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Effect of complexing ligands on the surface adsorption, internalization, and bioresponse of copper and cadmium in a soil bacterium, *Pseudomonas putida*

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HIGHLIGHTS

- We tested the bioavailability of copper or cadmium complexes to a soil bacterium.
- Adsorption, internalization, and bioresponse were evaluated.
- *Pseudomonas putida* was sensitive to the metal complexes.
- *P. putida* was in particular sensitive to Cu and Cd supplied as a citrate complex.

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ABSTRACT

Environmental quality criteria for metals toxic to soil and water organisms, using the free ion activity model or the biotic ligand model, are based on the concept that the major form of the metal available to the organism is the free metal ion, yet various metal complexes are bioavailable to a variety of soil and water organisms. We test here whether neutral copper or cadmium sulfates, negatively-charged copper or cadmium citrates and positively-charged copper acetate and cadmium chloride are bioavailable to a soil bacterium, *Pseudomonas putida*. Adsorption onto the cell surface and uptake into the periplasm and cytoplasm of this Gram-negative root colonizing bacterium was studied by adding a single concentration of Cu or Cd and varying the concentration of the ligands to complex 10–100% of the metal. Metal association from the complexes on and within the cell was defined using selective extraction procedures and compared with free ion controls using the Langmuir isotherm. Cellular responses also were assessed using a *P. putida* biosensor. Both uptake and bioresponse methodologies showed that *P. putida* was sensitive to the metal complexes. In particular, the bioresponse to Cu and Cd supplied as a citrate complex occurred with activities of free metal ions two orders of magnitude lower than for the control. We concluded that the tested metal complexes for Cu and Cd are taken up into the cell, where they trigger a bioresponse. We also discuss the implications of these findings on interactions between soil and water organisms and nanoparticles that release metal ions.

1. Introduction

The consequences of exposing soil bacteria to toxic metals need to be included in hazard and risk evaluation in soil environments. Microbes drive carbon and other nutrient cycles, affect plant health, and are the base of the food chain. Environmental quality criteria for toxic metals are based on the concept that the only form of metals in water that is available to organisms is the free metal ion. The free ion activity model (FIAM), first proposed for uptake of transition metals through fish gills (Morel, 1983), and the modified biotic ligand model (BLM), where competition between these toxic transition metals and Ca and Mg is considered, are validated for metal responses in algae and bacteria. For instance, both adsorbed and intracellular Cd in *Sinorhizobium meliloti*, a Gram-negative soil bacterium, relates to the free ion concentration (Slaveykova et al., 2009, 2010). Likewise, Ore et al. (2010) demonstrate that Cu ion activity explains toxicity in a bioluminescent biosensor strain of *Nitrosomonas europaea*. Inhibition of nitrification in a lab bioreactor correlates to Ni and Cd free ion levels rather than metals complexed with EDTA, NTA or citrate (Hu et al., 2002).

However, the bioavailability of metals is not limited to the free ion. Cu-dissolved organic matter complexes from Cu-contaminated

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manure are sensed by a *Pseudomonas fluorescens* biosensor (Brandt et al., 2008) and affect functional community response (Brandt et al., 2010). Nybroe et al. (2008) observe that a Cu–EDTA complex is not detected by this biosensor, whereas Cu–citrate and complexes formed with dissolved organic matter (DOM) are sensed. Likewise, Maderova et al. (2011) reported that Cu complexes in soil pore water caused a response in their *P. fluorescens* biosensor. The complexed metals enter cells through channels other than those required for hydrated metal ions ($M(H_2O)_n^{z+}$). Complexed $ZnHPO_4^0$ is transported into *Arthrobacter* via phosphate transporters (Moberly et al., 2010). Cd and Cu diethyldithiocarbamates are lipid soluble (Poldoski, 1979) and diffuse passively (Keung et al., 2008). Copper 8-hydroxy-quinoline oxine, a common fungicide and chelating agent, passively diffuses through cell membranes (Phinney and Bruland, 1994, 1997), as will neutrally-charged inorganic ligand complexes ($AgCl^o$, $CdCl_2^o$) (Campbell, 1995; Campbell et al., 2002). Metals that form hydrophilic complexes, such as copper and cadmium citrate, remain attached to the citrate as M–citrate$^-$ and enter algal cells through an anionic transport system (Campbell et al., 2002). In addition, other factors such as natural organic matter and microbial products affect the total body burden as demonstrated by algal studies of lead uptake (Slaveykova and Wilkinson, 2002; Lamelas et al., 2005; Slaveykova et al., 2010) and bacterial studies of Cd uptake (Smiejan et al., 2003; Kola and Wilkinson, 2005; Slaveykova et al., 2010). Slaveykova (2007) demonstrated that increased availability of Pb to algae was due to the formation of a ternary complex between Pb(II), fulvic acid, and the cell surface. Thus, modeling the system is more varied and dynamic than predicted by a simple equilibrium model.

Copper and Cd ions differ in their environmental behavior and biological response. Pabst et al. (2010) demonstrated differences in the surface adsorption and uptake of Cu^{2+} and Cd^{2+} for cells of *Pseudomonas putida* in a controlled background of 0.01 M KNO_3. The periplasmic and cytoplasmic loading for Cu was higher than for Cd, which was mainly associated with the cell surface. Overlapping as well as distinct responses to the Cu and Cd ions in gene expression is documented in *P. putida* cells challenged with these ions (Miller et al., 2009).

Copper is taken up by bacteria via dedicated mechanisms because it is essential at low doses for cell function (Miller et al., 2009). In contrast, Cd ions enter the cells by usurping other transporters, such as those for Zn and Mn ions (Benters et al., 1997; Silver, 1998; Hao et al., 1999). Copper is borderline between hard and soft acids complexing with a variety of inorganic ligands and N-containing organic functional groups. Cadmium is a soft acid that forms complexes primarily with sulfur containing compounds, the most important process in Cd toxicity.

In this study, we further our research by examining the binding and uptake of Cu and Cd, when present as complexes, to bacterial cells. Often, due to the experimental designs of reported studies, the concentration of the metal and the activity of the free ion covary, so it is difficult to sort out effects that support or refute the BLM or FIAM. This study was designed using one initial concentration of Cu and Cd and varying the free ion activity by adding appropriate concentrations of various ligands to generate solutions with 10–100% complexation of the metal. Neutral metal sulfate ($CdSO_4^o$, $CuSO_4^o$), positively charged Cd chloride and Cu acetate ($CdCl^+$, $CuCH_3COO^+$) and negatively charged metal citrate ($CdC_3H_5O(COO)_3^-$, $CuC_3H_5O(COO)_3^-$) complexes were formed to compare effects of charge on cell surface binding and cell entry. Cu and Cd loading from these complexes onto the bacterial cell surface and their accumulation in the periplasm and cytoplasm for a soil bacterium were studied using a strain of *P. putida*, a Gram negative root colonizing soil bacterium. Comparisons between the association of Cu and Cd with *P. putida* as affected by the presence of the ligands versus the free ions were made using Langmuir isotherms.

To complement these chemical studies, we examined the effects of the complexes compared to the free ions on a *P. putida* biosensor used previously to demonstrate toxicity of Cu, Zn and Ag ions (Gajjar et al., 2009). The biosensor contained a fusion of the promoter at locus PP_0588, encoding a protein that binds to heavy metals, with genes *luxAB*, endowing light output based on energy production of the cell (Gajjar et al., 2009). Transcripts from this gene accumulate after Cu treatment of the wild type strain in a defined medium when Cu–citrate complexes are present (Miller et al., 2009). Light output from this biosensor increases when cells in water suspension are exposed to low levels of Zn (0.05 and 0.1 mg L^{-1}) due to promoter activation (Gajjar et al., 2009). Toxicity of a treatment, such as with Cu greater than 0.02 mM or Ag more than 0.01 mM, is indicated by reduced light output (Gajjar et al., 2009).

This study examines the partitioning of Cu and Cd in the presence of ligands forming positive, negative and neutral complexes onto and into the bacterial cell and the bioresponse of the bacterial biosensor to the internalized metal. Both of these methodologies demonstrate that the complexed metals were perceived by the pseudomonads.

2. Materials and methods

2.1. Bacterial stocks

P. putida isolate Corvallis (Anderson and Jasalavich, 1979) and the *P. putida* biosensor strain (Gajjar et al., 2009) were grown to stationary phase in liquid minimal medium (MM) containing per L: K_2HPO_4 10.5 g, KH_2PO_4 4.5 g, sodium citrate ($2H_2O$) 0.5 g, $(NH_4)_2SO_4$ 1.0 g, sucrose 20 g and $MgSO_4$ 0.1 g (Gajjar et al., 2009). This medium was inoculated with stock cultures stored in 15% glycerol at −80 °C. Chemicals used were Mallinckrodt Analyzed Reagents (AR), KY.

2.2. Metal solutions: preparation and tests of cell loading

The metal solutions for the cell loading studies were prepared at concentrations of 0.08 mM (5 mg L^{-1}) of free Cu^{2+} using $Cu(NO_3)_2$ and 0.09 mM (10 mg L^{-1}) free Cd^{2+} using $Cd(NO_3)_2$ in a background of 0.01 M KNO_3. Nitrate does not form complexes with Cu or Cd (GEOCHEM-PC) (Parker et al., 1995). The ligands were added as potassium salts to generate solutions with 10%, 40%, 70%, and 100% of the added metals as free ions as determined by geochemical modeling using MINEQL + 4.5 (Schecher and McAvoy, 1998) (Table 1). Solutions of free Cu (8, 16, 31, 47, 80, 160 μM) and Cd (4, 9, 18, 27, 45, 71, 89 μM), prepared with the nitrate salts, were used to compare adsorption and uptake behavior of the metals without the presence of complexing ligands.

Table 1
Ligand concentrations added to obtain the associate free Cu and Cd activities.

Ligands				
Copper				
% Free	Activity (μM)	SO_4^{2-} (mM)	Acetate (mM)	Citrate (mM)
100	78.7	0	0	0
70	55.1	1.86	3.74	0.022
40	31.5	6.88	12.7	0.048
10	7.9	41.6	54.6	0.080
Cadmium				
% Free	Activity (μM)	SO_4^{2-} (mM)	Chloride (mM)	Citrate (mM)
100	89.3	0	0	0
70	62.5	1.73	4.79	0.16
40	35.7	6.27	14.7	0.54
10	8.9	29.7	67.2	3.0

All solutions were analyzed by Atomic Absorption Spectrometry (AA, PerkinElmer AAnalyst 800) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500c). The linear range of the AA for Cu was from 0.002 to 0.08 mM (0.1–5 mg L^{-1}) and for Cd, 0.0004–0.02 mM (0.04–2 mg L^{-1}). The linear range for the ICP–MS was 7.8×10^{-5} to 0.02 mM for Cu and 4.5×10^{-5} to 0.009 mM for Cd. Detection limit on a mass basis was 0.23 mmole kg^{-1} Cu and 0.13 mmole kg^{-1} Cd by ICP-MS.

In the attempt to directly measure the free ion activity with Cd^{2+} and Cu^{2+} ion selective electrodes (ISEs), problems with poor sensitivity, high minimal detection levels and poor response with citrate (Shuttleworth and Unz, 1993) made routine measurements impractical. The ISE results from the copper and cadmium solutions with the ligands were used to verify model predictions of equilibrium solution activities. The coefficient of determination between ISE and modeled free ion activity in the equilibrated solutions was 0.980, confirming that the modeled results were predictive of the true free ion activity in solution. Cu and Cd remained complexed in solution throughout the study.

2.3. Preparation of cells for loading studies

Cells were prepared as described in Pabst et al. (2010). Briefly, thawed cells from frozen stocks (200 l) were grown to early stationary phase in 50 mL of MM with shaking at 220 rpm in a 28 ± 2 °C incubator. The cells were washed after centrifugation in sterile deionized water (DI), centrifuged again and resuspended to 10^8 colony forming units mL^{-1} in 15 mL of the metal solutions, or in 0.01 M KNO_3 as a control. The samples were shaken at 220 rpm at 25 ± 1 °C for 30 min, and then centrifuged to pellet the cells and obtain a supernatant. The initial pH was 5.25 ± 0.37 for the Cu-complex solutions and 5.94 ± 0.23 for the Cd-complex solutions; pH remained within 0.2 pH units throughout the study without addition of buffers. Experiments were performed in triplicate.

2.4. Analysis: solution phase

After centrifugation, the supernatant was removed from the centrifuge tube with a pipette and filtered through a 0.2 μm nylon filter and the solution was analyzed for Cu or Cd using the AA or ICP-MS. Free ion activity of Cu^{2+} and Cd^{2+} in solution at equilibrium was calculated using MINTEQ from concentration determined by AA or ICP-MS and verified on selected samples by ISE.

2.5. Analysis: cell compartmentalization

A series of extractions was used to determine the proportion of Cu and Cd associated with surface exchange sites (q_{exch}), within the periplasmic space (q_{per}), and within the cytoplasm (q_{cyt}) as described by Pabst et al. (2010). Surface accumulations were extracted with Ca ions. The periplasmic extract was released from the Ca-treated cells by exposure to EDTA (tetrasodium salt dehydrate) (Vaara, 1992). Dimkpa et al. (2011) confirmed that the EDTA treatment did not lyse the pseudomonad cells. This EDTA extraction would include metals tightly bound to the outer membrane, i.e. metals not exchanged with the Ca in the first step, and metal in the periplasm. The cytoplasmic loading was determined after digestion of the remaining cell pellet with nitric acid (trace metal grade, Fisher Science, PA). Each sample was filtered, and Cu and Cd were analyzed by AA or ICP-MS. The centrifuge tubes were weighed between each extraction step to determine the mass of cells and liquid associated with the cells. The mass balance expressed as a percent was between 90% and 105% when the initial solution concentration and the sum of extracted metals were compared.

2.6. Quality control

Each test included: (1) 0.01 M KNO_3 with no metals or cells, (2) 0.01 M KNO_3 with cells but no metals, and (3) 0.01 M KNO_3 with no cells but with the test metal. These controls were included to provide: (1) contamination levels for Cu or Cd through the procedures, (2) metal levels in unexposed cells, and (3) any losses of Cu and Cd not due to cell adsorption/uptake throughout the experimental procedures. All blanks were below the detection limit and there was no loss of Cu or Cd to laboratory glassware.

2.7. Langmuir model

The Langmuir model was used as a tool to compare the results for the distribution of free metal ions to *P. putida* cells in solution with the different complexing ligands. The model was used for goodness of fit and for statistical comparison of the behavior of Cu and Cd complexes versus free ions but not for interpretation of mechanistic details. The Langmuir model (Eq. (1)) describes the non-linear relationship between the activity of the free ion in solution at equilibrium (C_{eq} mmole L^{-1}) and the amount of metal (q) on the surface exchange sites (q_{exch}), in the periplasmic space (q_{per}), or in the cytoplasm (q_{cyt}) in mmole kg^{-1} (Playle et al., 1993).

$$q = \frac{Q^o b C_{eq}}{1 + b C_{eq}} \quad (1)$$

where Q^o is a sorption maximum and b is the magnitude of the initial slope.

The best-fit model was obtained by directly fitting data to the Langmuir model by minimizing the residual sum of squares (Bolster and Hornberger, 2007). Joint confidence regions were calculated by creating a table of critical sums of squares for various Q and b values. The corresponding F value for the degrees of freedom indicates the 95% joint confidence regions. The joint confidence regions were used to determine if the presence of the ligand affected the distribution of Cu and Cd for q_{exch}, q_{per}, and q_{cyt}.

2.8. Analysis of metal bioavailability with the P. putida biosensor

The bioavailability studies were performed with suspensions of the *P. putida* biosensor cells in water or complexing solutions (0.05 M citrate, 0.4 M sulfate or 0.4 M chloride) at 10^8 cells mL^{-1} with and without the presence of metal. The initial total metal ion concentrations were 1.6, 16, 160 μM Cu and 0.89, 8.9, and 89 μM Cd. The addition of 0.05 M citrate to the metal solutions resulted in 100% complexation (GEOCHEM-PC). Sulfate addition (0.4 M) complexed 63% of the Cu and 67% of the Cd. Addition of KCl complexed 96% of the Cd.

Cells were challenged at the early exponential phase, after regrowth of overnight cultures in the MM as described above, at a cell density 1×10^8 cells mL^{-1}. Measurement of light output generated by the presence of *luxAB* genes within the biosensor (Gajjar et al., 2009) was performed after 60 min in a LMaxII384 Molecular Device luminometer with Softmax Pro V. 4.7 software. Each treatment condition was performed with at least three different experiments, each with triplicate readings of one sample. The data from the luminometer readings were normalized to the light output from the appropriate control (background solution without metal) and these values were plotted against the free ion activity predicted by GEOCHEM-PC.

3. Results

All Cu and Cd adsorption experiments were performed at a pH of 5.6 ± 0.2 where the cell surface had a charge of 322 ± 3.6 H^+

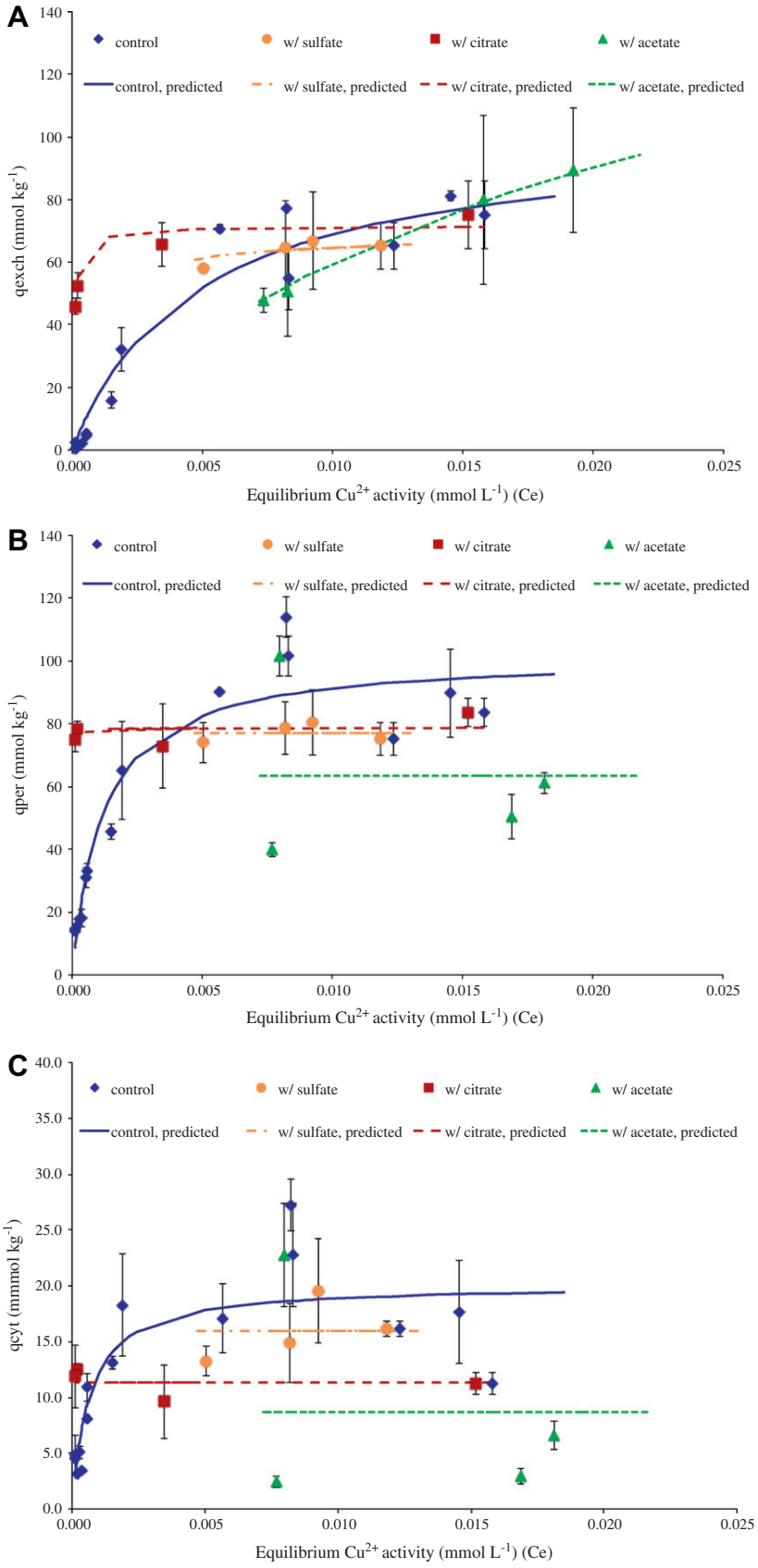

Fig. 1. Sorption isotherms of Cu associated with surface exchangeable (exch) (A), periplasmic (per) (B) and cytoplasmic sites (cyt) (C) and free Cu ion activity in solutions for the no ligand control and in the presence of sulfate, acetate and citrate. The points are averages of experimental data ($n = 3$) with 95% confidence interval error bars. The lines are the Langmuir model predictions.

Table 2
The coefficient of determination (r^2), and sorption maximum (Q) and binding affinity (b) calculated from q_{exch}, q_{per}, q_{cyt} for the Langmuir model for Cu sorption in the no ligand control and in the presence of acetate, sulfate or citrate. Q and b joint confidence region are followed by the same letter are not statistically different ($\alpha = 0.05$).

q_{exch}	Control		Acetate		Sulfate		Citrate	
r^2	0.930	a	0.647	a	0.048	b open[a]	0.795	b
Q mmol kg^{-1}	103		184		68.7		71.4	
b	208		47.9		0		14300	
q_{per}								
r^2	0.886	a	0.037	b open	0.000	b open	0.045	b open
Q mmol kg^{-1}	102		63.3		77.0		78.7	
b	851		0		0		0	
q_{cyt}								
r^2	0.669	a	0.020	c open	0.017	b open	0.003	c open
Q mmol kg^{-1}	20.1		8.69		15.9		11.3	
b	1531		0		0		0	

[a] Open indicates no upper 95% confidence value; slope is not significantly different from 0.

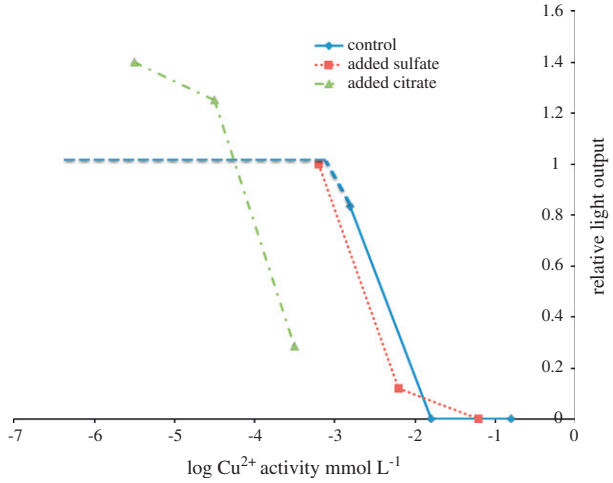

Fig. 2. Response of the pseudomonad biosensor to Cu^{2+} activity and Cu-complexes. Light output from the biosensor is reported as relative light units based on normalization of the test output versus output from cells not challenged with metal at 60 min of study. The biosensor cells were exposed to Cu complexed with sulfate or to citrate generating the different levels of free ion activity. These data are from one of three independently performed studies that produced the same result. The blue dash line is the projected behavior of the control; no bioresponse occurs below log Cu^{2+} activity of −2.8 mM.

$mmol_c$ kg^{-1} dry weight (Pabst et al., 2010). At this pH, carboxylic, phosphoric and sulfhydryl functional groups on the cell would be available for exchange reactions with Cu or Cd ions (Pabst et al., 2010). The pH remained within ±0.2 pH units throughout the interactions of the metal with the bacterial cells.

3.1. Adsorption and uptake of copper

Exposure to the maximum dose, 0.08 mM Cu L^{-1} (5 mg Cu L^{-1}), did not result in loss of cell culturability of the stationary phase cells (Pabst et al., 2010). Other researchers have used similar concentrations of Cu with various pseudomonad strains without death of the cells or cell wall damage (Langley and Beveridge, 1999). All cells exposed to Cu, whether as the free ion or as complexes, became blue in color that was stable when washed with Ca but lost with the EDTA extraction, consistent with disruption of the outer membrane. The blue coloration of the Cu-treated cells was not due to Cu precipitation since Cu would not form a solid phase at the pH of this study.

If the free ion is the only species of Cu that interacts with the bacterial cell, through surface adsorption and uptake, the slope (b) and the y intercept (Q) from the Langmuir model (Eq. (1)), describing the relationship between equilibrium free Cu^{2+} activity in solution and cell associated Cu (surface adsorbed, in the periplasm, in the cytoplasm), would be the same with and without added ligands. Experimental and modeled data are shown in Fig. 1 and Table 2.

For Cu surface adsorption in the presence of acetate, the Q and b values were not different from the Cu ion response in the control, showing this system followed the FIAM model; only the free metal ion was adsorbed (Fig. 1A and Table 2). The adsorption of Cu in the presence of citrate followed the Langmuir model, but the Q and b values were statistically different from the control. There was no fit to the Langmuir model in the presence of sulfate; the slope (b) was not statistically different from zero, showing that the interaction of Cu with the cell surface did not respond to increasing activity of free Cu^{2+}. The non-fit of the isotherm is a clear indication that the presence of the ligand, in this case sulfate, changes the interaction of Cu with the bacterial surface so that it is not described by the free ion activity alone.

The addition of citrate or sulfate, generating solutions with low activity of free Cu^{2+}, resulted in a higher amount of Cu associated with the cell surface than observed in the no ligand control (Fig. 1A). For example, at a solution equilibrium activity of 1×10^{-4} mM Cu^{2+}, the surface-associated concentration of Cu was 44 mmole kg^{-1} with citrate but only 2.3 mmole kg^{-1} for the control. The Q values for both citrate and sulfate complexes were similar, about 70 mmole kg^{-1}, and were equivalent to the surface-bound Cu in the control at the highest dosing of Cu^{2+} (0.08 mM Cu^{2+} initial activity or 0.015 mM equilibrium activity). The initial concentration of Cu added to all ligand systems was 0.08 mM; the adsorption of Cu in the presence of citrate and sulfate responded to the concentration added, not to the activity of free Cu^{2+} in solution in the presence of the ligands.

Copper was taken up into the periplasm and the cytoplasm from the control and in the presences of the complexes, although the concentrations of Cu in both compartments were less in the presence of the ligands (Fig. 1B and C, Table 2). The Langmuir isotherm well described the loading of free Cu^{2+} in the control into these compartments, as demonstrated in Pabst et al. (2010), but the isotherm did not fit Cu uptake in the presence of any of the complexes. The slopes (b) were not different than zero; there was no relationship between equilibrium Cu^{2+} solution activity and the amount of Cu in the periplasm or cytoplasm when acetate, sulfate or citrate were present; uptake of Cu was not responding to free ion activity in the presence of the ligands.

3.2. Metabolic effects from Cu complexes in the pseudomonad biosensor

To evaluate the bioresponse of the Cu^{2+} versus Cu complexes in this study, the light output from the biosensor cells was normal-

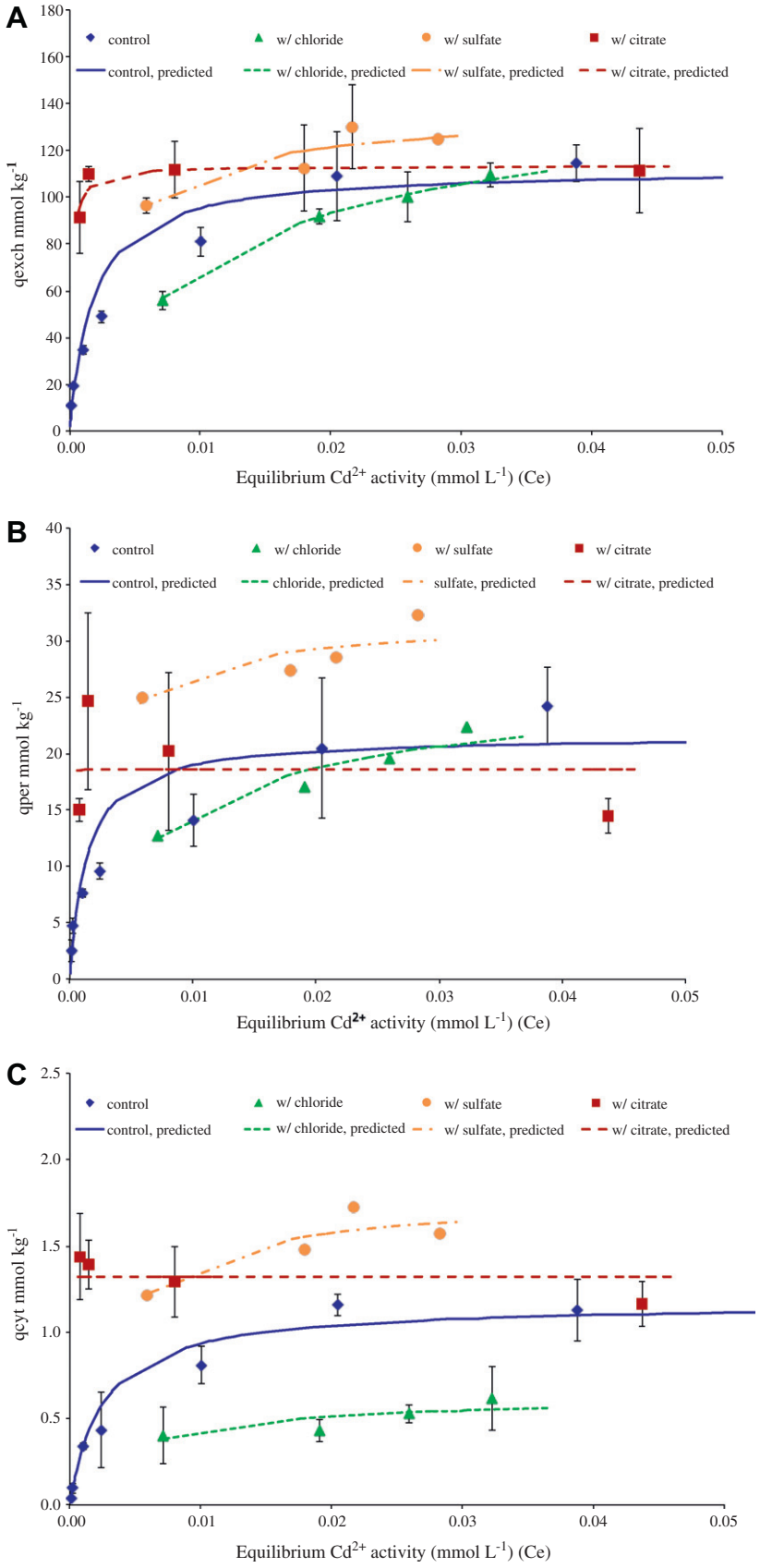

Fig. 3. Sorption isotherms of Cu associated with surface exchangeable (exch) (A), periplasmic (per) (B) and cytoplasmic sites (cyt) (C) and free Cu ion activity in solutions for the no ligand control and in the presence of sulfate, acetate and citrate. The points are averages of experimental data ($n = 3$) with 95% confidence interval error bars. The lines are the Langmuir model predictions.

Table 3
The coefficient of determination (r^2), and sorption maximum (Q) and binding affinity (b) calculated from q_{exch}, q_{per}, q_{cyt} for the Langmuir model for Cd sorption in the no ligand control and in the presence of acetate, sulfate or citrate. Q and b joint confidence region are followed by the same letter are not statistically different ($\alpha = 0.05$).

q_{exch}	Control		Chloride		Sulfate		Citrate	
r^2	0.903	a	0.918	a	0.529	b	0.379	c open
Q mmole kg^{-1}	108		145		137		114	
b	381		91.2		399		0	
q_{per}								
r^2	0.626	b[a]	0.721	b	0.207	a open	0.019	b open[b]
Q mmol kg^{-1}	19.6		26.2		31.9		18.7	
b	613		128		0		0	
q_{cyt}								
r^2	0.777	a	0.256	b open	0.303	c open	0.006	c open
Q mmol kg^{-1}	1.08		0.63		1.8		1.3	
b	414		0		0		0	

[a] Indicates only the Q values are the same.
[b] (Open) indicates no upper 95% confidence value; slope is not significantly different from 0.

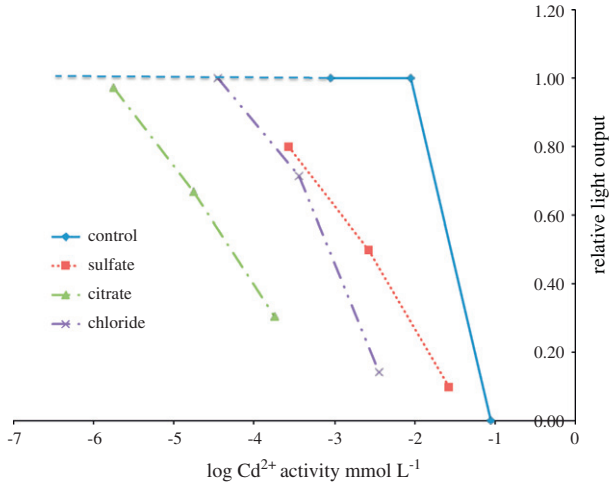

Fig. 4. Response of the pseudomonad biosensor to Cd^{2+} activity and Cd-complexes. Light output from the biosensor is reported as relative light units based on normalization of the test output versus output from cells not challenged with metal at 60 min of exposure. The biosensor cells were exposed to Cd complexed with sulfate, citrate and chloride generating the different levels of free ion activity. These data are from one of three independently performed studies that produced the same result. The blue dash line is the projected behavior of the control; no bioresponse occurred below log Cd^{2+} activity of -1.0 mM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ized to the value for each appropriate control (no Cu^{2+} added) at 60 min of exposure and plotted against the calculated initial log Cu^{2+} activity for each solution (Fig. 2). Thus, altered light output from the normalized value of 1 denoted response of the biosensor. Acetate complexes could not be assessed by this method; death of the biosensor cells occurred at the levels required for complexation with this experimental design. With the addition of sulfate to the system, the bioresponse corresponded to the toxicity caused by exposure to the free Cu ion alone (Fig. 2); bioresponse followed the FIAM. The addition of the complexing solution of potassium citrate (without Cu) increased light output from the biosensor because catabolism of the citrate provided an energy source to the cell, thus causing light output to exceed that of the control cells lacking citrate. When Cu citrate was added, the biosensor displayed a toxic response, shown by reduction in light output, at predicted free Cu ion activity two orders of magnitude lower than for response to the free ion. Cu–citrate complexes aided bioavailability of Cu to promote the biosensor response. Cu complexes with sulfate or citrate would dissociate in the periplasm/cytoplasm to permit delivery of Cu to the sensor for a biological response.

3.3. Adsorption and uptake of cadmium

Exposure of *P. putida* to 0.09 mmole L^{-1} Cd (10 mg Cd L^{-1}) did not affect culturability of the cells. The loading of Cd^{2+} in the control to the cell surface, the periplasmic space and the cytoplasm for the free ion fitted well to the Langmuir isotherm (Fig. 3 and Table 3), confirming the findings of Pabst et al. (2010).

In the presence of increasing concentrations of Cl, the adsorption of Cd onto the cell surface and transport into the periplasmic space was governed by the free ion activity of Cd in solution (Fig. 3A and B and Table 3). However, the concentration of Cd in the cytoplasm from the Cd chloride complex was not supportive of the FIAM. The zero slope (Fig. 3C) showed that the Cd concentration within the cell was 0.6 mmol kg^{-1} regardless of solution activity.

As with the response for the Cu ligands, both the citrate- and sulfate–Cd complexes produced greater loading at the cell surface than is explained by the free ion activity, especially at the low metal solution activity (Fig. 3A). The neutrally charged $CdSO_4^o$ complex associated with the cell surface followed the Langmuir isotherm (Fig. 3), but the Q and b values were statistically higher than for the free ion (Table 3). With the negatively-charged Cd[citrate]$^-$ complex, association of Cd with the cell wall was independent of the equilibrium solution activity; the slope was not significantly different from zero. Regardless of solution activity, 114 mmole kg^{-1} was associated with the surface. Complexing Cd with citrate or sulfate led to metal uptake into the periplasm and cytoplasm that was independent of free ion-activity; uptake was controlled by the Cd concentration, not the free ion activity.

3.4. Metabolic effects from Cd complexes in the pseudomonad biosensor

The biosensor responded to free Cd, as well as to the Cd complexes, with loss in light output. The responses of the biosensor to Cd in the presence of sulfate, citrate and chloride ligands occurred at Cd activities lower than when the ion was not complexed (Fig. 4). As observed with Cu, toxicity was highest with the citrate complex. These data demonstrated that free Cd activity was not predictive of the bioresponse when the metal was present with the ligands.

4. Discussion

These findings illustrated that bacterial cell loading and the bioresponse of Cu and Cd were not controlled exclusively by the free metal ion activity in solution when these metals were provided as complexes with organic (citrate, acetate) or inorganic

(chloride, sulfate) ligands. Copper and Cd were distributed into all cell compartments from the cell surface, the periplasm and the cytoplasm in the presence of the ligands. With Cu, the blue pigmentation of the cells treated with free and complexed Cu was indicative of the Cu being associated with periplasmic binding proteins that are thought to play a key role in copper homeostasis (Canters, 1986; Cha and Cooksey, 1991). The fact that the cells held more Cu in the periplasm and the cytoplasm than Cd, which remained largely surface bound, reflects the essential requirement of Cu for the cell versus the toxicity of Cd.

For the partitioning studies, we held the concentration of Cu or Cd added to the cells constant; altering the concentration of the added ligands controlled the activity of the free ion in solution. By this approach, we avoided complicating the process of interpreting whether the FIAM was supported when both the concentration of the added metal and the activity of the free ion increased across the test solutions. Brandt et al. (2008) also designed their studies on the effect of dissolved organic matter complexes on the bioavailability of Cu to *P. fluorescens* to avoid covarying descriptors of Cu in solution.

The FIAM was supported for Cu surface adsorption in the presence of acetate (Cu-acetate$^+$), indicating that only the free metal was perceived. Also, surface adsorption was predicted by the free ion concentration with chloride as the ligand for Cd (Cd–Cl$^+$). In contrast the adsorption of Cu and Cd in the presence of citrate or sulfate did not follow the activity of the free metal. The metal associated with the cell surface was much higher than that associated with the free ion activities of Cu or Cd, especially at the low doses. If binding was due only to dissociation of the metals at the cell surface, then Cu-sulfate ($-\log K = 2.4$), Cu-acetate ($-\log K = 2.2$), Cd-sulfate ($-\log K = 2.3$), and Cd-chloride ($-\log K = 2.0$) would behave similarly and be quite different to Cu–citrate ($-\log K = 7.2$) and Cd–citrate ($-\log = 5.0$). Because these relationships were not observed, we speculated that differential binding of the complex to cell surface features must account for the varied responses. Complexes with citrate and sulfate could be binding to the cell surface through anion transporters (Campbell et al., 2002), thus accounting for the metal being perceived as the total concentration rather than as the free metal ion.

With the exception of the Cd-chloride complex, the amount of Cu or Cd associated with the periplasm and the cytoplasm in the presence of ligands was independent of the activity of the free metal ion in solution; the slopes of the isotherms were zero. For Cu, the predicted maximal burden in the periplasm and the cytoplasm was lower with the complexes than with the free ion, perhaps indicating that penetration through the two cell membrane layers was restricted with Cu in ligand form. With Cd, complexation with the designated ligands either increased or had no effect on periplasmic and cytoplasmic loading. It is likely that transport into the cell of the complexes rather than the free metal was involved. Specific channels for the citrate complexes (Errecalde and Campbell, 2000; Campbell et al., 2002) and passive diffusion of neutral complexes such as $CuSO_4$ (Campbell, 1995; Campbell et al., 2002) would constitute uptake mechanisms not controlled by the free ion activity. However, other factors in addition to transport could be involved: dissociation into ions free from the ligand, efflux from the cytoplasm through specific protective ATP-driven pumps and association of free ions with periplasmic and cytoplasmic binding proteins. The detection of blue-colored cells upon exposure to each of the Cu-ligands indicated that some degree of dissociation into the free ions was occurring, at least in the periplasm, to associate with binding proteins in this cellular space.

Availability of ions from the complexes also was indicated by the toxicity response observed with the biosensor pseudomonad. Of significance is the finding that in the presence of citrate, both Cu and Cd were much more toxic than the free metal ion. Lighthart (1980) tested *Pseudomonas* sp. and Errecalde and Campbell (2000) tested *Selenastrum capriconutum* and found that growth decreased and Cd uptake increased with increasing citrate concentration. Joshi-Tope and Francis (1995) stated that metals that form bidentate complexes with citrate (Fe(III), Ni- and Zn-) were transported and degraded in *P. fluorescens*, although metals forming tridentate complexes with citrate (Cd-, Cu-) were not transported. However, Krom et al. (2000) determined that citrate uptake in *Bacillus subtilis* was stimulated when complexed with divalent cations; Cd and Cu were not included in this study. They deduced that CitM transported Mg, Ni, Co, Zn, and Mn citrate complexes that had effective ionic radii ranging between 65 and 80 pm. Complexes with Ca, Sr, and Ba with larger ionic radii between 99 and 134 pm were transported by the CitH transporter. CitH also transported Pb- and Cd–citrate complexes, with ionic radii 97 and 119 pm, in *Enterococcus faecalis* and *B. subtilis* and Cu, with ionic radius 73 pm, in *B. subtilis* (Blancato et al., 2006). Blancato et al. (2006) concluded that it was the size rather than the state of the metal complex that determined transport. Nybroe et al. (2008) demonstrated that for pseudomonads, citrate was not degraded to release free Cu ions that then caused a bioresponse. They referenced Joshi-Tope and Francis (1995) as reporting that Cu–citrate complexes are not degraded nor are the complexes taken-up by *P. fluorescens*. Yet both this present study and Nybroe et al. (2008) showed a bioresponse to Cu–citrate. The status of citrate uptake in the pseudomonad cells used in these studies is not resolved. Inspection of the genome of isolate *P. putida* KT2440 showed the presence of four genes involved in citrate uptake, one of these genes encoded an outer membrane acceptor for Fe(III) citrate. Currently, the location of the Cu-sensor that regulates gene expression to maintain Cu homeostasis is not resolved in the pseudomonads. Nybroe et al. (2008) proposed that the process could involve a cytoplasmic membrane-bound sensor kinase that could respond to cytoplasmic or periplasmic free Cu. From our results we speculate that the citrate complexes promoted delivery of both Cu and Cd so that they were sensed. This finding means that the risk to bacterial function would be underestimated if only the free metal ion concentration was considered.

The advent of nanotechnology complicates the scenario even more. With CuO and ZnO nanoparticles, metal release from the nanoparticles means that they act as primary sources of ions (Heinlaan et al., 2008; Gajjar et al., 2009; Studer et al., 2010; Dimkpa et al., 2011). Ions would thus radiate from these sources to affect cellular function dependent on the location of the nanoparticle within the cell (i.e. the wall, the periplasm or the cytoplasm). In *E coli*, internalization of ZnO nanoparticles (Brayner et al., 2006; Applerot et al., 2009) and Cd quantum dots (Hirschey et al., 2006) has been reported. Cd quantum dots also were found in the cytoplasm of *Photobacterium phosphoreum* (Wang et al., 2010). TiO_2 and Al_2O_3 nanoparticles attached to the cell surfaces of *Cupriavididu metallidurans* and *E. coli* MG1655 and translocation to the periplasm occurred (Simon-Deckers et al., 2009). Kakinen et al. (2011) showed by using a Cu-specific biosensor that Cu release was detected from CuO nanoparticles. However, they also demonstrated that media components influenced the extent of release of ions from the CuO nanoparticles (Kakinen et al., 2011), complicating the process of defining bioavailability. Thus, these studies with nanoparticles support the conclusion based on our uptake and biosensor studies that risks are incorrectly estimated when using free metal ion concentrations only.

5. Conclusion

The results of the measured accumulation levels and bioavailability studies performed with a biosensor both demonstrated that,

in studies of risk assessment, metal associations with ligands as well as the free ions should be considered when evaluating bacterial toxicity potential. This situation may be especially important in aqueous and soil systems where root exudates could supply citrate or acetate and in waters with high chloride or sulfate ion content. Thus the FIAM or the modified BLM may still not provide an adequate risk factor evaluation for Cu and Cd.

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