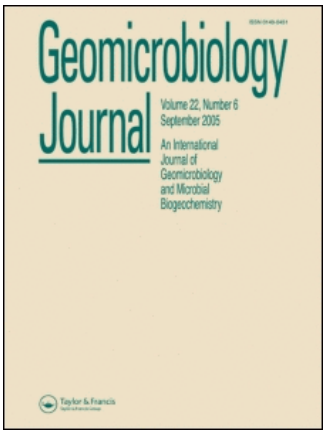

Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713722957

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Online publication date: 24 March 2011

To cite this Article Petrash, D. A. , Lalonde, S. V. , Raudsepp, M. and Konhauser, K. O.(2011) 'Assessing the Importance of Organic Matrix Materials in Biofilm Chemical Reactivity: Insights from Proton and Cadmium Adsorption onto the Commercially Available Biopolymer Alginate', Geomicrobiology Journal, 28: 3, 266 — 273
To link to this Article: DOI: 10.1080/01490451.2010.498298
URL: http://dx.doi.org/10.1080/01490451.2010.498298

Geomicrobiology Journal, 28:266–273, 2011

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490451.2010.498298

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Assessing the Importance of Organic Matrix Materials in Biofilm Chemical Reactivity: Insights from Proton and Cadmium Adsorption onto the Commercially Available Biopolymer Alginate

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Biofilms coat the exterior of most water-exposed interfaces, from the surfaces of sediments and rocks to the interior walls of fluid transport systems and even medical and dental apparatus. Composed of a diverse assemblage of microbial species growing in a matrix of extracellular polymeric substances (EPS), biofilms are well-known for their ability to sorb metals and nucleate mineral phases. In this study, purified alginate, a major polysaccharide component of some algal and bacterial EPS, was studied to ascertain its chemical reactivity towards dissolved cadmium and protons, and thus better constrain its role in overall EPS reactivity. FTIR analysis and compositional constraints based on known molecular structure indicate that alginate's geochemical behaviour is dominated by a single carboxyl functional group. Correspondingly, potentiometric titration data were best fit using a single functional group acidity constant (pK_a) and site concentration of 3.98 ± 0.01 and 1.728 ± 0.02 mol/kg, respectively, which are in agreement with typical carboxyl acidity (pK_a 3–6) and carboxyl functional group concentration based on alginate polymer composition. The logarithm of the Cd-carboxyl complexation constant (log K) was determined to be −0.52 ± 0.22, lower than carboxyl-Cd stability constants reported from independent studies of isolated microbes. Together, these results place important constraints on organic matrix contributions to overall biofilm reactivity.

Keywords extracellular polymeric substances, alginate, biofilms, titration, metal adsorption

INTRODUCTION

Microbial biofilms are complex communities comprised of a heterogeneous population of microorganisms, organic matrix materials, and associated authigenic minerals. Structural integrity within the microbial aggregate is established by means of biopolymers secreted by the microbes inhabiting the biofilm, the so-called extracellular polymeric substances (EPS), which account for 50 to 90% of biofilm organic matter (Flemming and Wingender 2001). Bacteria, algae, and fungi are all known to produce EPS, and although the composition of EPS is diverse, they typically consist of repeated chains of modified polysaccharides and their monomeric units, including carboxyl-rich uronic acids, with proteins, lipids, and nucleic acids typically present as relatively minor components (Flemming and Wingender 2001; Gadd 1993; Hunt 1986; Sutherland 2001).

Reliable thermodynamic parameters describing the adsorptive properties of EPS biopolymers are required to understand the role of EPS in a variety of geochemical processes, such as in the bioaccumulation of metals (e.g., Bhaskar and Bhosle 2006), mineral precipitation (e.g., Konhauser et al. 1994), and the use of microbial biomass in contaminated site bioremediation (e.g., Doshi et al. 2008) and wastewater treatment processes (e.g., Guibaud et al. 2006). Several studies have previously examined metal adsorption to bacteria, algae and fungi, with and without EPS, and they rather consistently show that removal of EPS can reduce metal binding capacity (Galun et al. 1983; Majidi et al. 1990; Lui and Fang 2002; Phoenix et al. 2002). However, due to spatial heterogeneities that arise during differentiation or stratification of regions within a single biofilm, the presence of authigenic and/or detrital mineral components, and the difficulties associated with isolating individual biofilm components (cells, EPS, minerals) in natural samples, the importance of individual EPS components towards overall EPS chemical reactivity is poorly understood.

Accordingly, in this work, we focused on the biopolymer alginate as an effort to better understand the geochemical role of a major polysaccharide constituent in EPS. While EPS composition varies significantly between microorganisms and for a single species according to factors such as growth rate, nutrient availability, and other stimuli (Christensen and Charaklis 1990), the common EPS component alginate is the most abundant marine biopolymer and the second most abundant biopolymer on Earth (Melvik and Dornish 2004). Alginate is a straight-chain, hydrophilic, polyuronic acid extensively secreted by brown

Received 26 April 2010; accepted 25 May 2010.
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algae and several species of bacteria, including *Pseudomonas sp. and Azotobacter sp.* (Sutherland 1995) All alginates, whether bacterial or algal, are composed by α-L-guluronate (G) and β-D-mannuronate (M) units linked through $1\rightarrow$ 4 glycosidic bonds (Dragget et al. 2005). The uronic acids occur as an irregular series of block structures including G-blocks (-G-G-G-G), M-blocks (-M-M-M-M), and M-G-blocks (-M-G-M-G) to form high molecular weight macromolecules ($>10^4$ Da) (Davies et al. 2003; Draget et al. 2005). The only difference between bacterial and algal alginates is that the mannuronic residues of the former are partially acetylated (Conti 1994; Draget et al. 2005; Saude and Hunter 2001; Sutherland 1990), with only minor effect on metal binding (Lee et al. 1996); thus we consider the commercially available alginate obtained from the brown algae *Macrocystis pyrifera* as a suitable experimental surrogate that may help to understand metal binding properties of alginates in general.

Recent studies focused on microbial surface reactivity have employed surface complexation modelling (SCM) to quantify, within a thermodynamic framework, the proton buffering and cadmium sorption capacity of diverse biomass, and to link metal sorption behaviour to the reactivity of specific surface functional groups at the cell-water interface (Borrok et al. 2004; Borrok et al. 2005; Daughney and Fein 1998; Daughney et al. 2001; Haas 2004; Yee and Fein 2001). Accordingly, in this study we employ potentiometric titrations and Cd metal sorption assays to develop a surface complexation model permitting direct comparison of the adsorptive properties of purified alginate with data obtained from other EPS studies.

MATERIAL AND METHODS

Materials and Preparation

All glassware was acid-washed in ~10% v/v HCl for 12 hours followed by three rinses, a 12 hour soak, and three more rinses with 18.2 MΩ water. All solutions were prepared gravimetrically and employed within 2 days of preparation. The purified alginate employed in this study, extracted from *Macrocystis pyrifera* (Sigma-Aldrich Canada, Oakville, ON), is a well characterized compound with a MW distribution of 80 to 120 kDa, and a mannuronic/guluronic acid ratio of ~1.56 (Sigma-Aldrich Canada, Oakville, ON).

Potentiometric Titrations

For alkalimetric titration, 0.1 g of alginic acid sodium salt was dissolved into 40 ml of electrolyte solution (0.01 M $NaNO_3$) and acidified with 2M HNO_3 to a pH of ~3. A double-junction glass pH electrode (Orion ROSS ultra, filled with 3M KCl) was calibrated using commercial pH buffers (Thermo Fisher Scientific, Nepean, ON; pH 2, 4, 7, 10, 12). The pH electrode was mounted into flasks containing the prepared alkalimetric titration solutions along with a magnetic stir bar, titrant dispenser, thermocouple, and N_2 gas line with diffusion stone bubbler. Solutions were adjusted to pH ~3 with 2M HCl, sealed with parafilm, and purged with N_2 for 30 minutes prior to, and throughout, titrations, to maintain a CO_2-free atmosphere in the flask.

Titrations were performed alkalimetrically from pH ~3 to 11 using a QC-Titrate autotitrator (Man-Tech Associates Inc., Guelph, Ontario) variably delivering CO_2-free 0.01 M NaOH for 0.1 pH increments with an average equilibration time between additions of ~30 s. Each addition of base occurred only after a pH electrode stability of 0.1 mV/s was attained for a typical total titration time of ~50 min. The proton buffering capacity of the dissolved alginate as a function of pH was determined by least-squares optimization as implemented in FITEQL (Herbelin and Westall 1999) and all reported charge excess values are relative to the titration starting point (pH $\approx$ 3.4).

Cadmium Adsorption

Cadmium adsorption behaviour of alginate was resolved using a dialysis separation technique, which allowed for the separation of free Cd^{2+} ions in solution from large organic molecules (10^4–10^5), with or without complexed Cd, by selective diffusion through a semi-permeable cellulose membrane. Sterilized dialysis tubing (10 mm diameter Spectra-Por cellulose membranes, 12–14 kDa cut-off, Cole-Parmer Canada Inc, Montreal, QC) was soaked for 30 min, rinsed three times with 18.2 MΩ water, and filled with ~ 1.1 mL of the electrolyte solution containing ~5 ppm of Cd and adjusted to a pH of 6.0, whereas the pH of the external solution was adjusted to values between 3 and 10 using either 0.1–1 M NaOH and HNO_3.

Filled dialysis bags were sealed with 12 mm polypropylene clips and then immersed in 50 ml polypropylene centrifuge tubes containing 40 ml of 0.01 M $NaNO_3$ electrolyte solution with ~5 ppm Cd and ~1 g/L biomass (alginate). By this approach the high molecular weight alginate macromolecules were excluded from the dialysis bag interior, while Cd^{2+} and other inorganic ions freely diffused through the membrane until chemical equilibrium was reached. The experimental solutions were left to equilibrate in an incubator at 25°C with constant rotary shaking (40 rpm) for a period of 24 hours, sufficient to achieve metal ion equilibrium between the diffusate and the retentate solutions (Truitt and Weber 1981). pH values of the retentate were recorded before and after the equilibration period, with the final pH value assumed to represent equilibrium between the diffusate and retentate. After the equilibration period the contents of the dialysis bags were collected, immediately diluted with 18.2 MΩ water, and acidified with concentrated trace-metal HNO_3.

Cadmium concentrations were determined by using a Perkin Elmer Elan6000 quadruple ICP-MS with a Cd detection limit of 4.2×10^{-6} ppm. The concentration of metal adsorbed to biomass in each vessel was calculated by subtracting the concentration of metal that remained in solution from the original Cd concentration in each experiment.

TABLE 1
Best-fit log Cd-carboxyl stability constants for metal adsorption experiments

Adsorption Experiment	Log K_{cdL}	*V(Y)*
A	−0.684	0.66
B	−0.576	0.570
C	−0.362	1.15
Average	−0.52 ± 0.22	

See text for calculation of uncertainties and description of the goodness-of-fit parameter $V(Y)$.

TABLE 2
Best-fit model parameters for alginate potentiometric titration data. See text for calculation of uncertainties and description of the goodness-of-fit parameter $V(Y)$

Titration number	pK_a	Site concentration (mmol/ g)	*V (Y)*
1	3.981	1.709	4.08
2	3.968	1.759	4.07
3	3.981	1.720	2.33
Average	3.98 ± 0.01	1.728 ± 0.02	

Surface Complexation Modelling

Surface complexation modeling was used to evaluate functional group acidity constants (pK_a), ligand concentrations, and the nature of the metal-organic ligand complexes in alginate solutions. The computer program FITEQL (Herbalin and Westall 1999), was used to fit thermodynamic parameters (site concentrations and acidity constants) of a discrete-site non-electrostatic surface complexation model to the potentiometric titration data. The charge balance in each titration step was calculated by the following charge balance equation:

$$[C_a - C_b] = [-Q] + [H^+] - [OH^-] \quad [1]$$

where $[C_a - C_b]$ is the concentration of acid added minus the concentration of base added; $[H^+]$ and $[OH^-]$ are theconcentrations of protons and hydroxyl ions, respectively, and $[-Q]$ is the negative charge excess due to deprotonation of alginate in solution, normalized per gram of biomass.

The functional group acidity constant, also called the proton stability constant, (K_a) is determined from the following equation:

$$K_a = [R - A^-][a_{H+}] \cdot ([R - AH^\circ])^{-1} \quad [2]$$

where R is the root organic molecule, AH^o is the protonated functional group, A^- is the deprotonated functional group and $\alpha_{H+}$is the proton activity. The pK_a value ($pK_a = -\log K_a$) is the pH at which [R-A^-] and [R-AH^o] are present at equivalent concentrations.

The computer program FITEQL was also used to model the Cd adsorption behaviour. Cd activities were determined from concentration data using coefficients calculated by the Davies equation. Adsorption of Cd by a deprotonated functional groups can be generalized by the equation:

$$Cd^{2+} + R - A^- \leftrightarrow R - A(Cd)^+ \quad [3]$$

where R-$A(Cd)^+$ is the Cd-organic complex. The equilibrium constant (K_{CdL}), reported as log K_{CdL} in Table 1, is given by following equation:

$$K_{CdL} = [R\text{ - }A(Cd)^{+1}] \cdot ([R\text{ - }A^-] \cdot [a_{Cd2+}])^{-1} \quad [4]$$

All the reported model parameters are the average of the triplicate experiments with one standard deviation uncertainty calculated as per Johnson et al. (2007) but reported as plus/minus the larger of the positive and negative uncertainties.

FTIR

FTIR spectroscopy was used to elucidate functional group identities in alginic acid sodium salt. The sample was placed on a salt plate (KCl) and focused using a Nic-Plan IR microscope attached to a Nicolet Magna-IR spectrometer 750. Spectra (from 500 to 4000 cm^{-1}) were acquired in absorbance mode and then converted into transmittance. Background spectra of water and of the glass slide were collected prior to measurement of biomass samples for baseline correction and normalization.

RESULTS AND DISCUSSION

Characterization of Alginate Functional Group Identities and Proton Reactivity

The relatively simple and well-characterized structure of the alginate monomers mannuronic acid and guluronic acid facilitates the identification of proton-reactive functional groups. On the basis of compositional constraints and proton reactivity over the pH 4–10 range (i.e., excluding hydroxyl protonation/deprotonation reactions), alginate reactivity should be limited to a single carboxyl group per alginate monomer. In the purified alginate there is an absence of other organic functional groups, typically considered proton-reactive in other biomasses (e.g., Beveridge and Murray 1980).

The FTIR spectrum of our alginate, shows striking similarities with the spectra of *Pseudomonas* EPS obtained by Freitas et al. (2009), with similar bands around 3200 cm^{-1}, 1600-1404 cm^{-1}, and 990–1300 cm^{-1}, representing OH, C=O, and C-O bonds, respectively (Fig. 1). In algal-derived alginate, however, there is an absence of the small band at 1732 cm^{-1} owing to the presence of acetyl in bacterial EPS (Freitas et al. 2009).

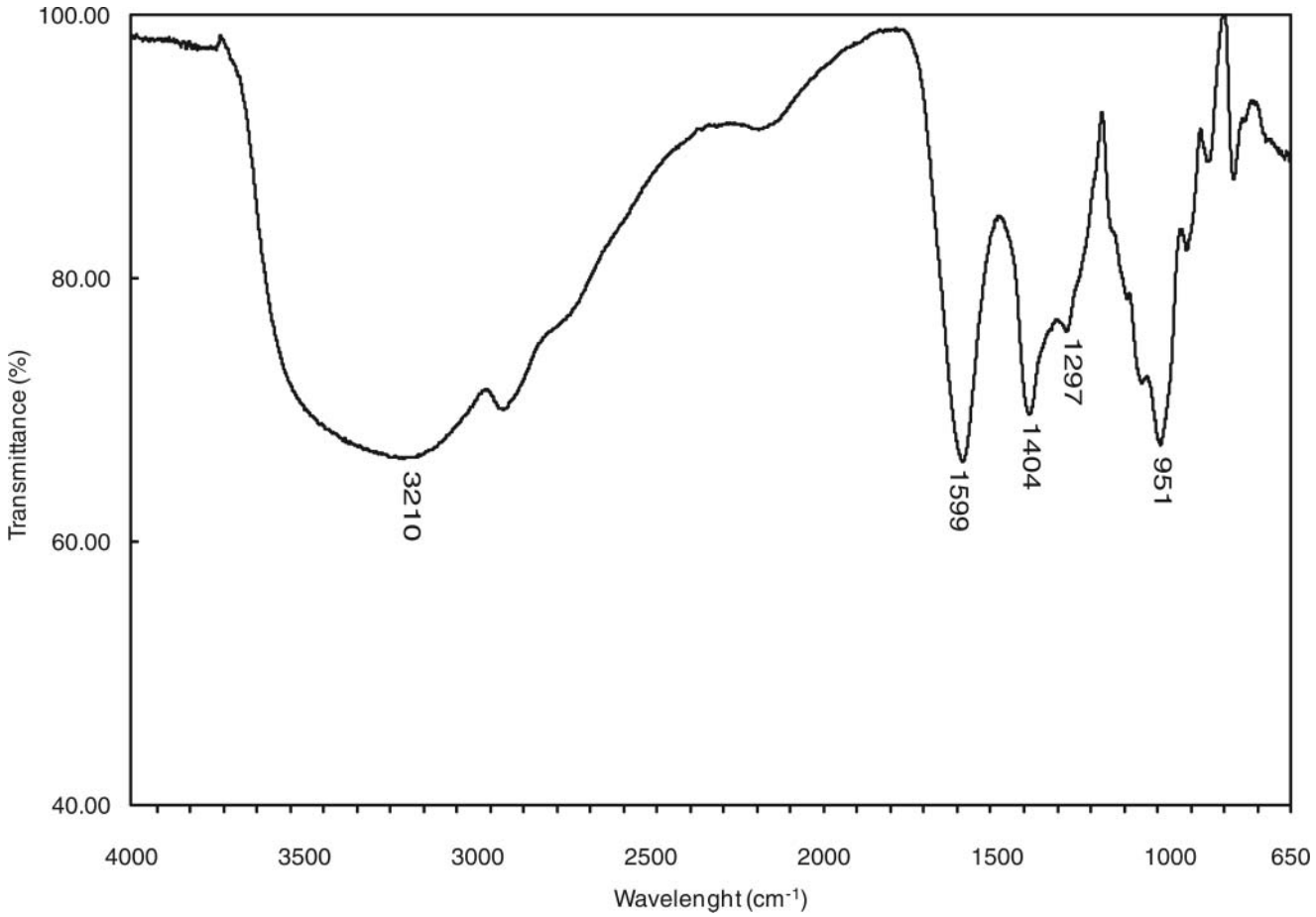

FIG. 1. FTIR spectra (percent transmittance) of alginic acid sodium salt. The labelled transmittance peaks have been interpreted as 3210: O-H; 1599: C=0 stretching; 1404: COO^- vibrating; 1026: C-OH stretching and 951: O-H deformation.

As expected, bands around 1160–1070 cm^{-1}, 1655–1645 cm^{-1} and 1450–1380 cm^{-1}, representative of phosphoryl, amino I and amino II functional groups, respectively (Eboigbodin and Biggs 2008; Ueshima et al. 2008; Won et al. 2005), are absent.

Potentiometric titration data of purified alginate similarly indicate that its acid-base behaviour can be accounted for by a single dominant carboxyl functional group. In the excess charge plot (Fig. 2), the slope at any given point can be interpreted as the instantaneous buffering capacity at that pH, and excess charge is equivalent to the cumulative surface concentration of deprotonated organic functional groups. For alginate, excess charge increases significantly in the pH 4–6 range, while from circumneutral to alkaline pH, a plateau is observed, indicating an absence of functional groups that are proton-reactive over this range. The excess charge data (Table 2) is best fit by a one-site model with an experimental apparent pK_{a1} value of 3.98 ± 0.01 ($V(Y) = 4.03$; (values less than 20 generally indicate good fit, but see Herbelin and Westall (1999) for discussion of the model variance function $V(Y)$).Our result is in agreement with (1) known carboxyl acidity constants (Smith and Martell 1982), (2) a previously reported value for alginate (4.0; Fourest and Volesky 1996), and (3) the alginate monomers guluronate and mannuronate (3.38 and 3.65, respectively; Haug et al. 1966).

With respect to functional group site concentrations, the excess charge data indicates that proton reactivity over the pH 4 to 10 range is 1.728 ± 0.027 mmol of protons per dry gram, as obtained from discrete site modeling using FITEQL (discussed here). The total proton reactivity of purified alginate reported here is comparable to the concentration of carboxyl sites determined by titration for alginate elsewhere (1.78 mmol/g; Fatin-Rouge et al. 2006; 1.96 mmol/g; Jodra and Mijangos 2003), but is lower than discrete site concentrations reported for various natural organic matter (4–24 mmol/g; Smith and Kramer 1999) (Table 3). This concentration is comparable to the average bacterial cell surface site concentration (0.32 mmol/wet gram, or approximately 2.56 mmol/dry gram, based on an average of 36 bacteria and consortia and a wet/dry weight ratio of 8; Borrok et al. 2005), but is restricted in proton reactivity to the pH range 3.5–5.5, in contrast to bacterial cells where the surface organic functional groups display a range of acidity constants corresponding to a variety of surface moieties (i.e., phosphoryl, sulfahydryl, amino, phenol, hydroxyl).

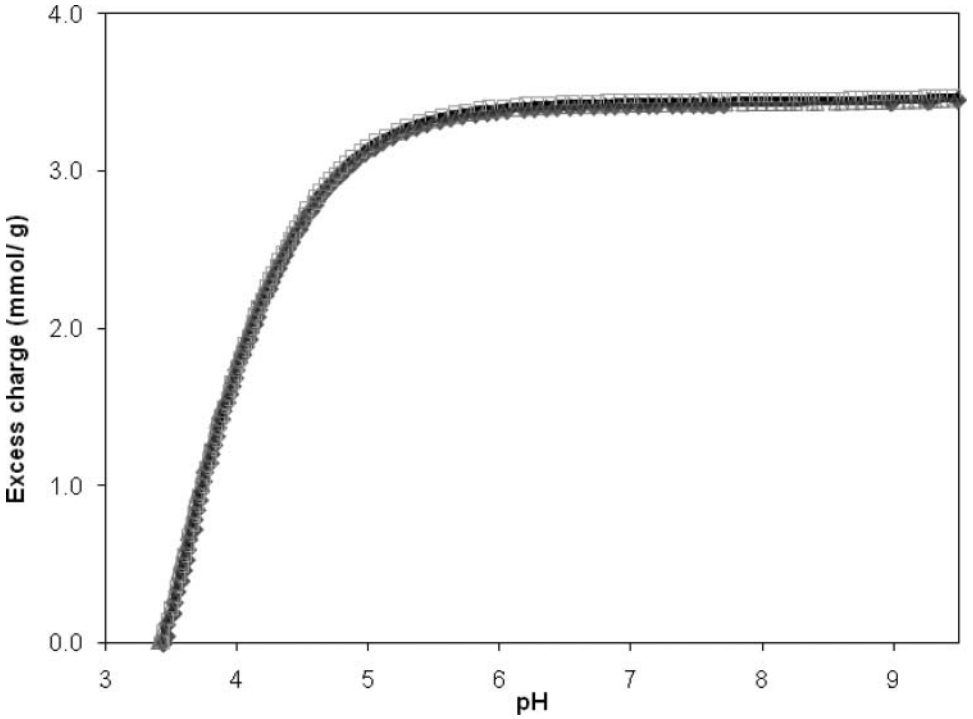

FIG. 2. Potentiometric titration data for three titrations of alginate in 0.01M $NaNO_3$(points). Single-site surface complexation model fits are also displayed for each titration (solid lines).

Cd Adsorption by Alginate

Parameters for the adsorption model were derived from the average carboxyl site density, its acidity constant, and experimental Cd adsorption data. The resulting Cd sorption isotherm (Fig. 3) was modeled under the assumption that sorption occurs solely to the carboxyl site identified by FTIR and titration. Hydroxyl groups are also present in alginate but they only become negatively charged at pH> 10 (Davies et al. 2003) and therefore do not play a role in metal binding over the experimental pH range. For the modeling of Cd adsorption data, a pH range of 3-8 was selected, as appreciable loss of Cd, presumably as the result Cd hydroxide precipitation, was apparent in blank experiments at pH values >8 (Fig. 3).

The Cd sorption isotherm (Fig. 3) strongly resembles the excess charge curve obtained from the proton titrations, highlighting the dependence of Cd adsorption on the availability of deprotonated carboxyl functional groups as a function of pH.

TABLE 3
pK_a values attributed to carboxyl functional groups and log Cd-carboxyl stability constants for various organic materials and microbial cell surfaces and comparison with alginate (this study)

Organic material or microbial species	Average pK_{a1}	Average cadmium stability constant (log K) attributed to pK_{a1}	Reference
Bacillus subtilis (bacteria)	4.82 ± 0.14	3.4	Fein et al. 1997
Mixtures of gram-negative and positive bacteria	5.0	3.60 ± 0.32	Yee and Fein 2001, 2003
Pseudokirchneriella subcapitata (algae)	3.9 ± 0.3	4.1 ± 0.5	Kaulbach et al. 2005
Fescue rubra (grass roots) *Geobacillus*	4.2 ± 0.1	3.5 ± 0.4	Ginn et al. 2008
Stearothermophilus (thermophilic bacteria)	3.73 ± 0.04	2.20 ± 0.16	Hetzer et al. 2006
Geobacillus thermocatenulatus (thermophilic bacteria)	3.84 ± 0.03	1.30 ± 0.22	Hetzer et al. 2006
EPS from 7 environmental bacterial strains		−1.72 ± 0.37	Guibaud et al. 2008
EPS from activated sludge		−2.16 ± 0.4	Guibaud et al. 2006
Purified alginate	3.98 ± 0.01	−0.52 ± 0.22	this study

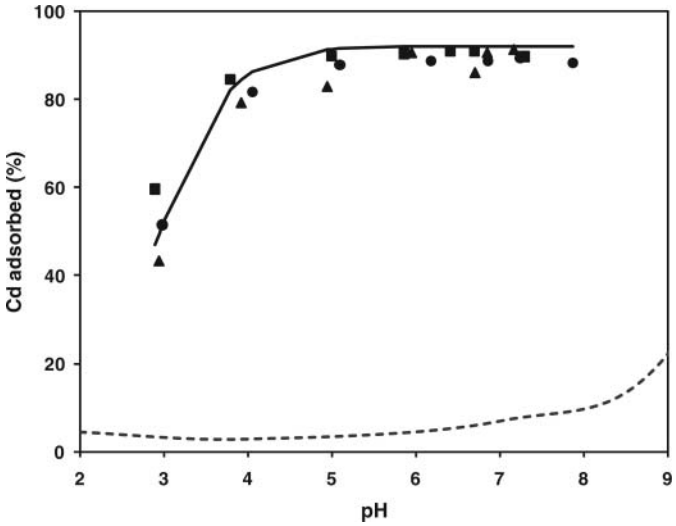

FIG. 3. Cd adsorption data (in triplicate as circles, squares, and triangles) and the average best-fit surface complexation model (solid line). The dashed curve represents an alginate-free filtered blank. Only data in the pH 3–8 range was employed for Cd adsorption modelling, as some Cd loss, likely due Cd hydroxide precipitation, was observed in blank experiments at more alkaline pH values.

At low pH, when competition between the metal adsorbate and protons for functional groups is high, very little metal is bound. With increasing pH, progressive deprotonation of carboxyl functional groups provides free ligands for metal sorption; by pH $\sim$ 5.5, close to 90% of Cd complexation by carboxyl functional groups was achieved. Conversely, pH values in our sorption experiments were not low enough to completely prevent metal adsorption, as the lowest pH experiments were conducted within a pH unit of the apparent carboxyl pK_a (Fig. 3); the capacity of alginate to form complexes under acid conditions has been previously described by Simsek–Ege et al. (2003).

The fit (solid line) of the FITEQL non-electrostatic Cd adsorption model is also shown in Figure 3. The average log K_{CdL}, -0.52 $\pm$ 0.22 (Table 1), is compared with values obtained for other reactive biomass in Table 3. The log K_{CdL} value determined here for alginate carboxyl groups is within the range previously reported for various bacterial EPS matrices, ranging from -2.14 to -1.00 (Guibaud et al. 2008), but is lower than the average log K_{CdL} of bacterial cell surfaces (Borrok et al. 2005).

A primary assumption in most studies of bacterial surface complexation is that carboxyl functional groups are responsible for the majority of metal adsorption at low pH. However, X-ray adsorption spectroscopy of Cd adsorbed to the surfaces of *Bacillus subtilis* at low pH has demonstrated that Cd was bound primarily to phosphoryl functional groups (Boyanov et al., 2003). Importantly, this means that studies which attribute all metal adsorption under acidic conditions to carboxyl ligands may, in fact, be overestimating the role of the carboxyl ligand in metal sorption. In such cases, the term [R–A$(Cd)^+$] in the calculation of K_{CdL} may actually comprise two or more functional groups (e.g., carboxyl and phosphoryl). In our study, this is excluded based on alginate compositional constraints, thereby allowing us to better assess the inherent reactivity of carboxyl groups towards Cd. In this regard, it is not surprising that previous studies employing more heterogeneous materials obtained higher K_{CdL} values.

Alternatively, the difference in carboxyl log K_{CdL} between alginate and other similarily-characterized biomass (Table 3) may be due to bidentate coordination of divalent metals by adjacent residues in alginate (Fourest and Volesky 1997; Davies et al. 2003). According to Davies et al. (2003), regions of the alginate polymer rich in guluronic acid (G-blocks), which display a higher selectivity for divalent metal ions, provide a multidentate environment for complexation, while in regions rich in mannuronic acid (M-blocks), complexation is thought to be predominantly unidentate. Detailed vibrational spectroscopy of alginate (i.e., Fourest and Volesky 1996; Filipiuk et al. 2004), provides some physical evidence for these assumptions.

However, Papageorgiou et al. (2010) recently proposed the reverse scenario for G-block and M-block metal complexation environments, and additionally, the nature of the coordination between divalent metal ions and heteropolymeric sequences (e.g., MGM and GMG blocks) is unexplored. Surface complexation models evaluating monodentate vs. bidentate complexation for M-blocks only, G-blocks only, and both M- and G-blocks simultaneously, provided neither increase in model goodness-of-fit (as indicated by the V(Y) parameter), nor log K_{CdL} values more comparable to previously reported Cd-carboxyl stability constants (data not shown). It is also important to note that molecular structure beyond the functional group may influence functional group pK_a and log K_{CdL}, and in this respect comparison with data obtained from microbial biomass possessing carboxyl moieties attached to a wide variety of macromolecular backbones entail some difficulties. Future studies aimed at better resolving the coordination environment of alginate-sorbed Cd may be able to distinguish between these competing scenarios.

CONCLUSIONS

By assessing the total functional group site concentration and acidity constant of purified alginate, and by investigating its cadmium adsorption behaviour, this work quantifies the potential role of alginate in metal sorption reactions involving EPS matrices. The commercially available alginate employed here has an apparent acidity constant (pK_a) of 3.98 $\pm$ 0.01 and an overall proton adsorptive capacity is 1.728 $\pm$ 0.02 mol/kg, lower than values reported for some natural organic matter, but comparable to bacterial cell surfaces; these values are attributed entirely to carboxyl functional groups based on alginate polymer composition. Finally, the cadmium-carboxyl stability constant determined here (log K_{CdL} = -0.52 $\pm$ 0.22) is comparatively lower than those obtained for carboxyl functional groups on more heterogeneous materials such as natural EPS and bacterial surfaces. Together, these results place important constraints on uronic acid contributions to overall EPS reactivity.

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