6.76 ± 0.05</td>
<td>7.19 10⁻⁵–2.83 10⁻⁴</td>
<td>2.15</td>
<td>0.084</td>
</tr>
<tr>
<td>Sphagnum sp.</td>
<td>Zinc</td>
<td>6.79 ± 0.08</td>
<td>7.34 10⁻⁵–2.88 10⁻³</td>
<td>2.05</td>
<td>0.059</td>
</tr>
<tr>
<td>Pseudoscleropodium purum</td>
<td>6.77 ± 0.05</td>
<td>7.19 10⁻⁵–2.85 10⁻³</td>
<td>2.25</td>
<td>0.095</td>
<td></td>
</tr>
<tr>
<td>Brachytecium rutabulum</td>
<td>6.78 ± 0.06</td>
<td>7.04 10⁻⁵–2.78 10⁻³</td>
<td>2.40</td>
<td>0.116</td>
<td></td>
</tr>
</tbody>
</table>

has a longer time to bind metals and increases the concentration of several metals inside the cells or cell walls [53]. Eventually, the difference in composition can also be explained by a different capacity to cation exchange, because of the differences in the chemical composition of the membranes and cell walls [54].

The concentrations of released metals during moss interaction with aqueous solution were significantly lower than those used in adsorption experiments. During the 9 h of solution exposure experiments, Sphagnum sp. and Hypnum sp. proved to be the most inert species in terms of both DOC and metal release, which is certainly linked to the specificity of their cell wall chemical composition as described below.

4.2. Acid–base properties of mosses

The amphoteric properties of the moss stem from acid–base dissociation of protonated organic moieties on the surface of the cell wall. The acid–base titration showed that Sphagnum sp. exhibits the highest excess of negative charges corresponding to its highest capacity for metal adsorption. The acid–base titration of 4 mosses demonstrated a certain variability of pKₘ among mosses likely linked to the different compositions of their cell walls. In this study, Hypnum sp., Sphagnum sp., P. purum and B. rutabulum showed pKₘ values ~4, 4.5–5.75, ~6–7.35, 8–9.15 and ~10. These pKₘ can be tentatively related with carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenol functional groups. Sphagnum sp. contains the highest amount of total binding sites, followed by P. purum (15% smaller), Hypnum sp. (25% smaller) and B. rutabulum (25% smaller). It is important to note that Sphagnum sp. exhibits the dominance of carboxyl, phosphoryl, amine functional groups, the main metal–binding moieties on the biological surfaces [19,48]. As such, Sphagnum sp. is the most efficient metal adsorbent given the carboxyl and phosphoryl groups are the primary metal–binding groups at a high concentration of metals [55,56] whereas the sulphhydryl and amine groups can be determinant especially under extreme pH conditions and low metal concentrations [57]. The pKₘ computed reported for different microorganisms are around ~ 3, 4–5, 6–7 and 9–10 with total binding sites around 0.044–0.113 mmol g⁻¹ of bacteria [39,48,57,58]. The relative percentage of functional groups for the different microorganisms inferred from surface titration (Fig. 6) showed that mosses possess a relatively higher percentage of carboxyl/phosphodiester, amine/polyphenol groups compared to bacteria [48,59] and cyanobacteria [43,60], whereas the number of carboxyl groups on mosses is smaller compared to bacteria. Lignin and cellulose represent the main organic composition of the mosses cell walls [61]. These polysaccharides contain alcohols, aldehydes, ketones, acids, phenolic and hydroxides as the main functional groups. Accordingly, the carboxylic and phenolic groups have been suggested to be responsible for the adsorption of metals on peat moss [62], similar to humic and fulvic acids [63].
References

Iron adsorption onto soil and aquatic bacteria: XAS structural study

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A B S T R A C T

Although the interaction between Fe and microorganisms has been extensively studied, the main physico-chemical factors controlling the mechanisms of Fe adsorption and precipitation on bacterial cell walls remain poorly understood. In this study, we quantified thermodynamic parameters of the Fe adsorption reaction and characterized the speciation of Fe adsorbed on the surface of cyanobacteria and soil heterotrophic bacteria. For this purpose, the molecular mechanisms of iron interaction with typical aquatic and soil bacteria were investigated by combining batch macroscopic adsorption experiments with atomic-level Fe K-edge X-ray absorption fine structure spectroscopy (XAFS). Three cyanobacteria species (Synechococcus sp., Planktothrix sp. and Gloeocapsa sp.) and aerobic heterotrophic soil rhizobacterium (Pseudomonas aureofaciens) were used for Fe3+ and Fe2+ adsorption experiments. These experiments were carried out for a wide range of initial iron concentration (4.5–57.3 μM) and pH (2.0–6.5). Surface adsorption data were rationalized using a Linear Programming Model (LPM), which allowed quantification of the surface adsorption constants and the number of binding sites. XAS (XANES and EXAFS) analysis of adsorbed iron demonstrated the predominance of O-coordinated Fe3+ species. Moreover, XANES data treatment using a linear combination fit of reference compounds suggested that the atomic environment of iron adsorbed onto soil bacterial surfaces was dominated by phosphoryl moieties with a lesser amount of carboxylates and some contribution of Fe(III)-oxy(hydr)oxide component. Complete oxidation of Fe(II) to Fe(III) was observed in the solid phase as determined by XANES analysis. Binding of Fe(III) to carboxylate groups was only significant for capsular cyanobacteria (Gloeocapsa sp.). The relative proportions of various Fe species at the cell surface determined by thermodynamic analysis of the macroscopic data and by XAS are in a good agreement. Our results suggest that, in the presence of surface organic ligands, the oxidation of divalent iron does occur, but the polymerization of formed Fe(III)oxy(hydr)oxides is partially inhibited and adsorbed iron in the form of both Fe–O–Fe polymers and individual Fe atoms attached to phosphoryl moieties. The presence of EPS reduces metal-cell binding capacity and enhances Fe polymerization at the bacterial surface.

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1. Introduction

Iron is one of the most abundant and biologically important metals and micronutrients in terrestrial and aquatic environments (Sunda and Huntaman, 1997; Maldonado and Price, 2001). The geochemical cycling of iron is known to control the primary productivity in natural waters. The major fraction of iron in aqueous solutions is represented by Fe(II), the most stable form of iron in oxygenated waters. Dissolved trivalent iron is present in solution in the form of organic and hydroxocomplexes (Gledhill and van den Berg, 1994; Donat and Bruoland, 1995; Wu and Luther, 1995) as well as in the form of organo-ferric colloids (de Baar and de Jong, 2001; Hassellöv and von der Kammer, 2008).

Microorganisms play a key role in the geochemical cycling of iron because they can serve as nucleation centres for iron adsorption and precipitation (Daughney et al., 2004). The mobility, reactivity and bioavailability of iron in aqueous solutions are affected by the adsorption and uptake processes at the interface between the microorganism and the aqueous solution (Geesey et al., 1977; Harvey et al., 1982; Mahmoon and Rao, 1993; Corapcioglu and Kim, 1995). Microorganisms have a high capacity for the adsorption of metal onto their cell walls (Beveridge and Murray, 1980; Gonçalves et al., 1987; Beveridge, 1989; Ledin et al., 1996; Barns and Nierzwicki-Bauer, 1997; Fein et al., 1997; Ledin et al., 1999; Wu et al., 2006) through a number of proton- and


THERMODYNAMIC MODELING OF U(VI) ADSORPTION ONTO BACTERIA: IMPLICATIONS FOR QUANTIFYING BIOAVAILABILITY RELATIONSHIPS

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

by

Ling Sheng

______________________________
Jeremy B. Fein, Director

Graduate Program in Civil Engineering and Geological Sciences
Notre Dame, Indiana

April 2013
2.4.2 Effect of Ca on the Rate of U(VI) Reduction

2.4.2.1 Quantifying U(VI) adsorption onto \textit{S. oneidensis}

We use our U(VI) adsorption measurements to determine the speciation of U(VI) on \textit{S. oneidensis} in order to determine if a relationship exists between cell wall uranyl speciation and U(VI) reduction rates in the U(VI) reduction experiments with Ca. We follow the modeling approach described by Gorman-Lewis et al. (2005) who measured U(VI) adsorption onto \textit{Bacillus subtilis} and used the results to determine the stability constants for the important U(VI)-bacterial surface complexes. Metal adsorption measurements conducted as a function of pH constrain the number of sites involved in metal binding, the pH range of influence, and the stability constants for the important metal-bacterial surface complexes. We used the program FITEQL (Herbelin and Westall, 1994) for the equilibrium thermodynamic modeling of the U adsorption data, accounting for aqueous speciation using reactions 14 to 33 listed in Appendix A. Activity coefficients for ions were calculated within FITEQL using the Davies equation. A discrete $pK_a$ 4-site non-electrostatic model was used to model the protonation behavior of the \textit{S. oneidensis} cell wall functional groups (Mishra et al., 2010). We refer to Sites 1-4 as the sites with $pK_a$ values of 3.3 ± 0.2, 4.8 ± 0.2, 6.7 ± 0.4, and 9.4 ± 0.5, respectively. The bacterial site density for each site was calculated according to the site densities of \textit{S. oneidensis} described by Mishra et al. (2010), which are $8.9(\pm2.6) \times 10^{-5}$, $1.3(\pm0.2) \times 10^{-4}$, $5.9(\pm3.3) \times 10^{-5}$ and $1.1(\pm0.6) \times 10^{-4}$ mol per gram of wet mass for sites 1-4, respectively.
determining the range of K values that account for the observed range of experimental values for the extent of adsorption. The average K values for Reactions 1-3 were used to generate the model curves that are plotted in Figure 2.3. The figure demonstrates that the set of average K values can account for the data well both as a function of pH and as a function of bacterial concentration.

<table>
<thead>
<tr>
<th></th>
<th>Log K (S. oneidensis)</th>
<th>Log K(b) (B. subtilis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\text{UO}_2^{2+} + R-L^2=(a) = [R-L2\cdot\text{UO}_2]^+$</td>
<td>$6.2 \pm 0.2$</td>
</tr>
<tr>
<td>2</td>
<td>$\text{UO}_2\text{CO}_3^0 + R-L^2=(a) = [R-L2\cdot\text{UO}_2\text{CO}_3]^-$</td>
<td>$6.9 \pm 0.3$</td>
</tr>
<tr>
<td>3</td>
<td>$\text{UO}_2(\text{CO}_3)_3^{4-} + R-L^3=(a) = [R-L3\cdot\text{UO}_2(\text{CO}_3)_3]^{5-}$</td>
<td>$7.3 \pm 0.2$</td>
</tr>
</tbody>
</table>

(a) R-L# represents S. oneidensis functional groups, Sites 1-4, with pK$_a$ values of $3.3 \pm 0.2$, $4.8 \pm 0.2$, $6.7 \pm 0.4$, and $9.4 \pm 0.5$, respectively (Mishra et al., 2010).
(b) Gorman-Lewis et al. (2005).
of the data for each Ca concentration in Figure 2.2b. The calculations of the U(VI) speciation in the systems of the U(VI) reduction experiments with Ca account for aqueous uranyl-hydroxide, -carbonate, -lactate, –acetate, and –bacterial surface complexation using the reactions and stability constants listed in Table 2.1 and Appendix A. The system was also constrained with mass balance constraints on dissolved carbonate, lactate, acetate, bacterial sites and U(VI) concentrations. Bacterial concentrations in the experiments were determined from the experimental suspensions, yielding an average experimental cell density of 3.2(±0.6) × 10^7 cells/mL. This cell density was transformed into the wet mass density by dividing by a conversion factor of 1.9(±0.6) × 10^{10} cells/g, which was determined based on our cell mass-cell counts transformation experiments, in which cells of specific wet mass were counted by direct cell counting method after suspended in specific volume of solution. Bacterial site concentrations were calculated using *S. oneidensis* site densities reported by Mishra et al. (2010). The calculated total binding site concentration was 0.61 mM for the Ca experiments. The U(VI) speciation in the system without Ca was calculated in a similar way to the procedures described above, but excluding all Ca-bearing reactions in Appendix A. The modeling results of the U(VI) reduction experiments with Ca indicate the presence and the concentrations of the important uranyl surface complexes, which are shown in Table 2.2.
TABLE 2.2
CALCULATED URANYL SURFACE COMPLEXES FORMED IN THE CA EXPERIMENTS

<table>
<thead>
<tr>
<th>[Ca] (mM)</th>
<th>[R-L2-UO₂]⁺</th>
<th>[R-L2-UO₂CO₃]⁻(a)</th>
<th>[R-L3-UO₂(CO₃)₂]⁻(a)</th>
<th>[R-L2-Ca₂UO₂(CO₃)₃]⁻(a)</th>
<th>Total Adsorbed U(VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.1 x 10⁻⁷</td>
<td>1.3 x 10⁻¹</td>
<td>1.0 x 10⁻¹</td>
<td>NONE</td>
<td>2.3 x 10⁻¹</td>
</tr>
<tr>
<td>0.5</td>
<td>1.2 x 10⁻⁷</td>
<td>5.6 x 10⁻²</td>
<td>1.0 x 10⁻¹</td>
<td>6.6 x 10⁻²</td>
<td>2.2 x 10⁻¹</td>
</tr>
<tr>
<td>1.5</td>
<td>1.3 x 10⁻⁸</td>
<td>6.0 x 10⁻³</td>
<td>9.7 x 10⁻²</td>
<td>1.6 x 10⁻¹</td>
<td>2.7 x 10⁻¹</td>
</tr>
<tr>
<td>2.5</td>
<td>4.6 x 10⁻⁹</td>
<td>2.0 x 10⁻³</td>
<td>8.8 x 10⁻²</td>
<td>1.8 x 10⁻¹</td>
<td>2.7 x 10⁻¹</td>
</tr>
<tr>
<td>5.0</td>
<td>1.2 x 10⁻⁹</td>
<td>4.8 x 10⁻⁴</td>
<td>6.3 x 10⁻²</td>
<td>2.0 x 10⁻¹</td>
<td>2.6 x 10⁻¹</td>
</tr>
</tbody>
</table>

(a) R-L#- represents *S. oneidensis* functional groups, Sites 1-4, with pKₐ values of 3.3 ± 0.2, 4.8 ± 0.2, 6.7 ± 0.4, and 9.4 ± 0.5, respectively (Mishra et al., 2010).
3.3.2 Thermodynamic Modeling

We use a thermodynamic surface complexation modeling approach to model the adsorption of U(VI) onto the cell surface of *S. oneidensis*. In this approach, U(VI) adsorption onto bacteria is modeled as interactions between a range of adsorbing aqueous uranyl species and specific sites on the bacterial cell wall. The adsorption reactions are modeled to involve discrete, negatively charged deprotonated sites, with the deprotonation reactions of the bacterial cell wall functional groups written as:

\[
R-L_n-H^0 \leftrightarrow R-L_n^- + H^+ \quad (2)
\]

where \( R \) is the bacterial cell wall macromolecule to which the functional groups are attached, and \( L_n \) represents a particular functional group type that is present on the bacterial cell wall. We use a non-electrostatic model (Fein et al., 2005), so the mass action equation for reaction (2) is expressed as:

\[
K_a = \frac{[R-L_n^-]a_{H^+}}{[R-L_n-H^0]} \quad (3)
\]

where \([R-L_n^-]\) and \([R-L_n-H^0]\) represent the concentration of deprotonated and protonated functional groups of each bacterial cell wall species in moles per liter of solution, respectively, and \( a_{H^+} \) represents the activity of \( H^+ \) in solution. We use the 4-site model of Mishra et al. (2010) to characterize the proton-active binding sites on *S. oneidensis*, with Sites 1-4 exhibiting \( pK_a \) values of 3.3 ± 0.2, 4.8 ± 0.2, 6.7 ± 0.4, and 9.4 ± 0.5, respectively. The corresponding site densities for Sites 1-4 are 8.9(±2.6) \times 10^{-5}, 1.3(±0.2) \times 10^{-4}, 5.9(±3.3) \times 10^{-5} and 1.1(±0.6) \times 10^{-4} moles per gram of wet mass, respectively.
TABLE 3.1
CALCULATED LOG K VALUES FOR URANYL SURFACE COMPLEXES FORMED ON THE CELL WALL OF S. ONEIDENSIS MR-1.

![Table with calculated log K values for uranyl surface complexes](image)

* Calculation of Log K value was not conducted for this dataset (see text).

(a) R-L\textsubscript{f} represents S. oneidensis MR-1 functional groups, Sites 1-4, with pH\textsubscript{a} values of 3.3 ± 0.2, 4.8 ± 0.2, 6.7 ± 0.4, and 9.4 ± 0.5, respectively (Mishra et al., 2010).
TABLE 4.1
CALCULATED URANYL SURFACE COMPLEXES AND AVERAGE REDUCTION RATE FOR EACH NAHCO₃ CONCENTRATION.

<table>
<thead>
<tr>
<th>[NaHCO₃] (mM)</th>
<th>[R-L⁻(^{(a)})]((\text{UO}_2\text{CO}_3\text{(OH)}_3))</th>
<th>[R-L¹⁻(^{(a)})]((\text{UO}_2\text{CO}_3))</th>
<th>Total Adsorbed U(VI) (^{(b)}) (mM)</th>
<th>Average Initial Reduction Rate (mM/h) ± 1σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>0.045</td>
<td>0.023</td>
<td>0.113</td>
<td>0.157 ± 0.014</td>
</tr>
<tr>
<td>5.0</td>
<td>0.024</td>
<td>0.022</td>
<td>0.071</td>
<td>0.104 ± 0.002</td>
</tr>
<tr>
<td>7.2</td>
<td>0.010</td>
<td>0.017</td>
<td>0.038</td>
<td>0.092 ± 0.010</td>
</tr>
<tr>
<td>11.9</td>
<td>0.001</td>
<td>0.009</td>
<td>0.011</td>
<td>0.073 ± 0.004</td>
</tr>
<tr>
<td>21.0</td>
<td>0.000</td>
<td>0.007</td>
<td>0.007</td>
<td>0.022 ± 0.005</td>
</tr>
<tr>
<td>30.0</td>
<td>0.000</td>
<td>0.001</td>
<td>0.001</td>
<td>0.027 ± 0.006</td>
</tr>
</tbody>
</table>

\(^{(a)}\) R-L#- represents \textit{S. oneidensis} functional groups, Sites 1 - 4, with pKₐ values of 3.3 ± 0.2, 4.8 ± 0.2, 6.7 ± 0.4, and 9.4 ± 0.5, respectively (Mishra et al., 2010).

\(^{(b)}\) Total adsorbed U(VI) includes all the uranyl surface complexes formed on the cell wall: [R-L¹⁻\((\text{UO}_2\text{CO}_3\text{(OH)}_3)\)\(^{1+}\)], [R-L¹⁻\((\text{UO}_2\text{CO}_3\text{(OH)}_4)\)\(^{1+}\)], [R-L¹⁻\((\text{UO}_2\text{CO}_3\text{(OH)}_5)\)\(^{1+}\)], [R-L¹⁻\((\text{UO}_2\text{CO}_3\text{(OH)}_6)\)\(^{1+}\)], [R-L¹⁻\((\text{UO}_2\text{CO}_3\text{(OH)}_7)\)\(^{1+}\)], [R-L²⁻\((\text{UO}_2\text{CO}_3\text{(OH)}_3)\)\(^{3+}\)], [R-L¹⁻\((\text{UO}_2\text{CO}_3)\)\(^{1+}\)] (Sheng and Fein, 2012).
**TABLE A.1**
AQUEOUS AND SURFACE SPECIATION REACTIONS USED IN THERMODYNAMIC MODELING.

<table>
<thead>
<tr>
<th>Reaction Description</th>
<th>( \text{Log } K \text{ (I=0)} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uranyl Carbonate Aqueous Complexation Reactions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 ( \text{UO}_2^{2+} + \text{CO}_3^{2-} = \text{UO}_2\text{CO}_3^{(aq)} )</td>
<td>9.94</td>
<td>(g)</td>
</tr>
<tr>
<td>25 ( \text{UO}_2^{2+} + 2\text{CO}_3^{2-} = \text{UO}_2(\text{CO}_3)_2^{2-} )</td>
<td>16.61</td>
<td>(g)</td>
</tr>
<tr>
<td>26 ( \text{UO}_2^{2+} + 3\text{CO}_3^{2-} = \text{UO}_2(\text{CO}_3)_3^{4-} )</td>
<td>21.84</td>
<td>(g)</td>
</tr>
<tr>
<td>27 ( 3\text{UO}_2^{2+} + 6\text{CO}_3^{2-} = \text{(UO}_2)_3(\text{CO}_3)_6^{6-} )</td>
<td>54.00</td>
<td>(g)</td>
</tr>
<tr>
<td>28 ( 2\text{UO}_2^{2+} + 3\text{H}_2\text{O} + \text{CO}_3^{2-} = (\text{UO}_2)_2\text{CO}_3(\text{OH})_3 + 3\text{H}^+ )</td>
<td>-0.86</td>
<td>(g)</td>
</tr>
<tr>
<td>29 ( 3\text{UO}_2^{2+} + 3\text{H}_2\text{O} + \text{CO}_3^{2-} = (\text{UO}_2)_3\text{CO}_3(\text{OH})_3^+ + 3\text{H}^+ )</td>
<td>0.65</td>
<td>(g)</td>
</tr>
<tr>
<td>30 ( 11\text{UO}_2^{2+} + 12\text{H}_2\text{O} + 6\text{CO}_3^{2-} = (\text{UO}<em>2)</em>{11}(\text{CO}_3)<em>6(\text{OH})</em>{12}^{2-} + 12\text{H}^+ )</td>
<td>36.41</td>
<td>(g)</td>
</tr>
<tr>
<td><strong>Other Reactions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31 ( \text{H}_2\text{O} = \text{H}^+ + \text{OH}^- )</td>
<td>-14.00</td>
<td>(d)</td>
</tr>
<tr>
<td>32 ( \text{H}_2\text{CO}_3 = \text{H}^+ + \text{HCO}_3^- )</td>
<td>-6.35</td>
<td>(d)</td>
</tr>
<tr>
<td>33 ( \text{H}_2\text{CO}_3 = 2\text{H}^+ + \text{HCO}_3^- )</td>
<td>-16.68</td>
<td>(d)</td>
</tr>
</tbody>
</table>

(a) R-L#- represents different \textit{S. oneidensis} funtional groups, Sites 1- 4, with pKa values of 3.3±0.2, 4.8±0.2, 6.7±0.4, and 9.4±0.5, respectively (Mishra et al., 2010).

(b) Gorman-Lewis et al., (2005).

(c) Dong and Brooks, (2006).

(d) Martell and Smith, (2001).

(e) Martell and Smith, (1977).

(f) The K values of these reactions have only been determined at ionic strength (I) = 1 (Martell and Smith, 1977). The K values were extrapolated to infinite dilution (I=0) here using the Davies equation.

(g) Guillaumont et al., (2003).
APPENDIX D:

URANYL SURFACE COMPLEXATION REACTIONS FOR *S. oneidensis* MR-1.

**TABLE D.1**

URANYL SURFACE COMPLEXATION REACTIONS FOR *S. oneidensis* MR-1.

<table>
<thead>
<tr>
<th>Uranyl Surface Complexation Reactions</th>
<th>Log K ± 2σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 $\text{UO}_2^{2+} + \text{R-L}_1^{-} \leftrightarrow [\text{R-L}_1^{-}\text{UO}_2]^+$</td>
<td>$4.5 \pm 0.5$ (b)</td>
</tr>
<tr>
<td>2 $(\text{UO}_2)_3(\text{OH})_5^+ + \text{R-L}_1^{-} \leftrightarrow [\text{R-L}_1^{-}(\text{UO}_2)_3(\text{OH})_5]^0$</td>
<td>$4.4 \pm 0.5$ (b)</td>
</tr>
<tr>
<td>3 $(\text{UO}_2)_4(\text{OH})_7^+ + \text{R-L}_1^{-} \leftrightarrow [\text{R-L}_1^{-}(\text{UO}_2)_4(\text{OH})_7]^0$</td>
<td>$4.9 \pm 0.4$ (b)</td>
</tr>
<tr>
<td>4 $(\text{UO}_2)_3(\text{OH})_4^- + \text{R-L}_1^{-} \leftrightarrow [\text{R-L}_1^{-}(\text{UO}_2)_3(\text{OH})_4]^{2-}$</td>
<td>$8.1 \pm 1.2$ (b)</td>
</tr>
<tr>
<td>5 $(\text{UO}_2)_2\text{CO}_3(\text{OH})_3^- + \text{R-L}_2^{-} \leftrightarrow [\text{R-L}_2^{-}(\text{UO}_2)_2\text{CO}_3(\text{OH})_3]^{2-}$</td>
<td>$4.1 \pm 0.6$ (b)</td>
</tr>
<tr>
<td>6 $\text{UO}_2\text{CO}_3^0 + \text{R-L}_1^{-} \leftrightarrow [\text{R-L}_1^{-}\text{UO}_2\text{CO}_3]$</td>
<td>$4.9 \pm 0.5$ (b)</td>
</tr>
</tbody>
</table>

(a) R-L#- represents *S. oneidensis* MR-1 functional groups, Sites 1-4, with pKₐ values of 3.3 ± 0.2, 4.8 ± 0.2, 6.7 ± 0.4, and 9.4 ± 0.5, respectively (Mishra et al., 2010).

(b) Sheng and Fein, (2012).


Application of the Surface Complexation Model to the Biosorption of Cu(II) and Pb(II) ions onto *Pseudomonas pseudoalcaligenes* Biomass

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ABSTRACT: In this study, a potentiometric titration was performed to investigate the surface acid–base properties of *Pseudomonas pseudoalcaligenes* isolated from activated sludge. Batch sorption as a function of pH was performed to explain the sorption behaviour of Cu(II) and Pb(II) ions onto the bacterial surface. The surface complexation approach in the frame of constant capacitance model was applied to determine the deprotonation constants, site concentrations and metal stability constants for the important surface-functional groups on the biomass. The optimized results showed that the three discrete sites–three pKas model, involving three distinct types of functional groups, namely, carboxyl, phosphate and hydroxyl, could actually fit the protonation of the bacterial functional groups, with average pKₐ values of 4.18, 6.31 and 9.18, and site concentrations of 0.526, 0.525 and 0.478 mmol/g, respectively. The two sites model provided the best fit for the biosorption of Cu(II) and Pb(II) ions onto the biomass by forming complexes with carboxyl and phosphate surface sites, with average log stability constants of 5.95 and 6.94 for Cu(II), and 6.64 and 8.08 for Pb(II), respectively. These results suggested that the surface complexation model yielded the accurate prediction for the biosorption behaviour of Cu(II) and Pb(II) ions on the bacterial biomass and the affinity of *P. pseudoalcaligenes* biomass for the adsorption of Cu(II) and Pb(II) ions was high enough to remove the metal ions from water.

1. INTRODUCTION

Heavy metal contamination is an important environmental issue because of its toxic effects, poor degradability and bioaccumulation of heavy metals (Naja and Volesky 2009; Srivastava and Goyal 2010). Metal speciation in both natural water and industrial wastewater is critical for predicting the mobility of ions and their impact on eco-environmental systems. As important metal-complexing agents, bacteria exhibit a high affinity towards metals owing to a variety of surface organic functional groups present on their cell walls.

It is a well-known fact that bacterial cell walls are mainly composed of polysaccharides, proteins and lipids that contain distinct surface organic functional groups such as amino, carboxylic, phosphate, hydroxyl sites (van der Wal *et al.* 1997; Pan *et al.* 2006, 2007). The main binding mechanisms of metal ions by different types of biomass involve complex formation, ion

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exchange, electrostatic attraction and inorganic micro-precipitation (Akthar et al. 1996; Bueno et al. 2008; Amirnia et al. 2012). Several studies have addressed the development of suitable mechanistic models to describe these surface reactions and explain biosorption (Fein et al. 1997; Yee and Fein 2001; Borrok et al. 2004a; Ginn and Fein 2008; Pabst et al. 2010; Kenney and Fein 2011; Moon and Peacock 2011; Amirnia et al. 2012). The empirical approaches based on distribution coefficients or semi-empirical Langmuir or Freundlich isotherms have been often used to describe metal sorption behaviour between solutions and solid substrates. Although these provide a direct measurement of sorption ability, the empirical or semi-empirical approach, depending on solution and substrate composition, cannot be applied outside of the specific parameters. Furthermore, the distribution approach does not consider the mass balance value, which misleads the prediction of metal speciation and mobility, and Langmuir or Freundlich isotherms does not account for the development of electrical charge at solid surfaces and the structure of adsorbed species (Koretsky 2000; Bethke and Brady 2002). In contrast, thermodynamically based surface complexation models (SCMs) provide explicit molecular descriptions of metal sorption using an equilibrium approach that defines surface species, chemical reactions, mass balances and charge balances. These models have a significant advantage over empirical or semi-empirical models; for example, once calibrated, they provide an accurate prediction of metal speciation under varying solution compositions (e.g. ionic strength, background electrolyte, competing ions).

Based on a set of molecular-scale thermodynamic reactions, SCMs have effectively been described to bind protons and metal ions onto various bacterial surfaces, including Bacillus subtilis (Fein et al. 1997; Fein and Delea 1999; Ginn and Fein 2009; Mishra et al. 2010; Moon and Peacock 2011), Bacillus licheniformis (Daughney and Fein 1998; Fowle et al. 2000) and Arthrobacter sp. (Pagnanelli et al. 2000). In addition, a number of investigations have reported similarities between bacterial species in their deprotonation and metal sorption behaviour. Yee and Fein (2001) suggested that a wide range of bacteria with Gram-positive and Gram-negative species exhibited similar proton- and Cd-binding behaviour and that a single set of stability constants of proton– and Cd–bacterial complexes was obtained using SCMs. By testing proton and Cd binding to the eight different consortia of bacteria grown from uncontaminated soil and water systems, Borrok et al. (2004b) observed similar affinities for proton and Cd towards all of the consortia, and the sorption behaviour was successfully modelled using a single set of stability constants. The universal bacterial sorption behaviour has also been supported by subsequent investigations with much larger range of bacteria grown from three natural water and soil locations (Johnson et al. 2007), as well as under extreme conditions including low and high pH values for Cd sorption (Kenney and Fein 2011). Although a number of bacterial species with large diversity exhibited broadly similar sorption behaviour, there were some indications that not all bacteria displayed the similar metal sorption behaviour. For example, the acidophilic species Acidiphilium angustum showed anomalous sorption behaviour to Pb and Cd, suggesting that the habitat of a species might significantly affect the cell wall functional group chemistry (Ginn and Fein 2008). The bacterial consortia from contaminated sites demonstrated significant enhancement in Cd sorption capacities compared with those grown from uncontaminated environment (Borrok et al. 2004c). An investigation of an archaea cell, Thermococcus zilligii, binding metals showed significantly less Cd adsorption capability than most bacteria (Daughney et al. 2010).

Therefore, more experimental evidences are still needed to supplement our present knowledge of metal sorption properties onto the bacterial biomass from diverse locations. A primary study indicated that Pseudomonas pseudoalcaligenes isolated from activated sludge exhibited a maximum adsorption capacity of 26 and 232 mg/g dry cells at pH 5 for Cu(II) and Pb(II) ions,
REFERENCES

CaCO₃ biomineralization on cyanobacterial surfaces: Insights from experiments with three Synechococcus strains

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A B S T R A C T

In the present paper, the impact of freshwater (ARC21 and LS0519) and marine (PCC8806) Synechococcus cyanobacteria on calcium carbonate (CaCO₃) precipitation has been examined in respect of the formation rates and morphology of crystals. Acid–base potentiometric titrations were employed to study surface functional groups, while CaCO₃ experiments have been carried out in presence and absence of cells at low to near-equilibrium conditions in respect to CaCO₃. During these experiments, the pH values have been monitored. Ca and alkalinity were measured and precipitates have been investigated by Raman spectroscopy and Atomic Force and Scanning Electron microscopy.

Our results showed that the Synechococcus strains exhibited different surface reactivity with total concentration of surface functional groups of 0.342 and 0.350 mM g⁻¹ of dry bact. for freshwater strains, and 0.662 mM g⁻¹ of dry bact. for the marine strain, which are on the same order of magnitude as that reported for bacterial cell surfaces. The marine strain showed the highest CaCO₃ formation rate with Ca²⁺ removal of 18 mM g⁻¹ dry bact. compared to 6–7 mM g⁻¹ dry bact. for freshwater strains. The morphological diversity in crystals has been linked to presence of specific functional groups. The linking cell surface properties to crystal morphologies and precipitation rates propose that bacterial surfaces may modulate CaCO₃ formation. Results of this work should allow better understanding of biomineralization in marine and freshwater systems as they define the precipitation rates in typical range of pH necessary for estimation of CaCO₃ formation by cyanobacterial communities.

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1. Introduction

Despite being the most abundant biologically formed mineral, CaCO₃ remains the focus of intensive research [1], and we are still far from understanding the natural biomineralization process of CaCO₃ in marine and freshwater systems [2,3]. Due to metabolic activities, microorganisms change the chemistry of their environment, thus inducing mineral formation. On the other hand, a large number of organic templates such as proteins and other biomolecules have been identified to control CaCO₃ formation. These macromolecules are present in microbial cell envelopes and many of them are highly negatively charged, so they can control crystal nucleation. Our understanding of the effect of microbial cell surface properties on CaCO₃ formation is still limited. So far, research has focused either on the role of different biomolecules [4], the role metabolic activity [5] or on the characterization of cell surface properties [6]. It remains unclear though whether there is a link between cell surface properties and crystal morphology.

Autophototrophic picocyanobacteria genera Synechococcus abundant in marine and freshwater environments, were found to be associated with deposition of CaCO₃ minerals [7,8]. While there is evidence that picocyanobacteria play a crucial role in CaCO₃ formation, the underlying mechanisms are a matter of controversial debates [9,10]. One hypothesis suggests that photosynthetic activity of bacterial cell leads to CaCO₃ formation by CO₂ uptake that influence calcium–carbonate equilibrium in the surrounding microenvironment, and thus shifts the chemical equilibrium in favor to CaCO₃ precipitation [7,11]. An alternative mechanism of CaCO₃ formation is that cell surfaces serve as a template for CaCO₃ nucleation, and crystal growth is possible due to oversaturation in respect of CaCO₃ in vicinity of cells [9]. Picocyanobacterial cells have a highly reactive surface due to their large surface area-to-volume ratio [3,12] that can be an ideal template for crystal nucleation [13]. The negatively charged functional groups at the cell surfaces can bind cations (i.e. Ca²⁺) to promote nucleation process and subsequently lead to mineral formation [9,14].

The proposed mechanism of CaCO₃ biomineralization suggests a formation of amorphous calcium carbonate (ACC) through aggregation of stable, amorphous, pre-critical clusters or particles is absorbed onto a surface, and crystallization of ACC to generate the final stable crystal product [15,16]. However, a recent study showed
experiments were determined from PHREEQC Interactive-Wateq4f database (Table S3).

For the biotic system, cell suspensions were immediately added to the flask with a syringe after the addition of CaCl₂ solution was completed. Aliquots of mineralization solution were sampled with sterile syringes and filtered through a 0.2 µm polycarbonate membrane filter (Nuclepore). The filters were rinsed with deionized water and stored in Petri-dishes at room temperature for further microscopic analysis. Filtrates were analyzed for dissolved calcium concentrations by flame atomic absorption spectroscopy (FAAS) via iCE 3500 Atomic Absorption Spectrometer (ThermoUnicam). Samples were taken at two intervals, referred to as Phase I, approximately 6 h after the beginning of experiments and Phase II, approximately 18–20 h from the beginning of experiments.

The apparent rate of CaCO₃ formation is calculated from the first derivative of dissolved [Ca²⁺] with respect to time

\[ R_{\text{CaCO}_3} = -\frac{d[\text{Ca}^{2+}]}{dt} \]

where \( R_{\text{CaCO}_3} \) is apparent rate of CaCO₃ formation, [Ca²⁺] is the dissolved calcium concentration and \( t \) is elapsed time. Calcium removal by biomass has been estimated as removed dissolved calcium normalized to weight of bacterial cells.

2.4. Scanning electron microscopy (SEM) and X-ray microanalysis

The deposited materials on the filters were examined using scanning electron microscope (S-5200 Hitachi, Japan) equipped with an energy-dispersive X-ray detector, with the operation acceleration voltage applied of 10 keV. Selected filters were coated with carbon for 15–20 s (Emitech K950X, Quorum Technologie). Energy-dispersive X-ray spectroscopy (EDX) was applied for elemental composition analysis of materials.

2.5. Atomic force microscopy (AFM) and Raman spectroscopy

AFM and Raman spectroscopy analysis for mineralization samples were performed using the NTEGRA Spectra system from NT-MDT (Russia) equipped with an inverted optical microscope (Olympus IX 71) coupled to AFM Ntegra Platform and Solar TII spectrometer. This system allows simultaneous acquisition of AFM and Raman images from transparent samples. Semi-contact AFM measurements were conducted in air using silicon cantilevers (NSG01, NT-MDT, Russia) with a spring constant of 5.1 N m⁻¹ and a frequency range of 87–230 kHz. Samples were imaged under ambient conditions (23 °C, 40–60% humidity). Raman spectra were acquired at 632.2 nm laser wavelength with 600 grooves per mm grating. The laser power was attenuated with neutral density filters built in the NTEGRA Spectra system to reduce fluorescence and sample destruction. The backscattered light was collected through a 100× oil immersion objective, 0.7 numerical aperture (NA) and redirected to a high-resolution 1600 × 200 pixels CCD camera (cooled at −70 °C). Raman spectra were acquired for 10 s per analytical window. The spectra were processed with NT-MDT SPM software (see Supplementary Materials for more details).

3. Results and discussion

3.1. Variation in cell surface characteristics of three Synechococcus strains

Fig. 1 shows the experimental data and modeled curves of acid–base titrations for three replicate titrations of ARC21 (Fig. 1(a)), LS0519 (Fig. 1(b)) and PCC8806 (Fig. 1(c)). The charged excess increased as pH evolves from 4 to 9, and modeled curves reflected experimental values well. The predominant sigmoidal shape of the titration curves signifies a buffering capacity due to the presence of bacterial cells. The forward and reverse titrations did not exhibit significant differences, indicating proton exchange reactions were fully reversible at the bacterial cell surface on the timescale of experiments. pK₅₆ spectra were generated as a result of LPM modeling and assigned to one of the five ligand classes based on the Five Electrostatic Binding Sites proposed by Cox et al. [23] (Fig. 1(d)–(f), Table S1).

The total ligand concentration \( \Sigma_L \) can be calculated from the modeling results for each bacterial strain. The results indicate significant differences in the composition of \( \Sigma_L \) between the three studied strains. The similarity in total ligand concentration \( \Sigma_L \) for the freshwater strains ARC21 (0.342 mM g⁻¹ of dry bact.) and LS0519 (0.350 mM g⁻¹ of dry bact.) was noticeable, while \( \Sigma_L \) for the marine strain PCC8806 was 0.662 mM g⁻¹ of dry bact., or a two-fold higher concentration than those determined for the freshwater strains (Table S1). Ligand compositions of ARC21 and LS0519 are different from one another despite their similarity in \( \Sigma_L \) values.

Carboxylic, phosphoryl and amine groups are common constituent of macromolecule components (i.e. protein, fatty acid and polysaccharide) of the cell membrane [23]. These functional groups can be protonated and dissociated depending on solution pH, and contributed to development of charges on the bacterial cell surface [22,24]. Among the three studied Synechococcus strains, PCC8806 have the highest total ligand concentration \( \Sigma_L \) and is expected to have a greater surface reactivity and binding capacity than the other two strains.

\( \Sigma_L \) for ARC21 and LS0519 are similar to another Gram-negative bacterium [25] (Table S2), while the three studied Synechococcus strains have lower \( \Sigma_L \) than previously reported cyanobacterial species (Table S2). The variability of these reported ligand concentrations among the bacteria could be caused by growth media, cell metabolic state and the age of the bacterial population [23].

3.2. pH dynamics in CaCO₃ biomineralization experiments

Dynamics of pH during biomineralization experiments in abiotic and three biotic systems is shown in Fig. 2. A reproducible pattern was observed in the abiotic control experiments with pH increased slightly over the course of the experiment (Fig. 2(a)). When compared with the abiotic control, distinctive pH profiles were obtained in the three biotic systems. pH values in the presence of ARC21 cells changed as small as 0.1 pH unit (Fig. 2(b)). In the experiments with LS0519, pH gradually increased by 0.3 pH units throughout the experimental time reached similar values of ca. 8.68 (Fig. 2(c)). pH trend was much more pronounced in PCC8806 (Fig. 2(d)) with an instantaneous increase from 8.4 to ca. 8.8 shortly after cell inoculation. Both the steepness and overall change in pH dynamics revealed the difference in CaCO₃ precipitation in the presence of bacterial strains. pH elevated slightly in abiotic experiments, whereas a drastic increase in pH followed by a pH drop were observed in PCC8806 experiments and pH changed steadily in experiments with ARC21 and LS0519. A similar pH evolution has also been reported in the experiments with PCC7924 [26].

The pH changes during the precipitation experiments (Fig. 1) have been caused by diverse processes that occurred both on the cell surfaces and in the bulk solution. The (de)-protonation process of surface functional groups may lead to pH changes, generation or decreasing alkalinity and impacts CaCO₃ formation. Indeed, the highest pH drift among three investigated strains


Redox-Controlled Changes in Cadmium Solubility and Solid-Phase Speciation in a Paddy Soil As Affected by Reducible Sulfate and Copper

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ABSTRACT: The solubility of Cd in contaminated paddy soils controls Cd uptake by rice, which is an important food safety issue. We investigated the solution and solid-phase dynamics of Cd in a paddy soil spiked with ∼20 mg kg⁻¹ Cd during 40 days of soil reduction followed by 28 days of soil reoxidation as a function of the amounts of sulfate available for microbial reduction and of Cu that competes with Cd for precipitation with biogenic sulfide. At an excess of sulfate over (Cd + Cu), dissolved Cd decreased during sulfate reduction and Cd was transformed into a poorly soluble phase identified as Cd-sulfide using Cd K-edge X-ray absorption spectroscopy (XAS). The extent of Cd-sulfide precipitation decreased with decreasing sulfate and increasing Cu contents, even if sulfate exceeded Cd. When both Cu and Cd exceeded sulfate, dissolved and mobilizable Cd remained elevated after 40 days of soil reduction. During soil reoxidation, Cd-sulfide was readily transformed back into more soluble species. Our data suggest that Cd-sulfide formation in flooded paddy soil may be limited when the amounts of Cd and other chalcophile metals significantly exceed reducible sulfate. Therefore, in multimetal contaminated paddy soils with low sulfate contents, Cd may remain labile during soil flooding, which enhances the risk for Cd transfer into rice.

INTRODUCTION

Cadmium (Cd) contamination of paddy soils due to the application of Cd-containing phosphate fertilizers or the use of contaminated irrigation water has been reported for several Asian countries, including China, Japan, Bangladesh, Thailand, Taiwan, and Korea.¹−⁶ Compared to other trace metals, Cd is rather mobile in soils.⁷ Consequently, Cd is more readily taken up by rice plants, where it can be translocated into the grain and thereby enter the human food chain.⁸,⁹ Long-term consumption of Cd-contaminated rice can cause serious human health problems such as the itai-itai disease.¹⁰ Because rice is the staple food for about half of the world’s population, Cd contamination of paddy soils and Cd uptake by rice have become an important food safety issue.⁸

Paddy soils are characterized by periodically changing redox conditions during rice cultivation, with prolonged flooding during the growing season and soil drainage prior to harvest.¹¹ Changes in soil redox state affect the solid-phase speciation and solubility of Cd and its availability for uptake by rice. During soil reduction, Cd may be mobilized by reductive dissolution of Mn(III/IV)- and Fe(III)-(oxyhydr)oxide sorbent phases and concomitant increases in dissolved Mn⁶⁺ and Fe⁵⁺ that compete with Cd for sorption sites.¹²,¹³ On the other hand, a decrease in Cd solubility or extractability during paddy soil reduction was observed in numerous laboratory studies and was attributed to the precipitation of Cd-sulfide under sulfate reducing conditions, albeit without spectroscopic confirmation.¹⁴−¹⁶ Also lower Cd transfer into rice plants and grains in flooded versus drained soil was suggested to be due to Cd-sulfide formation under reducing conditions.⁵,¹⁷

Direct spectroscopic insight into solid-phase Cd speciation and the relevance of Cd-sulfide formation in flooded paddy soils is limited. Recently, Khaokaew et al.¹⁸ used Cd K-edge X-ray absorption spectroscopy (XAS) to monitor changes in Cd speciation during flooding of a highly contaminated alkaline paddy soil (142 mg kg⁻¹ Cd). Over 150 days of flooding, Cd-carbonate represented the dominant Cd fraction, and only a minor fraction of the soil Cd became sequestered into Cd-sulfide. In a microcosm flooding experiment with a contaminated floodplain soil (30 mg kg⁻¹ Cd),¹²,¹⁹ on the other hand, we observed the formation of Cd-containing Cu-rich metal-sulfide nanoparticles in pore water by electron microscopy and concluded from soil extractions that most Cd...
exceeded dissolved Cd concentrations by 2–4 orders of magnitude. In the medium-sulfate series without Cu spike (MS-LCu, Figure 1d), Cd removal from solution started already after the first day of incubation, in line with the earlier start of sulfate reduction. The Cd decrease rate of 88 nmol L$^{-1}$ d$^{-1}$ was about twice as high as in the corresponding series with Cu spike (MS-HCu), and Cd was completely removed from solution after only 10 days. The faster removal of dissolved Cd in the absence of spiked Cu suggests that Cu competed with Cd for sulfide during metal-sulfide precipitation. The dependence of the decrease of dissolved Cd on sulfate availability and content of competing Cu is supported by the good correlation between the Cd decrease rates of the four treatments and the molar ratio of initial soil sulfate over the sum of soil Cu and Cd contents ($R^2 = 0.92$; Supporting Information, Figure S3).

Changes in Cd extractability during soil reduction reflected the observed Cd solution dynamics in the four treatments. In the MS-LCu series (Figure 1h), Cd was completely removed from the mobile fraction within the first 10 days and only 42% remained Na-acetate-extractable, which was in line with the faster decrease in Cd solubility and the assumed enhanced precipitation of Cd-sulfide in the absence of Cu. In contrast, in the Cu-spiked series (Figure 1e–g), Cd remained in the mobile and easily mobilizable fraction during the first 10 days of reduction. The slight increase of Na-acetate-extractable Cd at the expense of CaCl$_2$-extractable Cd, as observed in all series, may be explained by stronger Cd adsorption on mineral surfaces as solution pH increased from 6.6 to 7.1. After 40 days of reduction, more than 91% of the Cd in the low-sulfate series (LS-HCu) remained extractable, in contrast to the high-sulfate series, where extractable Cd decreased to 18% (MS-HCu) and 5% (HS-HCu), respectively. In the medium-sulfate series without Cu spike (MS-LCu), Cd became nonmobilizable during soil reduction.

During reoxidation, dissolved Cd slightly increased, which we attributed to oxidative dissolution of metal-sulfides. Over 4 weeks of reoxidation, however, Cd concentrations remained below the initial values prior to soil reduction (Figure 1a–d). This may be due to effective readsoption of Cd on freshly precipitated Fe- and Mn-oxides, especially at the relatively high pH values during the initial stage of soil reoxidation. Notably, in all Cu-spiked series Cd concentration slightly increased after 2 weeks, concomitant to a decrease in dissolved Cu concentrations (Supporting Information, Figure S9), which pointed to a competition between Cu and Cd for binding sites. Within 28 days of soil reoxidation, Cd was completely repartitioned into the mobile and easily mobilizable fractions (Figure 1e–h). However, the easily mobilizable (Na-acetate-extractable) Cd fraction remained higher than after the 2-day equilibration period, in line with the lower dissolved Cd levels.

Changes in Cd Speciation by X-ray Absorption Spectroscopy. Although the observed changes in Cd solubility and extractability were in good agreement with expected trends, these macroscopic results provided no molecular-level insight into Cd speciation changes during soil reduction and reoxidation. We therefore used Cd K-edge X-ray absorption spectroscopy to obtain direct information on solid-phase Cd speciation changes, an approach that has not previously been applied to Cd speciation at such low Cd levels in soils. Figure 2a–d shows the $k^2$-weighted EXAFS spectra of selected soil samples, and their Fourier-transformed EXAFS magnitudes in comparison to the spectra of three Cd reference compounds (Cd-carboxyl, Cd-thiol (pH9), and CdS). The positions of the first-shell peaks in the Fourier-transformed EXAFS spectra of Cd-carboxyl and CdS reflect the difference between first-shell O at ∼2.3 Å and first-shell S at ∼2.5 Å (Figure 2c; Supporting Information, Figure S1). Accordingly, visual inspection of the Fourier-transformed EXAFS spectra indicated a shift from dominantly O-coordinated Cd in the 2-day equilibrated samples to dominantly S-coordinated Cd after the 40-day reduction period and back to dominantly O-coordination Cd after the 28-day reoxidation period (Figure 2d).

To further evaluate these trends, we analyzed the sample EXAFS spectra by linear combination fitting (LCF) using Cd-carboxyl, Cd-thiol (pH9), and CdS as references (LCF spectra in Figure 2b and d, LCF fractions in Figures 2e, and Supporting Information, Table S5). The LCF analysis of the sample EXAFS spectra was complemented and supported by shell-fits and by LCF analysis of the corresponding XANES spectra (Supporting Information, section S4). In the EXAFS LCF analysis Cd-carboxyl served as a proxy for O-coordinated Cd adsorbed to natural organic matter, clay minerals, and metal oxides (Supporting Information, section S1). According to shell-fit results (Supporting Information, Table S1), Cd in these references is coordinated by ∼6 O atoms at a distance of 2.27–2.30 Å. In the crystalline CdS reference, Cd is coordinated by 4 first-shell S atoms at a distance of 2.52 Å and 12 s-shell Cd atoms at ∼4.1 Å (Supporting Information, Table S1). The Cd-thiol (pH9) reference is characterized by a similar first-shell S coordination (∼3.2 S at 2.52 Å) as crystalline CdS, but with a minor contribution from first-shell O (∼1.6 O at 2.32 Å) and without the contribution from second-shell Cd atoms (Supporting Information, Table S1). Second-shell Cd–Cd contribution may be strongly reduced or even absent in freshly precipitated nanoparticulate CdS, which may also exhibit a low degree of crystallinity and some isomorphic replacement of S$^{2−}$ by O$^{2−}$ or OH$^{−}$. Therefore, nanometer-sized and/or poorly crystalline Cd-sulfide may have been represented by a combination of the Cd-thiol (pH9) and CdS references in LCF analysis. In addition, the Cd-thiol (pH9) reference also served as a proxy for Cd complexed by reduced organic S groups of natural organic matter or bacterial cell walls adsorbed to metal sulfides. The interpretation of the LCF results with respect to S-coordinated Cd therefore required the concomitant consideration of the chemical extraction data. As metal binding to thiolate ligands is strongly pH-dependent, with low pH favoring the displacement of metal ions, we assumed thiol-bound Cd in our samples to be Na-acetate-extractable, while Cd-sulfide phases are expected to be nonextractable in the two extraction steps.

LCF analysis of three 2-day equilibrated soil samples on average returned 76% Cd-carboxyl and 24% Cd-thiol (Figure 2e; Supporting Information; Table S5). These fractions compared to ∼75% CaCl$_2$ and ∼25% Na-acetate-extractable soil Cd in the 2-day equilibrated samples, which suggested the presence of thiol-bound Cd. Over the course of soil reduction, the S-coordinated Cd fractions increased (Figure 2) as dissolved Cd and extractable Cd decreased (Figure 1). Accordingly, LCF analysis of a larger set of sample XANES spectra revealed a close linear relation between increasing S-coordinated Cd and decreasing dissolved Cd ($R^2 = 0.87$; Supporting Information, Figure S8b). In combination with the decrease of extractable Cd fractions, these trends suggested that Cd sequestration into less soluble form was dominantly due to formation of Cd-sulfide precipitates. After 40 days of soil
reduction, the CdS and Cd-thiol references accounted for 100% of the total Cd in the MS-LCu, HS-HCu, and MS-HCu series and for 90% of the total soil Cd in the LS-HCu series. The highest CdS fraction was found for the MS-LCu (69%) and the HS-HCu (64%) series, followed by the MS-HCu (51%) and LS-HCu (30%) series. In shell-fits, this trend was reflected by decreasing second-shell Cd−Cd (from CdS) and increasing first-shell Cd−O coordination with decreasing (Cd + Cu)/sulfate ratio (see Supporting Information, Table S7). The fact that Cd in the 40-day reduced sample from the MS-LCu series with the lowest (Cd + Cu)/sulfate ratio was neither CaCl2- nor Na-acetate-extractable (Figure 1h) suggested a near-complete sequestration of Cd in a Cd-sulfide precipitate. The presence of 31% Cd-thiol derived from LCF analysis was therefore interpreted to account for a very small Cd crystallite size and/or low crystallinity. From the shell-fit of this EXAFS spectrum, a low second-shell Cd coordination number of 4.2 ± 1.7 was derived (Supporting Information, Table S7), indicating a CdS crystallite size in the range of a few nanometers only. In the LS-HCu series with the highest (Cd + Cu)/sulfate ratio, in contrast, most Cd in the 40-day reduced sample was still extractable (Figure 1e). This suggested that a significant portion of the LCF-derived Cd-thiol fraction in this sample effectively represented Cd bound by reduced organic S groups. An increase of the Cd-thiol fraction from the 2-day equilibrated to the 40-day reduced sample could be due to the growth of bacterial biomass and an increase in Cd-binding by sulphydryl-groups on bacterial cell-walls or due to increasing Cd complexation by thiol groups in humic acid newly formed by reaction with bisulfide. Another explanation could be that some Cd adsorbed to the surface of newly formed CuS and was therefore more labile, similar to Cd adsorbed onto mackinawite. Based on the Cd speciation discussed for the series MS-LCu and LS-HCu, S-coordinated Cd in the intermediate series HS-HCu and MS-HCu represented mainly nanoparticulate Cd-sulfide and minor fractions of thiol-bound Cd, in line with Na-acetate-extractable Cd fractions (5% in HS-HCu; 18% in MS-HCu).

Upon reoxidation, the fraction of S-coordinated Cd species decreased rapidly in all treatments (Supporting Information, Table S5 and Figure S8a) and Cd speciation after 28 days of reoxidation closely resembled Cd speciation after soil equilibration (Figure 2e), irrespective of the different amounts of Cd-sulfide formed during soil reduction. The fast decrease in S-coordinated Cd suggests that all S-coordinated Cd species formed during soil reduction were readily oxidizable.

**Solubility and Solid-Phase Speciation of Cd as Affected by Reducible Sulfate and Cu.** Considering the Cu- and Cd-spiked series with varying reducible sulfate content (HS-HCu, MS-HCu, LS-HCu), our results clearly show that lower reducible sulfate contents may result in limited Cd-sulfide formation and thereby reduced Cd immobilization over the course of soil reduction. Furthermore, the comparison of the series without and with spiked Cu (MS-HCu, MS-LCu) clearly revealed that chalcophile Cu can reduce the extent of Cd-sulfide formation and Cd immobilization due to competitive metal sulfide precipitation. Nevertheless, even in the treatments with an excess of Cu over reducible sulfate (LS-HCu, MS-HCu), a significant fraction of the soil Cd was still sequestered into Cd-sulfide during soil flooding, despite the orders of magnitude lower stability of CdS versus CuS, and the ~25-fold lower soil Cd versus Cu contents. This can be attributed to the fact that Cd forms weaker complexes with NOM than Cu and also has a lower adsorption affinity for clay and oxide mineral surfaces. This is reflected by the lower extractability of Cu (<1% CaCl2-extractable) compared to Cd (>70% CaCl2-extractable) in the equilibrated Cu- and Cd-spiked soils. Furthermore, part of the Cu(II) in the Cu(II)-spiked soils became rapidly reduced to Cu(I) after soil flooding, which resulted in strong Cu(I) binding to NOM and metallic Cu formation via disproportionation. These transformation reactions may have further attenuated the competition of Cu with Cd for reaction with biogenic sulfide.

According to Cd EXAFS results, the Cd-sulfide formed during soil reduction exhibited a very small crystallite size (few nm) but may also have been of poor crystallinity or partly even amorphous, as concluded for CuS in the Cu-spiked series based on Cu EXAFS data. On the other hand, our Cd EXAFS results provided clear evidence for second-shell Cd−Cd coordination in Cd-sulfide, suggesting that at least a fraction of the Cd was present in a pure Cd-sulfide phase. Co-precipitation of Cu and Cd with sulfide was previously shown to lead to the formation of separate CdS and CuS phases rather than the formation of a mixed phase, which was attributed the large difference in the atomic radius between Cu and Cd. Accordingly, the formation of Cu-rich Cd-containing metal sulfide nanoparticles with sizes in the range of a few tens of nanometers that we previously observed in a contaminated floodplain soil may result from the aggregation of extremely small but chemically pure CuS and CdS crystallites. Notably, in the present study, Cd-sulfide readily dissolved during soil reoxidation, whereas CuS was more resistant to oxidative dissolution, also hinting to the sequestration of Cu and Cd into separate but possibly coaggregated metal-sulfides.

In contrast to a recent study on Cd speciation in a highly Cd contaminated alkaline paddy soil, we did not observe any Cd-carbonate formation in our incubation experiment, most probably due to the weakly acidic pH of the soil investigated here and the absence of calcite as a Cd-carbonate template. Conversely, CdS formation in the study of Khaokaew et al. may also have been limited by the amounts of reducible sulfate.

**Environmental Implications.** Our X-ray spectroscopic results clearly confirmed that decreasing Cd solubility and extractability during soil flooding can be related to formation of Cd-sulfide. Although, Cd-sulfide formation occurred even at Cu/sulfate ratios larger than unity, our results suggested that when the amounts of Cd and competing chalcophile cations such as Cu significantly exceed the amount reducible sulfate, Cd solubility and extractability may remain high even under anoxic conditions. Rice growth under waterlogged conditions thus may not prevent Cd transfer into rice in multimetal contaminated paddy soils with low sulfate content. Furthermore, our results suggest that Cd-sulfide formed during soil flooding periods readily dissolves during soil drainage and oxidation, most likely due to its amorphous or nanoparticulate character. This has to be considered in the discussion of water management strategies such as alternate wetting and drying where rapid Cd-sulfide dissolution during short drainage periods may lead to enhanced Cd solubility and phytoavailability. The dependency of Cd dynamics on reducible sulfate and Cu described in this study may also control Cd mobility in periodically flooded riparian soils with low sulfate contents, where elevated dissolved Cd concentrations may result in enhanced Cd release into surface and groundwater.
The dynamic nature of bacterial surfaces: Implications for metal–membrane interaction

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Abstract

Bacterial envelopes are chemically complex, diverse structures. Chemical and physical influences from cellular microenvironments force lipids, proteins, and sugars to organize dynamically. This constant reorganization serves to maintain compartmentalization and function, but also affects the influence of charged functional groups that drive electrochemical interactions with metal ions. The interactions of metal species with cell walls are of particular interest because (i) metals must be taken up or excluded to maintain cell function, and (ii) electrochemical interactions between charged metals and anionic ligands are inevitable. In this review we explore the associations of metals with metal-reactive ligands found within bacterial envelopes, and outward to include those within biofilm matrices. The mechanisms that underpin metal binding to these ligands have not been well considered with respect to the dynamic organization of the biological structures themselves. Bacteria respond sensitively and rapidly to growth environment with de novo syntheses of chemical constituents, which can impact metal interactions. We discuss causes of membrane chemical variability as observed in laboratory experiments, and offer consequences for this adaptability in natural settings. The structural impacts of metal ion associations with bacterial envelopes are often overlooked. This review explores how dynamic bacterial surface chemistry influences metal binding and, in turn, how metal ions impact membrane organization in laboratory and natural conditions.

Keywords: Bacterial membranes, membrane chemistry, metals, complexation chemistry, biofilms

Introduction

Bacteria necessarily interact with environmental metals. These cell-metal associations occur wherever bacteria are found; in higher organisms, in soils and plant rhizospheres, in aquatic sediments contaminated with toxic metals, and in atmospheric dusts. The first point of interaction for metals with planktonic bacteria is the cell envelope. These structures are chemically diverse, and serve a multitude of functions that are essential for cell survival. Cytoplasmic membranes are semi-permeable and contain transport proteins to enable the passing of molecules and ions, including metals, into the cell (Shabala et al., 2009; Reyes et al., 2009; Ruiz et al., 2009). They are also sites for chemical reception (Weber and Silverman, 1988; Gunsalus and Park, 1994; Celani and Vergassola, 2010; Hazelbauer and Lai, 2010), DNA replication and cell division (Cooper, 1991; Kaguni, 2006; Mott and Berger, 2007), motility (Kaiser, 2000; Rajagopala et al., 2007), and respiration (Richardson, 2000; Myers and Myers, 2001; Shi et al., 2007). At the most basic level, bacterial envelopes protect intracellular components from highly variable environments that include toxic metal species. This is accomplished in part by ionogenic constituents of the cell providing reactive groups that readily interact with chemically diverse metals (Beveridge and Koval, 1989; Beveridge, 1989; Beveridge, 2005).

Metal binding depends not only on the metal chemical species, but also on the environmental metal concentration, redox conditions, and pH. The cell surface chemistry strongly impacts metal associations. Chemical compositions of structural and functional components (Beveridge, 1989; Beveridge, 2005), cell respiration (Urrutia Mera et al., 1992; Daughney et al., 2001), the excretion of extracellular material (Ferris et al., 1989;
The metal binding capacity of MRGs
Several models are available for evaluating metal adsorption as a function of the electrostatic potential, including the constant capacitance model (CCM), the diffuse layer model (DLM), the triple layer model (TLM), and the NICA-Donnan model. Readers interested in a thorough review of the CCM, DLM, and TLM are directed to the excellent review by Goldberg et al. (2008). The CCM has been used extensively in bacterial surface thermodynamics modeling. For the purpose of fitting the CCM to data from bacterial titrations, Haas et al. (2001) used a capacitance (C) of 1.0 Fm⁻² for the gram-negative bacterium S. putrefaciens. This was based on the relationship between capacitance, the Born solvation coefficient of the electrolyte (\(\omega_{\text{ML}}\)) in cal·mol⁻¹ and the aqueous effective radius (\(r_{\text{e,ML}}\)) in Å (Sahai and Sverjensky, 1997):

\[
C = 5.929 - 32.928 \left( \frac{1}{\omega_{\text{ML}} r_{\text{e,ML}}} \right)
\]  

Using curve-fitting software, Fein et al. (1997) determined that the capacitance relating membrane charge of B. subtilis to surface potential was 8.0 Fm⁻², whereas that value calculated from (9) should be 1.2 Fm⁻². Bacterial membranes behave as polyelectrolyte brushes, so it is, therefore, preferable to assess the electrostatic potential between membranes and the bulk ions using a Donnan model (Yee et al., 2004) rather than a capacitance relationship conceived for a 2-dimensional system, such as the planar face of a mineral (Grahame, 1947). The Donnan model reflects a distribution of charges that arise throughout the volume of a membrane. This is in contrast to surface electrostatic models that consider charges arising on an aforementioned plane. At low ionic strength treatments, there is a strong potential between the charges in the membrane and the bulk solution, attracting counterions from the bulk solution and shifting the effective binding coefficients (Lyklema et al., 2005). The inverse is also true, in which high electrolyte salt concentrations screen effective charge potential between the cell and the bulk solution. This creates a plateau in the magnitude of counterion attraction, despite increasing charge density (Dague et al., 2006). Screening of the charge potential may be responsible for the absence of electrostatic effects that some researchers have observed.

The metal binding capacities of bacterial envelopes are often determined through batch experiments in which suspensions of bacteria are exposed to metals and the remaining metal concentrations in the supernatant are measured. These are typically performed after titrations have been used to quantify site densities and acidity constants of MRGs. Batch experiments can be conducted across a range of pH values at fixed metal concentrations (Fowle et al., 2000; Daughney et al., 2001; MacLean et al., 2004; Kulczycki et al., 2005; Kenny and Fein, 2011), or at a fixed pH across a spectrum of metal concentrations (Mishra et al., 2010) to quantify metal adsorption to bacterial envelopes. Spectroscopy studies may be used to support batch experiments. Using EXAFS, Mishra et al. (2010) were able to demonstrate that phosphoryl, carboxyl, and sulhydryl MRGs are the dominant moieties for Cd binding in high, nominal, and low metal:bacteria ratios respectively. In a novel approach to characterizing LPS affinity for bulk solution cations, Barkleit et al. (2008) omitted batch experiments but instead used titrations with and without 0.1 mM uranyl (UO₂²⁺) concentrations to determine metal stability constants. This experiment was supported by time-resolved laser-induced fluorescence spectroscopy (TRLFS), which demonstrated that the metal forms mono and bidentate complexes with phosphate MRGs at relatively low and high pH values, respectively. Whether using titration methods or batch experiments, an understanding of the aqueous metal species, as described in Section 3, is necessary for solving mass balance equations that represent the partitioning of the metal between aqueous and adsorbed fates. Titration and batch experiments supported by spectroscopic techniques offer excellent means of characterizing total-surface functional groups because they can be used to examine whole cells. Alternatively, chromatographic and gel separation techniques are capable of separating and characterizing extracted proteins, lipids, or carbohydrates. While these techniques provide physicochemical details about individual components, they do not provide much information about exposed surface ligands in a heterogenous membrane environment.

Sources of MRG variability in laboratory experiments
The dynamic nature of the bacterial surface is due, in part, to constant fluctuation in its biochemical composition. This also includes de novo syntheses of extracellular polysaccharides and proteins. Alterations in membrane protein, LPS, capsular or extracellular polymeric substance (EPS) synthesis, motility or respiration appendages, or biofilm lifestyles all alter available MRGs. In the case of EPS, the environment typically regulates biosynthesis indirectly. The following section reviews the occasional subtle cellular responses of bacteria to changes in environment, and seeks to identify unpredictable or unforeseen sources of surface MRG variability.

Culture conditions
Cell walls are usually the first lines of defense that planktonic bacteria have against their environment. Bacteria alter their membrane chemistry in response to environmental stimuli, for protective and metabolic purposes. Bacterial stress responses are not the focus of this review, but many bacterial surface modifications will impact cell surface MRG composition. Kapetas et al. (2011) observed that when bacteria have a greater buffering capacity when allowed to equilibrate longer between the addition of titrant during potentiometric titration experiments. The


Solid-phase cadmium speciation in soil using $L_3$-edge XANES spectroscopy with partial least-squares regression

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Cadmium (Cd) has a high toxicity and resolving its speciation in soil is challenging but essential for estimating the environmental risk. In this study partial least-square (PLS) regression was tested for its capability to deconvolute Cd $L_3$-edge X-ray absorption near-edge structure (XANES) spectra of multi-compound mixtures. For this, a library of Cd reference compound spectra and a spectrum of a soil sample were acquired. A good coefficient of determination ($R^2$) of Cd compounds in mixtures was obtained for the PLS model using binary and ternary mixtures of various Cd reference compounds proving the validity of this approach. In order to describe complex systems like soil, multi-compound mixtures of a variety of Cd compounds must be included in the PLS model. The obtained PLS regression model was then applied to a highly Cd-contaminated soil revealing Cd$_3$(PO$_4$)$_2$ (36.1%), Cd(NO$_3$)$_2$.4H$_2$O (24.5%), Cd(OH)$_2$ (21.7%), CdCO$_3$ (17.1%) and CdCl$_2$ (0.4%). These preliminary results proved that PLS regression is a promising approach for a direct determination of Cd speciation in the solid phase of a soil sample.

Keywords: X-ray absorption; PLS regression; mixtures; Cd; speciation.

1. Introduction

High cadmium (Cd) concentrations are becoming a more and more serious environmental problem in various agro-ecosystems and urban areas, as the increasing use of Cd-bearing phosphorus (P) fertilizer and industrial pollution leads to Cd enrichments in soil (McLaughlin & Singh, 1999). The heavy metal Cd is known for its high toxicity and can, especially in its mobile forms, easily be accumulated in the food chain by plant and animal uptake (Adriano, 2001) with the risk of serious health (e.g. consumers) and economic (e.g. farmers) problems. A first step for risk assessment is the determination of total Cd concentrations in soil. Knowledge about the abundance and behavior of Cd species is fundamental to estimating their fate in the environment, associated risks to human health, and to develop strategies for Cd remediation or immobilization. Sequential extractions have been developed to characterize Cd pools in environmental samples (e.g. Tessier et al., 1979; Zeien & Brümmer, 1989). However, these extractions provide assignments to Cd pools of different solubility rather than chemically defined Cd speciation sensu strictu. Furthermore, involved successive extraction procedures can alter the Cd species in the sample (Bertsch et al., 1997). To overcome such analytical limitations, X-ray absorption spectroscopy (XAS) can be applied, which enables an element-specific and non-invasive direct speciation of Cd in the solid phase (Fendorf & Sparks, 1996). In general, a Cd XANES spectrum is the weighted sum of signals from all Cd species within the sample. Various approaches were applied to deconvolute the spectrum of an unknown sample into proportions of Cd species. The majority of the previously published studies used extended X-ray absorption fine-structure (EXAFS) spectroscopy at the Cd K-edge to investigate Cd complexes in various types of environmental samples by fitting the $\chi(k)$ model function to the EXAFS oscillation by using least-square methods (Sery et al., 1996; Pickering et al., 1999; Mishra et al., 2009; Jalilehvand et al., 2009; Mah & Jalilehvand, 2010; Demchenko et al., 2010). However, there are only a limited number of beamlines available that are capable of providing a stable flux at the necessary energy range of the Cd K-edge (~27 keV). Thus, access to those beamlines is restricted by the limited number of possible users. For this reason, attempts were made to use other edges for Cd speciation. The three Cd L-edges are in the energy range between 4018 and 3538 eV, which is available at a great number of beamlines. The capability of Cd L-edge XANES for Cd speciation has been reported in several studies.
(e.g. Pickering et al., 1999; Isaure et al., 2006; Jalilehvand et al., 2009), although the sensitivity of the Cd L-edge is lower compared with the Cd K-edge being amenable at concentrations as low as 1 mg kg\(^{-1}\) (Mishra et al., 2010). By contrast, the energy resolution at the Cd L-edge is higher than for the Cd K-edge. Most studies used the XANES region of the Cd L\(_{3}\)-edge, as the EXAFS region is distorted by the feature of the potassium (K) K-edge superimposing the EXAFS oscillation of the Cd L\(_{3}\)-edge. Therefore, Cd L\(_{3}\)-edge EXAFS is not suitable for those environmental samples that contain K.

A common approach for XANES data evaluation is the so-called ‘fingerprint approach’. In this, spectra of known reference compounds are visually compared with the spectrum of unknown composition to assign major constituents. Most of the previously published studies evaluated Cd L\(_{3}\)-edge spectra using this approach as a first step before using more sophisticated methods (e.g. Pickering et al., 1999; Isaure et al., 2006; Jalilehvand et al., 2009). This method gives a first indication of the qualitative composition of the sample. A more sophisticated method, which also enables a quantitative estimation of the Cd composition, is linear combination fitting (LCF). This approach involves finding the best combination of Cd reference compounds spectra which fit the sample spectra when weighted and summed. For example, Isaure et al. (2006) used LCF to discriminate Cd bound to O and N ligands from S ligands in plant samples. In complex matrices such as sediment and soil samples many different Cd species may be abundant. Therefore, some researchers have applied principal components analysis (PCA) and an adjacent target transformation in order to preselect major reference compounds for subsequent LCF analysis. This approach was successfully applied to analyze the effect of calcium (Ca) addition on the Cd speciation in tobacco plants (Isaure et al., 2010) and to investigate the interactions and sorption mechanisms of Cd to red mud (Luo et al., 2011).

Partial least-square (PLS) regression is another approach to evaluate spectral data. By using this multivariate calibration method latent variables are computed to model independent variables. By correlating them with dependent variables, linear calibration models are constructed enabling prediction of proportions of individual compounds in spectra (Lindberg et al., 1983). So far, PLS regression has been extensively applied to evaluate infrared spectroscopic data for analyzing the composition of multi-compound samples (e.g. Brown et al., 2004; Janik et al., 2009). However, this method has been applied only rarely to XANES data (Kuno et al., 1999; Kuno & Matsuo, 2000; Yasoshima et al., 2001); these authors tested the capability of PLS regression to estimate the proportions of iron (Fe)-bearing minerals in mixtures and then they successfully applied the obtained PLS model to estuarine sediment samples.

The objectives of the present study were (i) to provide a comprehensive library of Cd L\(_{3}\)-edge XANES spectra for subsequent studies, (ii) to test the capability of PLS regression to estimate the proportions of known multi-compound Cd mixtures, and (iii) to apply this approach to quantitatively estimate the Cd forms in a soil sample.

## 2. Experimental

### 2.1. Cd reference compounds

For XANES analysis 12 reagent-grade Cd reference standards were purchased from different chemical suppliers: Cd(OH)\(_2\), CdO, CdCO\(_3\), Cd(CH\(_3\)COO)\(_2\) (Cd-Org), and Cd\(_3\)(PO\(_4\))\(_2\) (Cd-P 1) were purchased from Alfa Aesar (76057 Karlsruhe, Germany), Cd(NO\(_3\))\(_2\)•2H\(_2\)O and Cd(CH\(_3\)COOO\(_2\))•2H\(_2\)O (Cd-Ac) from J. T. Baker (72973 Pfulingen, Germany), CdCl\(_2\) was obtained from Fluka (30926 Seelze, Germany), CdSO\(_4\) and 3CdSO\(_4\)•8H\(_2\)O were purchased from Merck (64293 Darmstadt, Germany). The Cd phosphate standard Cd\(_5\)H\(_3\)(PO\(_4\))\(_4\)•4H\(_2\)O (Cd-P 2) was synthesized by applying a slightly modified procedure as previously published by Matsuk et al. (2008). Briefly, CdCl\(_2\) and K\(_2\)HPO\(_4\) were added to 65 ml deionized water to yield a concentration of [Cd\(_{\text{aq}}\)] = 4.80 mM and [PO\(_4\)\(_{\text{aq}}\)] = 3.52 mM, respectively. Both solutions were dropwise mixed (~5 ml min\(^{-1}\)) under continuous stirring to a 500 ml beaker containing 370 ml deionized water. The pH of the solution was kept constant at 5.00 throughout the reaction using potassium hydroxide and nitric acid. The resulting precipitate was filtered and washed with deionized water and subsequently dried at 313 K. All reference compounds were used without further purification and ground in an agate stone mortar and pestle. Solid binary and ternary mixtures of various Cd standards were prepared in various proportions on a Cd atomic mass basis. Furthermore, artificial binary to senary mixtures were mathematically calculated using the normalized spectra of all reference compounds. For this, spectra were summed in various proportions and the resulting spectra were normalized as described below.

### 2.2. XANES data collection and data treatment

The Cd L\(_{3}\)-edge XANES measurements were performed at the Canadian Light Source, Canada, a 2.9 GeV third-generation synchrotron source, on the soft X-ray micro-characterization beamline (SXMB) covering an energy range of 1.7 to 10 keV (Hu et al., 2010). The air-dried and homogenized samples were spread as thin film onto a double-sided carbon tape and mounted onto a copper sample holder before being placed in the vacuum chamber. A Si(111) double-crystal monochromator (\(\Delta E/E \approx 10^{-4}\)) was used for all measurements. All XANES spectra of reference compounds were recorded in total electron yield mode to avoid self-absorption effects, and the soil sample was measured in fluorescence yield mode. All fluorescence yield spectra were recorded within the energy range between 3500 and 3650 eV using a silicon drift detector. The chosen step size was of 0.7 eV (3500 to 3530 eV), 0.2 eV (3530 to 3580 eV) and 0.8 (3580 to 3650 eV). Furthermore, a dwell time of 1 s for reference compounds and 10 s for the soil sample were chosen. Three to four scans were recorded, and the beam was moved to a ‘fresh’ sample spot after each scan to avoid X-ray-induced changes in the sample. Afterwards, spectra of these individual scans were averaged to improve the signal-to-noise ratio and then background corrected by subtracting a linear
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References
Chemical and structural status of copper associated with oxygenic and anoxygenic phototrophs and heterotrophs: possible evolutionary consequences


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ABSTRACT

Copper adsorption on the surface and intracellular uptake inside the cells of four representative taxons of soil and aquatic micro-organisms: aerobic rhizospheric heterotrophs (Pseudomonas aureofaciens), anoxygenic (Rhodovulum steppense) and oxygenic (cyanobacteria Gloeocapsa sp. and freshwater diatoms Navicula minima) phototrophs were studied in a wide range of pH, copper concentration, and time of exposure. Chemical status of adsorbed and assimilated Cu was investigated using in situ X-ray absorption spectroscopy. In case of adsorbed copper, XANES spectra demonstrated significant fractions of Cu(I) likely in the form of tri-coordinate complexes with O\(^{\sim}\)Na and/or S ligands. Upon short-term reversible adsorption at all four studied micro-organisms’ cell surface, Cu(II) is coordinated by 4.0 ± 0.5 planar oxygens at an average distance of 1.97 ± 0.02 Å, which is tentatively assigned to the carboxylate groups. The atomic environment of copper incorporated into diatoms and cyanobacteria during long-term growth is similar to that of the adsorbed metal with slightly shorter distances to the first O/N neighbor (1.95 Å). In contrast to the common view of Cu status in phototrophic micro-organisms, XAFS failed to detect sulfur in the nearest atomic environment of Cu assimilated by freshwater plankton (cyanobacteria) and periphyton (diatoms). The appearance of S in Cu 1st coordination shell at 2.27–2.32 Å was revealed only after long-term interaction of Cu with anoxygenic phototrophs (and Cu uptake by soil heterotrophs), suggesting Cu scavenging in the form of sulfhydryl, histidine/carboxyl or a mixture of carboxylate and sulfhydryl complexes. These new structural constraints suggest that adsorbed Cu(II) is partially reduced to Cu(I) already at the cell surface, where as intracellular Cu uptake and storage occur in the form of both Cu(I)-S linked proteins and Cu(II) carboxylates. Obtained results allow to better understand how, in the course of biological evolution, micro-organisms elaborated various mechanisms of Cu uptake and storage, from passive adsorption and uptake to active, protein-controlled surface reduction, and intracellular storage.

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INTRODUCTION

Compared with other essential oligoelements, copper is particularly interesting as it combines versatile chemical behavior and coordination chemistry with multiple oxidation states (+1 and +2) both in solution and in solid. Among all bioactive metals, Cu exhibits by far the lowest concentration in cells (Andreni et al., 2008). The extreme toxicity of elevated copper concentrations to all aquatic photosynthetic microorganisms is usually explained to be due to blocking and reducing the thiol sites on proteins (e.g., Chang & Reinfelder, 2000). In particular, Cu\(^{2+}\) may bind to SH-group-containing glutathione (Kachur et al., 1998), and phytochelatin production in algae is known to be induced by the presence of Cu in response to the toxicity of the metal (Le Faucheur et al., 2005; Le Faucheur et al., 2006). The copper resistance systems of bacteria such as Pseudomonas involve the production of proteins that bind copper in the periplasm and close to the outer membrane (Cooksey, 1993, 1994). Because of the high Cu affinity to thiol groups of cell proteins such as glutathi-
solutions (e.g., glutamate, oxalate, citrate, phosphate) does not reveal clear similarities. Tentatively, they might be attributed to 1–2 C or P atoms at 2.6–3.2 Å in the three samples (NMIN-2, Gl-3, and Ps-5), implying that incorporated Cu binds carboxylate or phosphoryl ligands, similarly to other intracellular metals (Pokrovsky et al., 2005, 2008c).

In contrast, for Cu incorporated into anoxicogenic phototrophs *Rhodovulum* sp. (Rh-3, Rh-2) and EPS-rich heterotrophs *Pseudomonas* (Ps-3), EXAFS analyses unambiguously detected the presence of ~2 sulfur atoms at ~2.30 Å, together with ~2 O/N atoms at 2.03–2.10 Å. The Cu-S and Cu-O/N distances are similar to those in proteins and other organic compounds containing tri- and four-coordinated Cu(I) and Cu(II) (e.g., Kau et al., 1987; Pickering et al., 1993; Pufahl et al., 1997; Poger et al., 2008). Although EXAFS is not capable of distinguishing Cu(I) from Cu(II) because of the similarity of their Cu-O/N and Cu-S bond lengths, LCA fits of XANES spectra of these samples yield ~80% of total copper as Cu(I). This compares favorably with the fraction of thiol complexes (X) derived from the number of S atoms from EXAFS spectra and assuming dominant Cu-S₂ stoichiometries (*X*ₐₙₛ = 100% × (2.0 ± 0.5)/3.0 = 70 ± 15%). Therefore, almost all incorporated Cu(I) in these samples is likely to be bound to thiol groups of intracellular proteins. The presence of next-nearest Cu atoms around the copper absorber in the form of Cu-S-Cu linkages with Cu-Cu distances of 2.7 ± 0.1 Å found in some proteins coordinating Cu in biological structures (Pickering et al., 1993; Poger et al., 2008) was not detected within the spectral statistics (≤~0.3 Cu atoms in the second shell) in *R. steppense* and *P. aeruginosa* (samples Rh-3 and Ps-3). It should be noted, however, that such linkages are often difficult to detect unambiguously even in a single-complex environment in isolated proteins owing to structural disorder caused by a large range of Cu-S distances and overlap in the EXAFS signal of Cu-Cu contributions with those of Cu-C from thiolate ligands (Pufahl et al., 1997; Poger et al., 2008).

EXAFS analysis of Cu chemical status in growth media of EPS-rich soil bacteria did not reveal significant differences between initial growth solution (Media-0) and cell supernatant solution collected after 28 h of growth at pH ~7 (Media-1). Both solutions show ~4 O/N atoms at ~1.99 Å (Table 2), together with the corresponding MS contributions at 3.2 Å (Fig. 7, not corrected for phase shift), similar to those in Cu(II)-O/N₄ reference aqueous solutions, whereas the next-nearest shell expected from Cu-O-C bonds at 2.6–3.3 Å is too weak to be quantified. This spectral pattern is in agreement with the dominant presence in these solutions of Cu²⁺ cation and/or Cu(II) bound to carboxyl or amide/amine ligands in a square planar geometry. Two important findings for the SP growth media are the following: i) sulfur is not detectable in the Cu first shell and ii) the fraction of Cu(I) deduced from the 8982-eV (<20–30%) is smaller than that in intracellular assimilated samples (Ps-3).

**DISCUSSION**

**Surface adsorption**

Our XAS results show that upon short-term adsorption at the freshwater diatom and cyanobacteria cell surface from aqueous solution of inert electrolyte, Cu remains coordinated in the nearest shell with 3–4 oxygen atoms at 1.98 ± 0.01 Å likely in a pseudo-square geometry. Existing chemical and thermodynamic arguments suggest essentially carboxylate binding as follows from surface complexation modeling which is based on macroscopic adsorption experiments (cf. Pokrovsky et al., 2008a,b; Gonzalez et al., 2010). Reversible surface adsorption of copper at circum-neutral pH on anoxicogenic phototrophs (sample Rh-1) and soil EPS-rich (sample Ps-1) and EPS-poor (sample Ps-4) bacteria yields essentially carboxylate environments, whereas the adsorption of Cu on EPS-rich soil bacteria in acidic solutions (Ps-2) brings about appearance of ~1 sulfur atom in the Cu first atomic shell. Sulfur-containing groups (-C-S- or -S-S-) in the EPS chains of rhizospheric bacteria of *Pseudomonas* genus were evidenced from macroscopic and spectroscopic observations (Emnova et al., 2006, 2007). As a result, proteins and EPS may form stable complexes adjacent to the outer membrane and participate in reversible adsorption of Cu(II) from solution on the surface. This is especially pronounced for Cu adsorption from acidic solution (sample Ps-2, Table 2 and Fig. 6). At such elevated acidities, binding to fully protonated carboxylate groups should be less significant than at neutral pH, and Cu binding may also occur to ligands other than carboxylate, such as sulfhydril moieties (Gardea-Torresdey et al., 1990), which have a much higher affinity for both Cu(I) and Cu(II).

The sulfhydril groups of proteins are also known to control reversible adsorption of Cd on *Bacillus subtilis* and *S. oneidensis* bacteria at low surface loadings (Mishra et al., 2010), comparable with those investigated in this study. In the case of Cd-bacteria interaction, production of Cd-binding sulfhydril groups at the cell surface may represent a component of toxicity response mechanism located either on the cell walls or within the cell, which may be initiated even by non-metabolizing cell (Mishra et al., 2010). Recently, adsorption of Cu(II) on *B. subtilis* has been studied using EXAFS (Moon & Peacock, 2011). At very high Cu concentration in solid, from 0.13 to 0.66 weight %, only monodentate, inner-sphere, Cu-carboxylate complex has been identified.

**Intracellular assimilation**

Copper assimilated by oxygenic phototrophs (samples NMIN-2, Gl-3) revealed a coordination environment with 3.0 ± 0.5 O/N atoms at 1.95 ± 0.01 Å, thus indicating slight but detectable shortening (by 0.04 ± 0.01 Å) of the average first-neighbor distances upon incorporation of Cu inside the cells, in agreement with formation of stiffer and less disordered


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Detailed Assessment of the Kinetics of Hg-Cell Association, Hg Methylation, and Methylmercury Degradation in Several Desulfovibrio Species

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The kinetics of inorganic Hg [Hg(II)] association, methylation, and methylmercury (MeHg) demethylation were examined for a group of Desulfovibrio species with and without MeHg production capability. We employed a detailed method for assessing MeHg production in cultures, including careful control of medium chemistry, cell density, and growth phase, plus mass balance of Hg(II), and MeHg during the assays. We tested the hypothesis that differences in Hg(II) sorption and/or uptake rates drive observed differences in methylation rates among Desulfovibrio species. Hg(II), associated rapidly and with high affinity to both methylating and nonmethylating species. MeHg production by Hg-methylating strains was rapid, plateauing after ~3 h. All MeHg produced was rapidly exported. We also tested the idea that all Desulfovibrio species are capable of Hg(II), methylation but that rapid demethylation masks its production, but we found this was not the case. Therefore, the underlying reason why MeHg production capability is not universal in the Desulfovibrio is not differences in Hg affinity for cells nor differences in the ability of strains to degrade MeHg. However, Hg methylation rates varied substantially between Hg-methylating Desulfovibrio species even in these controlled experiments and after normalization to cell density. Thus, biological differences may drive cross-species differences in Hg methylation rates. As part of this study, we identified four new Hg methylators (Desulfovibrio aespoeensis, D. alkalitolerans, D. psychrotolerans, and D. sulfodismutans) and four nonmethylating species (Desulfovibrio alcohlovorans, D. tunisiensis, D. carminophilus, and D. piger) in our ongoing effort to generate a library of strains for Hg methylation genomics.

Methylmercury production is associated with Hg pollution. Methylmercury production is an anaerobic process that occurs in saturated soils and wetlands, decaying periphyton mats, aquatic bottom sediments, and anaerobic bottom waters. Early investigations, prior to the advent of modern methylmercury (MeHg) analyses, reported a wide variety of aerobic and anaerobic Gram-positive and Gram-negative bacteria, and fungi to be capable of MeHg production. However, subsequent studies with pure cultures have conclusively demonstrated a role only for sulfate-reducing bacteria (SRB) and iron-reducing bacteria (FeRB; principally Geobacter spp.) belonging to the Deltaproteobacteria. Many field studies, using selective microbial stimulants and inhibitory strains and FeRB as the dominant Hg methylators in natural aquatic systems, though recent studies have hypothesized that methanogens may be significant in some systems.

Only a subset of SRB and FeRB are capable of Hg methylation, but why this is the case remains unclear. Early work by Choi and Bartha (13) suggested that Hg methylation was a "metabolic mistake" of SRB utilizing the acetyl coenzyme A (acetyl-CoA) pathway for carbon metabolism. Subsequent studies have indicated that Hg methylation capability is not restricted to SRB possessing the acetyl-CoA pathway (20). At present, it is not possible to conclusively identify the methyltransferase or methyl donor in SRB or other Deltaproteobacteria responsible for in vivo Hg methylation. Hg methylation occurs intracellularly (23), and significant effort has therefore been devoted to elucidating the mechanism(s) of Hg uptake by Hg-methylating bacteria.

Passive diffusion of neutral HgS species has been hypothesized to control Hg uptake in SRB (4, 18), while more recent work suggests a role for facilitated transport of specific Hg-amino acid complexes in sulfide-free solutions (51, 52). Further muddying this picture are recent studies demonstrating that HgS or HgS-disolved organic matter nanoparticles or clusters are bioavailable to Hg-methylating bacteria (29, 60). The mechanism of uptake of these Hg-S species is not yet known. To summarize, significant gaps exist in our understanding of the diversity of microorganisms capable of Hg methylation, the bioavailability of Hg for uptake and the mechanism of uptake, and the mechanism(s) of enzymatic Hg methylation.

In this study, we compared methylation rates among a group of Desulfovibrio species. Desulfovibrio spp. are prevalent in the environment; Desulfovibrio is the best-studied genus of Hg methylators, and a wide variety of Desulfovibrio type strains are available in culture collections. The strains used in this study include Desulfovibrio species previously tested for MeHg production potential, plus several newly tested strains. Importantly, the rate measurements were done in highly controlled, chemically defined, short-
nonmethylating *Desulfovibrio* strains. *Desulfovibrio* spp. do not appear to contain the *mer* operon (3), a genetic system that includes an active Hg transporter (3), and the mechanism of Hg uptake by methylating and nonmethylating *Desulfovibrio* strains alike remains unknown.

**Kinetics of Hg methylation and export.** For the Hg-methylating *Desulfovibrio* strains, MeHg was immediately and rapidly produced. Hg(II) -cell association rates generally exceeded initial Hg methylation rates by an order of magnitude (Table 2), and so Hg(II), association with the cell surface probably did not limit the overall rate of Hg methylation. However, because these experiments did not distinguish Hg(II), uptake from sorption, we cannot rule out the possibility that intracellular uptake was rate limiting. Interestingly, we observed no time lag in MeHg production, as previously reported by Schaefer et al. (52). Because methylation occurs intracellularly (23) the lack of a time lag in Hg methylation suggests fairly rapid intracellular Hg uptake.

MeHg was rapidly exported from cells after production. Throughout the assays, 75 to 100% of total MeHg was always in the filter-passing phase (Fig. 1). Further, we saw no evidence for the buildup of a cell-associated MeHg pool. Excreted MeHg had high affinity of Hg(II) for these cell surfaces. Similar findings have been observed previously in longer-term experiments (23, 29) but have yet to be adequately explained. Possible explanations for the low affinity of MeHg for the cell surface include selectivity of cell surface receptors and transporters for specific Hg(II)-ligand complexes compared to MeHg-ligand complexes and/or inertness of MeHg complexes with regard to ligand exchange reactions at the cell surface. MeHg generally has lower particle affinity in the environment than Hg(II) (32, 35).

Mass balance clearly showed the persistence of particulate Hg(II), that was not converted to MeHg (Fig. 1). One explanation for incomplete conversion of Hg(II), to MeHg is limitation in the methyl donor. We tested this idea by conducting washed-cell methylation assays with starved cells. Assays were conducted with *D. desulfuricans* ND132 in a minimal salts medium without a carbon source, electron donor, electron acceptor, or essential nutrients (only 0.171 M NaCl, 10 mM MOPS, and 0.5 mM D-cysteine). The kinetics of Hg-cell association and methylation in these starved assays were comparable to assays in more complete medium (Hg methylation rates of 0.027 ± 0.001 zmol cell⁻¹ min⁻¹ for starved cells versus 0.014 ± 0.001 zmol cell⁻¹ min⁻¹ for cells in minimal medium [see Fig. S6 in the supplemental material]). The fact that starved cells methylate Hg as fast as cells provided with energy-generating substrates suggests that energy requirements for methylation of low-nM levels of Hg(II), are small. Hg methylation may be supported by either endogenous metabolism or low concentrations of residual energy-generating substrates remaining after cell washing. Further, Hg methylation rates by *D. desulfuricans* ND132 in washed-cell assays were proportional to added HgCl₂ concentration over the entire concentration range examined, ~0.25 to 40 nM Hg(II), (see Fig. S6). Taken together, these findings argue against the interpretation that incomplete Hg methylation is due to depletion of the methyl donor(s) to Hg(II),.

Alternatively, uptake capacity for Hg(II), may be limited by precipitation of Hg(II), into a form unavailable for uptake and/or sorption of Hg(II), to cell surfaces in a location or form unavailable for intracellular uptake. Precipitation of Hg(II), or strong sorption of Hg(II), to cell surface sorption sites may serve as "sinks" for Hg(II), lowering the total pool of bioavailable Hg(II),. X-ray-based spectroscopic investigations might provide definitive information regarding the nature of Hg(II), interactions with SRB cell surfaces (42, 43), but significant improvements in method detection limits would be required to probe Hg(II), speciation at the cell surface at environmentally relevant concentrations (sub-nanomolar).

**MeHg degradation kinetics.** We examined demethylation kinetics for three *Desulfovibrio* strains (one methylator and two nonmethylators) and found no evidence for significant demethylation by any of the bacteria (Fig. 5). Previous studies (including a study with *D. desulfuricans* ND132 in our laboratory) reported demethylation rates of ~3 to 20%/day for various SRB in batch growth assays (11, 23). Given these demethylation rates, it is possible that our assays were simply too short to measure low demethylation rates (a demethylation rate of 9%/day would be

![FIG 5 No appreciable Hg demethylation was observed in ~8-h incubations of either an Hg-methylating strain (*D. desulfuricans* ND132) or nonmethylating strains (*D. carboxiniphilus* and *D. desulfuricans* Essex6). The data for this demethylation experiment were collected under conditions of growth stage, cell density, and total Hg addition (~1 nM) similar to those in the association/methylation experiments whose results are shown in Fig. 1 and 2. Filter-passing total Hg (THg) or MeHg is the THg or MeHg that passed a 0.2-μm filter.](http://aem.asm.org/)

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34. Schafer JK, et al. 2011. Active transport, substrate specificity, and meth-
BIOMINERALIZATION AND BIOSORPTION INVOLVING BACTERIA:
METAL PHOSPHATE PRECIPITATION AND MERCURY ADSORPTION EXPERIMENTS

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

by

Sarrah M. Dunham-Cheatham

______________________________
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Graduate Program in Civil and Environmental Engineering and Earth Sciences
Notre Dame, Indiana
August, 2012
1997, 2002; Borrok et al., 2004, 2007; Wu et al., 2006). The cell wall of a bacterium contains proton-active functional groups, such as carboxyl, phosphoryl, hydroxyl, amino, and sulf hydryl groups (Beveridge and Murray, 1976; Degens and Ittekkot, 1982; Guiné et al., 2006; Madigan et al., 2009; Mishra et al., 2009, 2010). When deprotonated, these functional groups have the ability to adsorb cations (e.g. metals, aqueous complexes) from solution (Beveridge and Murray, 1976; Ledin et al., 1996; Fortin and Beveridge, 1997; Warren and Ferris, 1998; Ohnuki et al., 2005; Borrok et al., 2007). It has been shown that adsorption of metals to bacterial surfaces is rapid (Fowle and Fein, 2000; Yee et al., 2000), dependent on solution pH (Fein, 2006), and reversible (Fowle and Fein, 2000). In addition to affecting metal mobility, metal adsorption likely represents the first step in bioavailability of metals to bacteria. According to the Biotic Ligand Model, the bioavailability of toxic metals, such as Hg, is a result of the adsorption of the metal to a biological surface of the living organism (Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004; van Leeuwen et al., 2005). Thus, it is important to construct quantitative models of Hg adsorption onto bacteria that are capable of accounting for Hg partitioning under a range of conditions of geologic and environmental interest. Mercury is of particular interest because it might exhibit different aqueous complexation behavior and/or form different types of bonds than other previously studied metals. For instance, because it is a B-type metal, Hg has a high affinity to bond with sulfur ligands (Reddy and Aiken, 2000; Ravichandran et al., 2004). Because bacterial cell walls contain sulf hydryl functional groups (Mishra et al., 2009, 2010) and natural organic matter contains sulfur compounds (Haitzer et al., 2003; Hertkorn et al., 2008), the affinity of Hg for sulfur compounds may have a significant effect on the behavior of Hg adsorption behavior in the presence of bacteria and natural organic matter.
experimental conditions, individual stability constants for Hg-bacterial surface complexes cannot be determined in the Cl-free system. In the presence of chloride, all of the bacterial species exhibit minimal Hg adsorption below pH 4, increasing adsorption between pH 4 and 8, and slightly decreasing extents of adsorption with increasing pH above 8. The low extent of adsorption at low pH suggests that $\text{HgCl}_2$\textsuperscript{0}, which dominates aqueous Hg speciation below pH 5.5, adsorbs only weakly. The increase in Hg adsorption above pH 4 is likely due to adsorption of $\text{HgCl(OH)}$\textsuperscript{0}, and is limited by site availability and transformation to $\text{Hg(OH)}_2$\textsuperscript{0} as pH increases. I use the adsorption data to determine stability constants of the $\text{HgCl(OH)}$- and $\text{Hg(OH)}_2$-bacterial cell envelope complexes, and the values enable estimations to be made for Hg adsorption behavior in bacteria-bearing geologic systems.

3.2 Introduction

Bacteria are present in soils and groundwater systems (Madigan et al., 2009), and adsorption onto bacterial cell envelope functional groups can affect the speciation, distribution and transport of heavy metals (Beveridge and Murray, 1976; Fortin et al., 1997; Ledin et al., 1999; Small et al., 1999; Daughney et al., 2002). Although the adsorption behaviors of a wide range of bacteria have been studied for a wide range of metals (e.g., Beveridge and Murray, 1976, 1980; Beveridge, 1989; Mullen et al., 1989; Fein et al., 1997, 2002; Borrok et al, 2004, 2007; Wu et al., 2006), Hg has received less attention. Recent studies have found that proton-active sulfhydryl functional groups exist on the surface of bacterial cell envelopes (Guine et al., 2006; Mishra et al., 2009; 2010). Many studies have demonstrated that Hg has a high binding affinity for sulfur compounds (Fuhr and Rabenstein, 1973; Blum and Bartha, 1980; Compeau and Bartha, 1987; Winfrey and Rudd, 1990; Benoit et al., 1999), and thus the adsorption of Hg to bacteria may be dominated by this type of binding. Due to the high affinity for this bond to
freshwater basal media, and incubating it for 5 days at 32 °C. Cells were pelleted by centrifugation at 8100g for 5 minutes, and rinsed 5 times with 0.1 M NaClO₄ stripped of dissolved oxygen by bubbling a 85%/5%/10% N₂/H₂/CO₂ gas mixture through it for 30 minutes. After washing, the three types of bacteria used in this study were then pelleted by centrifugation at 8100g for 60 minutes to remove excess water to determine the wet mass so that suspensions of known bacterial concentration could be created. All bacterial concentrations in this study are given in terms of gm wet biomass L⁻¹.

3.3.1.2 Bacterial Potentiometric Titrations

Surface complexation modeling requires determination of bacterial cell envelope site concentrations and acidity constants. These parameters have been determined previously for B. subtilis (Fein et al., 2005) and S. oneidensis MR-1 (Mishra et al., 2010), but they have not been determined for G. sulfurreducens. To obtain these values, four replicate potentiometric titrations of G. sulfurreducens cells (100 gm L⁻¹) were conducted in 0.1 M NaClO₄ under a N₂ atmosphere with an automated burette assembly. The biomass suspension was prepared using washed biomass and 0.1 M NaClO₄ that was purged with N₂ gas for 30 minutes prior to the preparation of the suspension. The suspension pH was measured using a glass electrode filled with 4 M KCl that was standardized using commercially supplied pH standards. The titrations were performed by measuring the pH after each addition of aliquots of commercially supplied volumetric standard of 1.030 M NaOH or 1.048 M HCl to the suspension. Acid or base additions were made only after a maximum drift of 0.01 mV/s was attained.

The biomass suspension was titrated first with HCl to achieve a pH of ~2.0. The suspension was then titrated with NaOH to a pH of ~10.0. Titrations of the electrolyte solution
\[ K_{\text{ads}} = \frac{[(R - A_i)(Hg \ species)^{(x-1)+}]}{a (Hg \ species^{x+}) [R - A_i^-]} \]

where \( K_{\text{ads}} \) is the thermodynamic equilibrium constant for Reaction 3, \( a \) represents the activity of the species in parentheses, and brackets represent concentrations in mol L\(^{-1}\). Acid/base potentiometric titration data provide constraints on the number of site types, their \( K_i \) values and their site concentrations; Hg adsorption measurements conducted as a function of pH constrain the number of sites involved in Hg binding, the pH range of influence, and the stability constants for the important Hg-bacterial cell envelope complexes. I used the program FITEQL 2.0 (Westall, 1982) for the equilibrium thermodynamic modeling of the adsorption data, using the aqueous speciation equilibria and equilibrium constants given in Table 9, and using the program’s activity coefficient calculations via the Davies equation.

3.4 Results & Discussion

3.4.1 Potentiometric Titrations

Potentiometric titrations of \( G. \ sulfurreducens \) biomass were performed in order to calculate site concentrations and pKa values for discrete proton-active cell envelope functional groups. \( G. \ sulfurreducens \) exhibits significant proton buffering behavior over the entire pH range studied. Each of the four replicate \( G. \ sulfurreducens \) sets of titration data is depicted in Figure 19. \( G. \ sulfurreducens \) exhibits a similar total buffering capacity \((C(a) - C(b) - [H^+] + [OH^-]) / m_a\) to that measured for other bacterial species. For example, between pH 3 and 9, \( G. \ sulfurreducens \) has a buffering capacity of \(3.5 \pm 0.6 \times 10^{-4} \text{ mol/g} \) (reported error represents 1σ uncertainty), compared to a value of \(3.0 \times 10^{-4} \text{ mol/g} \) for \( Bacillus \ subtilis \) (Fein et al., 2005), \(3.1 \times 10^{-4} \) for \( Shewanella \ oneidensis \) (Mishra et al., 2010), and \(1.27 \times 10^{-4} \) and \(2.23 \times 10^{-4} \text{ mol/g} \) for \( Acidiphilium \).
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
<th>Site 4</th>
<th>[sites]_{total}</th>
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<tr>
<td></td>
<td>Site Concentrations (mol sites / gm bacteria)</td>
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<tr>
<td><strong>B. subtilis</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.1 ± 1.6 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>1.1 ± 0.36 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>4.4 ± 1.3 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>7.4 ± 2.1 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>3.1 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td><strong>S. oneidensis</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.9 ± 2.6 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>1.3 ± 0.20 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>5.9 ± 3.3 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>1.1 ± 0.60 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.9 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<td><strong>G. sulfurreducens</strong></td>
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<td>9.1 ± 0.41 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>4.1 ± 0.24 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<td>-4.8 ± 0.1</td>
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<tr>
<td><strong>S. oneidensis</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>-8.8 ± 0.3</td>
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Reported uncertainties are 1σ errors.

(a) Fein et al., 2005.

(b) Mishra et al., 2010
4.2 Introduction

Heavy metals, such as Hg, adsorb to proton-active functional groups on bacterial cell envelopes (e.g., Beveridge and Murray, 1976; Fortin and Beveridge, 1997; Daughney et al., 2002; Fein, 2006; Kenney and Fein, 2011), affecting the speciation and distribution of these metals in geologic systems. Recent studies (e.g., Guiné et al., 2006; Mishra et al. 2009; 2010) have shown that at least some bacterial cell envelopes contain proton-active sulfhydryl functional groups. Because Hg binds readily and strongly to sulfur compounds (Compeau and Bartha, 1987; Winfrey and Rudd, 1990; Benoit et al., 1999), bacterial adsorption of Hg may dramatically affect the distribution, transport and fate of Hg in geologic systems.

Natural organic matter (NOM) is present in nearly every near-surface geologic system, and complexation reactions between metals and NOM can dramatically change the behavior of the metals in the environment (McDowell, 2003; Ravichandran, 2004). NOM molecules contain a range of functional group types, including carboxyl, phenol, amino, and sulfhydryl groups, that have the potential to create highly stable complexes with metal ions across the pH range (Ephraim, 1992; Ravichandran et al., 1999; Drexel et al., 2002; Haitzer et al., 2002; Croué et al., 2003; Ravichandran, 2004). Hg binds strongly to the sulfhydryl groups present within the NOM structure (Dong et al., 2011; Muresan et al., 2011). The relative thermodynamic stabilities of Hg-NOM and Hg-bacteria complexes are not well known. Depending on these relative stabilities, the formation of metal-NOM complexes may decrease adsorption of Hg to bacteria cell envelopes due to a competitive ligand effect, or under certain conditions may increase adsorption of Hg to bacteria due to ternary complexation with NOM. For example, investigating Pb, Cu, and Ni separately, Borrok et al. (2007) found that ternary metal-FA-bacteria complexes form, and that the importance of the complexes is strongly affected by pH. Conversely, Wightman and Fein (2001) found that the presence of NOM decreases the amount of Cd
where \( \text{Hg species}^{x^+} \) represents the specific aqueous Hg species considered, \( \text{R-A}_i^- \) represents the deprotonated cell or FA binding site, \( \text{(R-A}_i^-)(\text{Hg species}) \) represents the Hg-bacterial cell envelope or Hg-FA complex, and the \( 'x' \) represents the charge of the aqueous Hg species.

Stability constants for each of the Hg-bacterial cell envelope and Hg-FA complexes are expressed as the corresponding mass action equation for Reaction (10):

\[
(11) \quad K_{ads} = \frac{[(R - A_i)(\text{Hg species})^{(x-1)^+}]}{a \ (\text{Hg species}^{x^+}) [R - A_i^-]}
\]

where \( K_{ads} \) is the thermodynamic equilibrium constant for Reaction (10), the square brackets represent concentrations in mol L\(^{-1}\), and \( a \) represents the activity of the species in parentheses.

I used FITEQL 2.0 (Westall, 1982) for the equilibrium thermodynamic modeling of the adsorption data, using the aqueous speciation equilibria and equilibrium constants given in Table 12, and using the Davies equation within FITEQL to calculate activity coefficients. Because all of my experiments were conducted at the same ionic strength, I applied a non-electrostatic model to account for the Hg adsorption data. Bacterial site concentrations and acidity constants used in the calculations for \textit{B. subtilis}, for \textit{S. oneidensis}, and for \textit{G. sulfurreducens} are from Fein et al. (2005), Mishra et al. (2010), and Dunham-Cheatham et al. (2012), respectively. The objective of the modeling exercise was not to construct precise site-specific mechanistic binding models, but rather to provide a quantitative means of estimating the competitive binding of bacteria and FA under a range of relative concentration conditions. Toward this end, because specific binding constants for Hg with each site type on the FA molecule are not known, I modeled Hg binding with the FA as a single complexation reaction between Hg\(^{2+}\) and the deprotonated form of a generic FA site. I assumed that this generic binding site exhibits an
**TABLE 12**

HG REACTIONS USED IN THE SPECIATION MODELING

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Log K</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{O} - \text{H}^+ = \text{OH}^-$</td>
<td>-14.00</td>
</tr>
<tr>
<td>$\text{H}_2\text{CO}_3^0 - \text{H}^+ = \text{HCO}_3^-$</td>
<td>-6.355</td>
</tr>
<tr>
<td>$\text{H}_2\text{CO}_3^0 - 2\text{H}^+ = \text{CO}_3^{2-}$</td>
<td>-16.67</td>
</tr>
<tr>
<td>$\text{H}_2\text{CO}_3^0 - \text{H}_2\text{O} = \text{CO}_2^0$</td>
<td>2.770</td>
</tr>
<tr>
<td>$\text{Na}^+ + \text{H}_2\text{CO}_3^0 - 2\text{H}^+ = \text{NaCO}_3^-$</td>
<td>-15.41</td>
</tr>
<tr>
<td>$\text{Na}^+ + \text{H}_2\text{CO}_3^0 - \text{H}^+ = \text{NaHCO}_3^0$</td>
<td>-6.60</td>
</tr>
<tr>
<td>$\text{Na}^+ + \text{H}_2\text{O} - \text{H}^+ = \text{NaOH}^0$</td>
<td>-14.2</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + \text{H}_2\text{O} - \text{H}^+ = \text{HgOH}^+$</td>
<td>-3.40</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + 2\text{H}_2\text{O} - 2\text{H}^+ = \text{Hg(OH)}_2^0$</td>
<td>-5.98</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + 3\text{H}_2\text{O} - 3\text{H}^+ = \text{Hg(OH)}_3^-$</td>
<td>-21.1</td>
</tr>
<tr>
<td>$2\text{Hg}^{2+} + \text{H}_2\text{O} - \text{H}^+ = \text{Hg}_2\text{(OH)}^{3+}$</td>
<td>-3.30</td>
</tr>
<tr>
<td>$3\text{Hg}^{2+} + 3\text{H}_2\text{O} - 3\text{H}^+ = \text{Hg}_3\text{(OH)}^{3+}$</td>
<td>-6.40</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + \text{H}_2\text{CO}_3^0 - 2\text{H}^+ = \text{HgCO}_3^{0}$</td>
<td>-3.91</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + \text{H}_2\text{CO}_3^0 - \text{H}^+ = \text{HgHCO}_3^+ \text{Hg}^{2+} + \text{H}_2\text{CO}_3^0 + \text{H}_2\text{O} - 3\text{H}^+ = \text{Hg(OH)CO}_3^-$</td>
<td>0.42</td>
</tr>
<tr>
<td>$\text{B}_3^+ + \text{H}^+ = \text{B}_3\text{H}_0^0$</td>
<td>3.30</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
</tr>
<tr>
<td>$\text{Shewanella oneidensis}$</td>
<td>3.30</td>
</tr>
<tr>
<td><strong>Geobacter sulfurreducens</strong></td>
<td>3.36</td>
</tr>
<tr>
<td>$\text{B}_3^+ + \text{H}^+ = \text{B}_3\text{H}_0^0$</td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>4.80</td>
</tr>
<tr>
<td><strong>Shewanella oneidensis</strong></td>
<td>4.80</td>
</tr>
<tr>
<td><strong>Geobacter sulfurreducens</strong></td>
<td>4.81</td>
</tr>
<tr>
<td>$\text{B}_3^+ + \text{H}^+ = \text{B}_3\text{H}_0^0$</td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>6.80</td>
</tr>
<tr>
<td><strong>Shewanella oneidensis</strong></td>
<td>6.70</td>
</tr>
<tr>
<td><strong>Geobacter sulfurreducens</strong></td>
<td>6.49</td>
</tr>
<tr>
<td>$\text{FA}^+ + \text{H}^+ = \text{FA-H}_0^0$</td>
<td>5.85</td>
</tr>
</tbody>
</table>

(a) Powell et al., 2005.
(b) Martell and Smith, 2001.
(c) Fein et al., 2005.
(d) Mishra et al., 2010.
(e) Dunham-Cheatham et al., 2012.
(f) Calculated as the average of all reported pKa values in Table 2 from Borrok and Fein (2004). Assumed total site concentration is the sum of the average site concentrations for the individual FA sites: 5.50 x 10^-3 moles of sites per gram of humic substance.
strongly with Hg (Xia et al., 1999; Hesterberg et al., 2001; Drexel et al., 2002; Haitzer et al., 2002; 2003), leading to effective competition with bacterial cell envelopes which also contain proton-active sulfhydryl functional groups (Guiné et al., 2006; Mishra et al., 2009; 2010). In the experimental systems, FA binding sites outnumber those present on the bacteria. For example, 50 mg L\(^{-1}\) FA corresponds to approximately 2.8 x 10\(^{-4}\) moles of sites L\(^{-1}\) (Borrok and Fein, 2004), while 0.2 gm L\(^{-1}\) \(B.\) subtilis biomass contains 4.7 x 10\(^{-5}\) total moles of sites L\(^{-1}\). At pH 8, 50 mg L\(^{-1}\) FA does diminish the extent of Hg adsorption, but only from approximately 70% (with no FA present) to 60%. It appears that given equal site concentrations, bacterial binding of Hg would dominate the competition with FA.

4.5 Discussion

The experimental results presented here suggest that bacterial cell envelope functional groups and FA functional groups exhibit reasonably similar binding affinities for Hg under the experimental conditions. Hg binding onto the bacterial cell envelopes is extensive, and although Hg binds strongly with FA, especially with the sulfhydryl groups present within FA (Xia et al., 1999; Hesterberg et al., 2001; Drexel et al., 2002; Haitzer et al., 2002; 2003), the presence of even up to 50 ppm FA with only 0.2 gm (wet mass) L\(^{-1}\) of bacteria does not cause the speciation of Hg to be dominated by the FA. The results strongly suggest that there is a fairly equal competition between the bacterial and FA binding sites for the available Hg.

In order to quantify the competitive binding, I used a semi-empirical surface complexation approach. First, I used the FA-free adsorption data at pH 4, 6, and 8 to solve for equilibrium constants for the following Hg\(^{2+}\) adsorption reactions, respectively:

\[
(12) R-A_1^- + Hg^{2+} \leftrightarrow R-A_1^-Hg^+
\]


SORPTION OF YTTRIUM AND THE RARE EARTH ELEMENTS ON THE
MARINE MACROALGA ULVA LACTUCA

By

Alison M. Zoll

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2011

Advisory Committee:
Assistant Professor Johan Schijf, Chair
Associate Professor Carys L. Mitchelmore
Professor Neil V. Blough
than those on organic substrates.

In order to better understand metal-organic interactions, two types of organic matter are commonly selected for study. Some utilize homogenized organic substrates such as natural organic matter standards or colloidal humic acid mixtures, while others focus on a single organism. Most metal-organic sorption studies in this latter category have focused on freshwater and terrestrial organisms, such as bacteria, ferns, fungi, and yeast (Fowle and Fein, 1999; Wang et al., 2001; Boyanov et al., 2003; Ding et al., 2005; Wei et al., 2005a, b; Naeem et al., 2006; Ha et al., 2010; Mishra et al., 2010). These studies have focused on identifying metal-binding functional groups and determining binding-site pK_a values (Table 1.1), and many of the organisms have a high affinity for metal ions in solution, making it easy to measure relative changes in metal concentration. Sorption modeling in these studies often use empirical partitioning approaches (i.e. Freundlich isotherms), where sorption is described in terms of a generic partition between the solution and the surface, without consideration for different types of surface sites or influence from solution chemistry. Unfortunately, this approach provides only limited mechanistic and stoichiometric information about surface and solution reactions (Davis and Kent, 1990).

Despite the wide range of organisms studied, there is remarkable consistency in the identity and properties of the functional groups that interact with trace metals. Similar pK_a values across Gram-negative bacteria, Gram-positive bacteria (which differ fundamentally in their extracellular molecular structure), and fungal species suggest similar functional groups are present to participate in metal sorption. Authors often assign similar site identities based on the similar pK_a values, consistent with the known
composition of cellular organic matter (Table 1.1). These functional group identities in some cases have been confirmed with spectroscopic techniques (for example, as in MISHRA et al., 2010). The four main metal-complexing functional groups generally identified in freshwater and terrestrial species are sulfonyls, carboxylates, phosphates, and amines or phenols (Fig. 1.1A).

Table 1.1: Summary of pKₐ values and possible site identities from potentiometric titrations of various model organic organisms.

<table>
<thead>
<tr>
<th></th>
<th>P. agglomeransᵃ</th>
<th>S. oneidensisᵇ</th>
<th>B. subtilisᶜ</th>
<th>S. cerevisiaeᵈ</th>
<th>possible site identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKₐ(1)</td>
<td>–</td>
<td>3.3 ± 0.2</td>
<td>3.3</td>
<td>3.4 ± 0.4</td>
<td>sulfonyl</td>
</tr>
<tr>
<td>pKₐ(2)</td>
<td>4.3 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>4.7</td>
<td>5.0 ± 0.2</td>
<td>carboxyl</td>
</tr>
<tr>
<td>pKₐ(3)</td>
<td>6.9 ± 0.5</td>
<td>6.7 ± 0.4</td>
<td>6.8</td>
<td>6.8 ± 0.4</td>
<td>phosphate</td>
</tr>
<tr>
<td>pKₐ(4)</td>
<td>8.9 ± 0.5</td>
<td>9.4 ± 0.5</td>
<td>8.9</td>
<td>8.9 ± 0.6</td>
<td>amine/phenol</td>
</tr>
</tbody>
</table>

ᵃ NGWENYA et al. (2003); ᵇ MISHRA et al. (2010); ᶜ FEIN et al. (2005); ᵈ NAEEM et al. (2006)

Figure 1.1. A. The most commonly identified organic functional groups responsible for metal complexation in bacteria and fungi. Shown from left to right are carboxyl, sulfonyl, phosphate, amine and carbolic acid, the simplest of phenols. Phenols include any compound where hydroxyl groups are bound to aromatic rings. B. Structure of sulfate functional groups, which are commonly found on marine macroalgae.
Sulfonyl groups generally have low pK\textsubscript{a}s, and have been found in both Gram-negative and Gram-positive bacteria to participate in cadmium sorption at low pH (BOYANOV et al., 2003; HA et al., 2010; MISHRA et al., 2010). Sulfonyl groups may also be present on *Saccharomyces cerevisiae* (a fungal species), whose low pK\textsubscript{a} matches those found in bacterial species for sulfonyl groups (NAEEM et al., 2006). Carboxylate groups participate in Cd sorption in Gram-positive and Gram-negative bacteria (*Bacillus subtilis*, *Shewanella oneidensis*, *Pantoea agglomerans*) and lead, copper and cadmium sorption on the fungal species *Aspergillus niger* (KAPOOR and VIRARAGHAVAN, 1997; BOYANOV et al., 2003; NGWENYA et al., 2003; HA et al., 2010; MISHRA et al., 2010). Carboxyl pK\textsubscript{a}s are usually found in the range of 4 – 6 on these species. Phosphate groups generally have pK\textsubscript{a}s ~ 6 and have been identified in metal sorption studies on bacteria and fungi as well (BOYANOV et al., 2003; NGWENYA et al., 2003; NAEEM et al., 2006; HA et al., 2010; MISHRA et al., 2010). High pK\textsubscript{a} (9 \text{–} 12) groups are typically attributed to nitrogen-containing amines or to phenol groups, which are common across all types of organic matter. High-pK\textsubscript{a} groups have been found to participate in sorption of many different metals (Cd, Pb, Th, U, Zn) on both bacteria and fungi (TSEZOS and VOLESKY, 1982a, b; GADD, 1990; KAPOOR and VIRARAGHAVAN, 1997; BOYANOV et al., 2003; NAEEM et al., 2006; HA et al., 2010; MISHRA et al., 2010).

Sorption studies with marine species are far less common than those for freshwater organisms, perhaps due to inherent difficulties associated in isolating and culturing organisms such as marine bacteria. The majority of marine work has focused on macroalgae, such as the brown algae *Sargassum fluitans*, *Petalonia fascia*, and *Colpomenia sinuosa*, as well as the green alga *Ulva fasciata* (SCHIEWER and VOLESKY,
coefficient $K_S$ (Eq. 1.4), which describes the binding of dissolved metals (M) to functional groups on a solid substrate (S), resulting in the formation of surface complexes (S-M):

$$K_S = \frac{[S-M]}{[M][S]}$$

Calculating meaningful values of $K_S$ requires properly quantifying the concentrations of all species in Eq. (1.4). As outlined above, the presence of colloidal material in the dissolved phase can confound proper estimates of dissolved metal concentrations, [M]. This has substantial implications for equilibrium models, as the dissolved metal concentration is not only used directly to calculate [M], but often the concentration of surface-bound metal, [S-M], as well (see for example QUINN et al., 2006a; NGWENYA et al., 2009; HA et al., 2010; MISHRA et al., 2010). In these cases, [S-M] is calculated by subtracting the equilibrium dissolved metal concentration ([M]) from the total metal concentration ([M]_init). This is generally more accurate than attempting to directly measure [S-M], so long as S is the only sorbent present in the experimental solution. The equilibrium dissolved metal concentration is usually measured by filtering with 0.22 µm syringe filters, which do not capture the colloidal fraction. Therefore, the presence of colloids will cause overestimation of [M] and underestimation of [S-M] and hence $K_S$. It should be noted that in metal sorption studies on inorganic surfaces, colloid-bound metals do not seem to be important, as has been demonstrated for YREE sorption on hydrous ferric oxide and manganese oxide (SCHIJF and MARSHALL, 2011; K. Marshall, pers. cmm.).

I investigated the effects of colloid-bound metal formation in sorption experiments on $U. lactuca$ at 0.05, 0.5 and 5.0 M ionic strength. I found that colloids
solutions is released almost immediately from the dried *U. lactuca* in the “short test” experiments. Therefore, it is possible that rinsing the dried standard may only remove a portion of the colloidal material. As metal sorption experiments may use either fresh or dried biomass (Texier et al., 2000; Turner et al., 2007, 2008; Mishra et al., 2010), it is prudent to always check for the presence of colloid-bound metals.

In addition to metal sorption studies, both living and dried *U. lactuca* have also been used in bioremediation and biomonitoring studies (Suzuki et al., 2005; El-Sikaily et al., 2007), where colloidal artifacts have been typically unaccounted for and unrecognized. The presence of colloid-bound metals could have serious implications for these efforts. Macroalgae such as *U. lactuca* have high surface reactivity, worldwide distribution, and a natural ability to grow well in polluted environments, making them ideal biomonitor for dissolved metals in coastal systems. Biomonitor can be used to monitor water quality and the bioavailability of dissolved metals by measuring metal concentrations from an organism’s tissue. If *U. lactuca* or other biomonitor release organic colloids that interact strongly with trace metals, it will greatly influence the relationship between tissue and ambient water concentrations. Metal-bound colloids could also have an effect on the bioavailability of the metals to other organisms in the surrounding area. Those who wish to use *U. lactuca* in bioremediation studies will also need to determine whether colloid-bound metals are present. Materials engineers have proposed using dried *U. lactuca* biomass as a biofilter in packed columns to treat metal-contaminated environments (Zeroual et al., 2003; Suzuki et al., 2005; Herrero et al., 2006; El-Sikaily et al., 2007), as a potential alternative to more expensive synthetic cation-exchange resins. BCR-279 is very similar to the biomass used in these studies, and
(Serrano et al., 2009), and YREE sorption on iron hydroxides (Quinn et al., 2006a; Schijf and Marshall, 2011). Davis et al. (1998) also argued that because the surface charge behavior of complex environmental samples is not well understood, NEMs are a more appropriate choice over electrostatic SCMs for modeling metal sorption on organic matter.

Although NEMs have been used to describe metal sorption on bacteria, fungi, soils, etc. (Fowle and Fein, 1999; Markai et al., 2003; Naeem et al., 2006; Deo et al., 2010; Mishra et al., 2010), these studies have utilized chemical equilibrium programs (e.g. FITEQL) to model sorption data and provide best-fit parameters. Programs such as these solve a system of equilibrium reactions and mass balance equations to provide a numerical fit of the data, and they require detailed knowledge of all components in the system being studied, including metal-binding site concentrations, protonation constants, and all reactions taking place. Many of these details are not known for most types of organic matter, which makes it difficult to use this approach without making a number of simplifying assumptions.

The majority of sorption studies, including those that utilize FITEQL, titrate the sorbent with a metal solution at constant pH, an approach lending itself to a description with generic (e.g. Langmuir, Freundlich, Frumkin etc.) sorption isotherms (Stumm and Morgan, 1996). These studies regard sorption as a bulk partitioning of the metal between the solution and the surface. Isotherms are fit to log-log plots of the degree of metal sorption as a function of dissolved metal concentrations, using site densities and conditional surface complexation constants as adjustable parameters. This is useful for complex organic substrates since the surface is treated as a generic, homogeneous
(pK_C ~ 9) also supports the presence of a phenol, which typically have pK_a values of ~ 9 – 10. Most studies have attributed high pK_a groups to amines (for example GONZALEZ-DAVILA et al., 1995; YEE et al., 2004), but phenols and amines share similar pK_a values and cannot be well distinguished by spectroscopic techniques (such as Extended X-ray Absorption Fine Structure). The unique chemical properties of the YREEs and the pattern of stability constants make it possible to distinguish these two groups, and group C here clearly suggests YREE binding to a phenol group. It is also more likely that the YREEs would bind with a phenol rather than an amine because the YREEs generally have a lower affinity for nitrogen-bearing groups.

Stability constants for MOH^{2+} binding to group C (log β_C^+) have a similar pattern to free-metal binding to group C, though the trend is somewhat suppressed, which could be due to different surface affinities for the MOH^{2+} vs. the M^{3+} species. It is likely that the free metal will have a slightly higher affinity for the surface due to its higher charge (3+) and smaller ionic radius.

The second functional group stability constant pattern (log β_B) did not match that of any known YREE complexation constants, but it is possible that group B is a phosphate complex. Unfortunately, there is no published pattern of measured YREE-phosphate stability constants available for comparison. Phosphate is a component of cell membrane phospholipids, and other work with microbial biomass have attributed mid-pK_a groups (pK_a s ~6) to a phosphate complex (BOYANOVA et al., 2003; NGWENYAYA et al., 2003; HA et al., 2010; MISHRA et al., 2010). Phosphate groups typically have pK_a values in the range of ~6 – 7, which fit well with the pK_B (~6) used in the NEM fits.

The similarity between log OAcβ_1 and log β_A is greater for 0.5 M than 5.0 M,
information about the system. Three monoprotic functional groups are present on the algal surface, where both free metal and hydrolyzed metal participate in sorption. This result also verifies conclusions in SCHIJF and EBLING (2010) finding the same number of functional groups, the same proton stoichiometry, and the same pKₐ values.

The final research objective was to use YREE patterns of stability constants to help determine the identity of *U. lactuca*'s functional groups. While this technique is a powerful one, the results are ultimately inferential in that a YREE pattern provides evidence for the presence of a functional group, but cannot conclusively determine a functional group’s structure. The LFERs suggest the presence of a carboxyl and phenol group, which are consistent with the known composition of organic matter, known functional group pKₐ values, and YREE chemical properties. However, the results ultimately cannot provide definitive structures for groups A and C. The technique is also limited in that if a YREE complexation constant pattern has not been measured for the type of functional group present, it cannot be identified (as was the case for group B). Nevertheless, LFERs for the YREEs provide important evidence for the presence of certain functional groups over others in the same pKₐ range, as is the case for phenol and amine groups.

One common method for determining functional group identities is to use spectroscopic techniques such as EXAFS in conjunction with metal sorption experiments (NGWENYA et al., 2009; MISHRA et al., 2010). Both approaches will generally compliment and inform one another so that together they offer a comprehensive picture of the thermodynamic and the structural properties of the system. EXAFS data provides information about a metal’s local bonding structure, such as interatomic distances and


Removal of Copper and Zinc Ions onto Biomodified Palm Shell Activated Carbon

Gulnaziya Issabayeva and Mohamed Kheireddine Aroua

Abstract—commercially produced in Malaysia granular palm shell activated carbon (PSAC) was biomodified with bacterial biomass (Bacillus subtilis) to produce a hybrid biosorbent of higher efficiency. The obtained biosorbent was evaluated in terms of adsorption capacity to remove copper and zinc metal ions from aqueous solutions. The adsorption capacity was evaluated in batch adsorption experiments where concentrations of metal ions varied from 20 to 350 mg/L. A range of pH from 3 to 6 of aqueous solutions containing metal ions was tested. Langmuir adsorption model was used to interpret the experimental data. Comparison of the adsorption data of the biomodified and original palm shell activated carbon showed higher uptake of metal ions by the hybrid biosorbent. A trend in metal ions uptake increase with the increase in the solution’s pH was observed. The surface characterization data indicated a decrease in the total surface area for the hybrid biosorbent; however the uptake of copper and zinc by it was at least equal to the original PSAC at pH 4 and 5. The highest capacity of the hybrid biosorbent was observed at pH 5 and comprised 22 mg/g and 19 mg/g for copper and zinc, respectively. The adsorption capacity at the lowest pH of 3 was significantly low. The experimental results facilitated identification of potential factors influencing the adsorption of copper and zinc onto biomodified and original palm shell activated carbon.

Keywords—Adsorption, biomodification, copper, zinc, palm shell carbon.

I. INTRODUCTION

An increased concentration of heavy metals in the environment is a result of the extensive development of many industries that use heavy metals in the goods/products manufacturing. Copper and zinc are the oldest metals ever used, like a bronze and a brass, and these materials have been very important in the development of human progress. For instance, building construction is the single largest market of copper consumption followed by electronics and electronic products, transportation, industrial machinery, and consumer products. Copper byproducts from manufacturing and obsolete copper products are easily recycled and contribute significantly to copper supply to industries. However, considerable amounts of copper are discharged into wastewater streams, although at lower concentrations but nevertheless polluting the aqueous environment. Zinc is also a heavy metal, it is also an essential element in bio-molecules and its uptake by living organisms is important. However, excessive concentrations of zinc from metal refining and manufacturing processes wastewaters result in damages to human health causing metabolism disruptions and arteriosclerosis [1]. Conventional technologies used for metals removal of wastewater such as precipitation, coagulation, ion-exchange and reverse osmosis are certainly efficient; however they are also expensive and often generate significant volumes of sludge, solid waste. The final residues are subject to landfill disposal or safe storage, and such practices increase overall stock of heavy metals in the environment. Development of modern technologies in the waste treatment/minimization field is often directed towards utilization of such materials that correspond to the basic requirements: effectiveness and low cost. The major economy sector of Malaysia is production of palm oil that has greatly expanded in the past decades making Malaysia a leading exporter worldwide. Palm shells are generated in great quantities in this sector and along with other byproducts of the production process are disposed as waste. It is only in recent years the research interest to palm shell waste has increased due to the fact that this material was shown to be an excellent source of high quality and low cost activated carbon production. Another plentiful source of low-cost biosorbents is biomass of various microorganisms. It was shown that microbial biomass can successfully remove metal ions from aqueous solutions via mechanisms of biosorption and bioaccumulation [2]-[6]. The objective of our study was to develop a hybrid biosorbent which combines excellent adsorptive properties of the palm shell activated carbon and sorption capacity of the bacterial biomass of Bacillus subtilis to remove copper and zinc ions in aqueous solution. In this paper, the results for copper and zinc removal at different pH of the solutions containing varying concentrations of metal ions are presented; also characterization data of the original and biomodified adsorbents are shown.

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surface area for the biomodified PSAC is lower than that of the original PSAC. Comparison of the adsorption capacities for copper and zinc removal, it could be noticed that zinc shows relatively higher predisposition to be adsorbed on the biomodified PSAC as compared to copper ions. Such observation perhaps relates to the properties of two metals which are quite similar in terms of atomic weight and radius but different in terms of electronegativity. Also, specific interactions between metal ions and surface groups present on the biosorbent surface play important role.

It is believed that the crucial aspect of the research on the development of a hybrid biosorbent is optimization of the biomodification procedure. The research is considered to be promising as the experimental data indicated important aspects of the biomodification procedure which require further modification and optimization to develop a high efficiency hybrid adsorbent for heavy metals removal in wastewaters.

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Adsorption behaviour of cadmium by Bacillus cereus M$^{16}$: some physical and biochemical studies

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ABSTRACT

Adsorption behaviour of cadmium from its aqueous solution by growing and non-growing cells of a mutant strain of Bacillus cereus M$^{16}$ has been studied to explore the possibility of the biomass to address environmental pollution due to this toxic metal. The results establish that about 75% and 88% of the cadmium can be removed by growing and non-growing cells of the selected strain respectively from its aqueous solution at pH 6.8, temperature 30°C, and 120 rpm shaking speed. For growing cells, inoculum size 2% and medium volume 50 mL is found to be optimum. The adsorption rate of cadmium on the biomass is very fast initially and attains equilibrium within 60 min following pseudo second-order rate model ($R^2 = 0.99$). The equilibrium adsorption isotherm can be best described by Langmuir–Freundlich dual model ($R^2 = 0.99$) indicating that both physisorption and chemisorption take place simultaneously. Cadmium can be desorbed from the loaded biomass using mineral acids (0.1 M).

Keywords: cadmium, biosorption, Bacillus cereus M$^{16}$, pseudo second order, Langmuir isotherm

INTRODUCTION

Pollution due to heavy metals has become a serious environmental problem in the recent years. Heavy metals, being non biodegradable, represent an important environmental problem due to their toxic effects and their tendency to accumulate throughout the food chain leads to serious ecological and health problems (Volesky and Holan, 1995; Bailey et al., 1999). One such heavy metal, cadmium, along with its compounds, is widely used in pigments, as heat stabilizers for plastics, for corrosion resistance of steel and cast iron, metal plating, phosphate fertilizer, mining, pigments, alloy industries, in soldering and brazing, and in the battery industry (Ni–Cd batteries) (Niragu and Sprague, 1987). Cadmium is highly toxic and there is some evidence that it is carcinogenic (Hiatt and Huff, 1975). The harmful effects of cadmium include a number of acute and chronic disorders, such as “itai-itai” disease, renal damage, emphysema, hypertension, and testicular atrophy (Nordberg et al., 1993). When ingested by human beings, cadmium that is not excreted immediately has a long half-life of several hundred days, so that a low-dose exposure over a long period of time can lead to a high body burden. According to the Environmental regulation authority in India maximum limit of cadmium for discharge into drinking water and water bodies is 0.01 and 2.0 ppm respectively (Bayrammoglu et al., 2002). Although over several decades the removal of toxic heavy metals from the industrial wastewaters used the most common physio-chemical processes such as lime precipitation, ion exchange, ultrafiltration or reverse osmosis, the cost-effectiveness of the processes were limited. Moreover, they often generated huge amount of metal-bearing sludge causing difficulties in disposal.

Bioaccumulation and biosorption possess good potential to replace conventional methods for the removal of dyes/metal (Volesky and Holan, 1995). The bioaccumulation process is defined as the transfer of organic or inorganic pollutants into the interior of living cells (Barron, 1995). The process involves use of growing cells in metal ion removal (Inthorn, 2001; Dursun et al., 2003). Conversely biosorption is a metabolically passive process which involves uptake of toxicants by dead or inactive biological materials. Both the processes has distinct advantages over conventional methods, viz., the process does not produce chemical sludge. It can be highly selective, more efficient, easy to operate, and hence cost effective for the treatment of large volume of wastewaters containing low metal concentrations (Deans and Dixon, 1992; Puranik and Paknikar, 1997).

Microorganisms have evolved various measures to respond to heavy metal stress. Interaction between metals and microbial cells can occur through adsorption to cell surfaces through metabolically assisted accumulation within
Adsorption behaviour of cadmium

the cell or as metal complexes with extracellular microbial metabolites. Moreover, the performance of a biosorbent depends on biomass characteristics and also the microenvironment of the target solution i.e., solution pH, temperature, interaction with co-ions, etc. (Krauter et al., 1996; Fein et al., 1997; Fein et al., 2001; Borrok et al., 2004; Mishra et al., 2009; Mishra et al., 2010; Tsezos, 1995).

The present work was undertaken to evaluate the biosorption (Cd²⁺) capacity of Bacillus cereus M¹¹⁶. The uptake capacity of the Bacillus cereus M¹¹⁶ was studied as a function of initial pH, dose of the biosorbent, initial metal ion concentration, time of contact. The study includes various other aspects such as the analysis of the mono-component biosorption data using Langmuir–Freundlich dual isotherm model, equilibrium kinetic studies following pseudo second order rate equations.

METHODS AND MATERIALS

Microorganism

The strain Bacillus cereus M¹¹⁶ (Bera et al., 2007) used in this study was isolated in our laboratory and maintained by monthly sub culturing in nutrient agar and stored at 4°C.

Chemicals

The chemicals and ingredients of the microbiological media used in this study were obtained from Merck, and HiMedia laboratory, India respectively.

Preparation of standard cadmium solution

A stock solution of cadmium (500 mg L⁻¹) was prepared by dissolving required amount of cadmium chloride in double distilled water. Standard solutions of metal ions were prepared by appropriate dilution of the stock solution having pH of the solutions adjusted with 0.1 M HCl or NaOH as required.

Estimation of cadmium

Cadmium ion concentration was measured by flame atomic absorption spectrophotometer with reference to calibration curves drawn from the standard cadmium stock solutions (0.2–6 mg L⁻¹). The amount of cadmium uptake by the biosorbent was calculated from the following mass balance equation,

q = (Cᵢ – Cᵢ) V/1000W

where q is the amount of metal ion uptake, Cᵢ the metal ion concentration after adsorption, Cᵢ the initial metal ion concentration, W the amount of biosorbent and V the volume of the solution.

Biosorption experiments

For the experiments with growing cells of Bacillus cereus M¹¹⁶ inoculum was prepared by transferring one loop-full of cell from a slant culture to 50 mL fermentation medium containing g L⁻¹: beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 5.0 in 250 mL Erlenmeyer flask and incubated at 30°C for 24 h, under shaking condition 120 rpm. The solution pH was adjusted to 6.0 by using 1N HCl or 1N NaOH. Fermentation medium (50 mL in 250 mL Erlenmeyer flask) containing 15 mg L⁻¹ Cd(II) ion was inoculated with 2% (v/v) inoculum and incubated at 30°C for 48 h under the similar conditions described above. Supernatant was collected by centrifuging the fermentation broth at 5500 rpm for 15 min. Residual concentration of cadmium in the supernatant was determined using atomic absorption spectrophotometer at 228 nm. Each of the experiment was repeated three times and the average values were calculated.

Experiments with resting cells were conducted using the same culture medium containing no Cd(II) ion for the production of Bacillus cereus M¹¹⁶ biomass under similar environmental conditions. Viable biomass was harvested by centrifugation at 5,500 rpm for 10 min at room temperature and washed twice with double distilled water. Washed cells were transferred to a 50 mL solution containing 25 mg L⁻¹ Cd(II) in a 250 mL Erlenmeyer flask. The percent metal bound was taken to be the difference between the control and the final concentration of the metal in the supernatant (Gardea-Torresdey et al., 1998). All experiments were carried out with washed cells (wet), but interpretations were cited on a dry-cell weight basis. For scientific interpretations, the sorbent material dry-weight basis is preferred (Volesky, 1999). Relationships between washed biomass and dry biomass were calculated. The ratio was found to be wet cell: dry cell = 5.5 : 1.

To investigate the effect of pH on the adsorption process, the solution pH was adjusted at a range of 4.5–7.5. Experiments with growing bacterial cell were carried out varying the initial metal ion concentration, inoculum volume and the volume of the medium at a range of 15–500 mg L⁻¹, 1–4% and 40–100 mL respectively at 120 rpm shaking speed and 30°C temperature. In the biosorption experiments with resting cells unless otherwise conditions stated, the initial metal ion concentration, temperature, biomass dose were 25 mg L⁻¹, 30°C and 500 mg (wet weight) respectively. Biosorption equilibrium isotherms were determined during contact of the biomass with varying concentrations (15–500 mg L⁻¹) of Cd(II). Kinetic studies were carried out with 25, 50 and 100 mg L⁻¹ cadmium solutions at pH 6.0 and 30°C temperature. The concentration of cadmium ion in the solution was determined at different time intervals up to 180 min. Each data point was obtained from individual flasks and therefore no correction was necessary due to withdrawal of sampling volume.

RESULTS AND DISCUSSION

Effect of environmental conditions on biosorption of cadmium using growing cells of Bacillus cereus M¹¹⁶

Effect of Cd(II) on specific growth rate of Bacillus cereus M¹¹⁶

50 mL nutrient broth medium in 250 mL Erlenmeyer flask was inoculated with 1 mL 24 h cell suspension of Bacillus


EXPERIMENTAL STUDIES OF GEOCHEMICAL REACTIONS THAT AFFECT
THE MOBILITY OF METALS AND NANOPARTICLES OF ENVIRONMENTAL
INTEREST

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

by

Lindsay Ann Seders

Jeremy B. Fein, Director

Graduate Program in Civil Engineering and Geological Sciences
Notre Dame, Indiana
October 2010
Despite the indications that bacterial exudates can affect geochemical processes, there has been virtually no work conducted to quantify their environmental concentrations or characteristics. The research described in Chapter 4 was designed to:

(1) use potentiometric titrations to determine the extent of proton binding exhibited by bacterial exudates made using a Gram-positive (*Bacillus subtilis*) and a Gram-negative (*Shewanella oneidensis*) bacterial species with a range of initial bacterial concentrations (40-100 g/L bacteria) and in solutions with a range of ionic strengths (0.01-0.3 M);

(2) calculate the acidity constants and the concentrations of proton binding sites found in the exudates using chemical equilibrium modeling;

(3) compare the calculated values for exudates produced from the two bacterial species and at different bacterial concentrations and ionic strengths in order to better understand how differences in cell wall characteristics may influence the properties of bacterial exudates and how changing solution chemistry may affect the production of bacterial exudates;

(4) compare the proton binding behavior of bacterial exudates with that determined previously for bacterial cells (Borrok et al., 2005; Fein et al., 2005; Mishra et al., 2010);

(5) use FTIR spectrometry to provide an initial qualitative description of the exudates from the two bacterial species.

The proton binding properties determined for the bacterial exudates in this study are important as they are among the first of their kind and will provide a framework for understanding the interactions of bacterial exudates with other components in geologic and engineered systems.
4.2. Materials and Methods

4.2.1. Experimental

4.2.1.1. Bacterial Growth and Exudate Preparation

The bacterial species used in these experiments were the aerobic, Gram-positive soil bacterium *Bacillus subtilis* and the facultatively anaerobic, Gram-negative bacterium *Shewanella oneidensis* MR-1, originally collected from lake sediment. The cell wall constituents and reactivity of *B. subtilis* (Beveridge and Murray, 1980; Fein et al., 2005) and *S. oneidensis* (Venkateswaran et al., 1999; Ha et al., 2010; Mishra et al., 2010) have been well characterized. *B. subtilis* and *S. oneidensis* were initially grown aerobically in 3 mL of trypticase soy broth (TSB) spiked with 0.5% yeast extract (YE) at 32°C with shaking for 24 hours. Bacteria were then transferred to 2 L of the TSB/YE solution to grow under the same conditions and harvested after 24 hours once reaching the stationary growth phase. The washing procedure consisted of centrifugation at 8500 rpm, followed by resuspension of the cells in a NaClO₄ solution of the desired ionic strength (0.01, 0.1, or 0.3 M), repeated five times. The bacteria were then suspended in NaClO₄ of the same ionic strength with which they were washed at a concentration of approximately 40, 80, or 100 g/L bacteria (wet mass). Each bacterial suspension was adjusted to pH 5.5 using aliquots of 6 M HNO₃ and rotated for 2.5 hours, with pH readjusted every 30 minutes. After rotating for 2.5 hours, the bacterial suspension was centrifuged at 10,000 rpm for 10-20 minutes to remove the bacterial cells. The supernatant or ‘exudate’ solution was decanted and used to conduct potentiometric titrations. A portion of the exudate solution was also diluted for measurements of the total organic carbon concentration ([TOC])
4.3.2. Exudate Titrations

Forward and reverse titrations of both *B. subtilis* and *S. oneidensis* exudate solutions (Figure 4.4 and Figure 4.6) showed full reversibility of the proton binding reactions on the timescale of the experiments. Experimental replicates from the same exudate solution, as well as from exudate solutions made at the same conditions but from a different bacterial batch, also generally showed good agreement (data not shown). The bacterial exudates exhibited a significant buffering capacity over the pH range examined in this study (2.5-10). In order to compare the buffering capacity of bacterial exudates to that typically exhibited by bacterial cells, the total buffering capacity of the exudate solutions from pH 3-9 was averaged for each combination of bacterial type, bacterial concentration, and ionic strength and compared to the total buffering capacity over the same pH range from potentiometric titrations performed previously on *B. subtilis* (Fein et al., 2005) and *S. oneidensis* (Mishra et al., 2010) bacterial cell suspensions (Table 4.1). This comparison is meaningful because the buffering capacity of the exudate solutions was normalized to the mass of bacteria used to generate those solutions, so the comparison relates the buffering capacity of a cell suspension to the buffering capacity of the exudate solution generated by that same concentration of cells.
TABLE 4.1
TOTAL BUFFERING CAPACITY (MOL/G) OF BACTERIAL EXUDATES AND BACTERIAL CELLS, CALCULATED FOR THE PH RANGE OF 3 TO 9.

<table>
<thead>
<tr>
<th>Bacterial exudates &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total buffering capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
</tr>
<tr>
<td>0.01 M, 40 g/L</td>
<td>7.8 x 10&lt;sup&gt;-6&lt;/sup&gt; ± 4.6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.01 M, 80 g/L</td>
<td>5.6 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 1.1 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.01 M, 100 g/L</td>
<td>7.2 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 6.0 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 40 g/L</td>
<td>6.0 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 3.2 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 80 g/L</td>
<td>4.4 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 2.6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 100 g/L</td>
<td>3.9 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 4.2 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.3 M, 100 g/L</td>
<td>2.8 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 2.4 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S. oneidensis</strong></td>
<td></td>
</tr>
<tr>
<td>0.01 M, 40 g/L</td>
<td>4.6 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 1.6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.01 M, 80 g/L</td>
<td>3.2 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 1.9 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.01 M, 100 g/L</td>
<td>3.0 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 4.6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 40 g/L</td>
<td>6.0 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 3.9 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 80 g/L</td>
<td>4.8 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 3.5 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 100 g/L</td>
<td>4.4 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 5.0 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.3 M, 100 g/L</td>
<td>6.0 x 10&lt;sup&gt;-5&lt;/sup&gt; ± 4.5 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial cells</th>
<th>Total buffering capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong> &lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0.01 M, 75-150 g/L</td>
<td>1.6 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 M, 75-150 g/L</td>
<td>2.8 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.3 M, 75-150 g/L</td>
<td>2.3 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S. oneidensis</strong> &lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0.1 M, 50 g/L</td>
<td>3.1 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup>This paper; <sup>b</sup>Fein et al., 2005; <sup>c</sup>Mishra et al., 2010
Furthermore, the average total site concentration calculated from 36 sets of potentiometric titration data involving either bacterial species or bacterial consortia is $3.2 \pm 1.0 \times 10^{-4}$ mol/g (Borrok et al., 2005). Thus, the total site concentration for bacterial cells varies over a relatively small range, which is significantly higher than the range observed in this study for bacterial exudates. The total site concentrations for the exudates are closer to the total site concentrations calculated by Kaulbach et al. (2005) from the algal species *P. subcapitata* ($2.77 \times 10^{-5} \pm 2.9 \times 10^{-6}$ mol/g).

4.4. Conclusions

Potentiometric titrations and chemical equilibrium modeling were used to characterize the proton binding of bacterial exudates from *B. subtilis* and *S. oneidensis* bacterial cells. The calculated total buffering capacity of the bacterial exudates decreases with increasing ionic strength for the *B. subtilis* exudates but increases for the *S. oneidensis* exudates. Total site concentrations, which are likely related to buffering capacity, also change with ionic strength following the same trends as the buffering capacities. Calculated pK$_{a(i)}$ values and FTIR spectra, however, do not vary significantly as a function of ionic strength or bacterial type and concentration. This is somewhat remarkable as the two bacterial species have very different cell surfaces.

The total site concentrations calculated for the bacterial exudate solutions are approximately an order of magnitude lower per gram of bacteria than those calculated previously for a range of bacterial species. These differences suggest that exudates, with their lower site concentrations, are less efficient at binding protons and metals than


