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Macroscopic and Spectroscopic Analysis of Lanthanide Adsorption to Bacterial Cells.


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Abstract

This study was designed to combine surface complexation modelling of macroscopic adsorption data with X-ray Absorption Spectroscopic (XAS) measurements to identify lanthanide sorption sites on the bacterial surface. The adsorption of selected representatives for light (La and Nd), middle (Sm and Gd) and heavy (Er and Yb) lanthanides was measured as a function of pH, and biomass samples exposed to 4 mg/L lanthanide at pH 3.5 and 6 were analysed using XAS. Surface complexation modelling was consistent with the light lanthanides adsorbing to phosphate sites, whereas the adsorption of middle and heavy lanthanides could be modelled equally well by carboxyl and phosphate sites. The existence of such mixed mode coordination was confirmed by Extended X-ray Absorption Fine Structure (EXAFS) analysis, which was also consistent with adsorption to phosphate sites at low pH, with secondary involvement of carboxyl sites at high adsorption density (high pH). Thus, the two approaches yield broadly consistent information with regard to surface site identity and lanthanide coordination environment. Furthermore, spectroscopic analysis suggests that coordination to phosphate sites is monodentate at the metal/biomass ratios used. Based on the best fitting $p$Ka site, we infer that the phosphate sites are located on N-acetylglucosamine phosphate, the most likely polymer on gram-negative cells with potential phosphate sites that deprotonate around neutral pH.

1 Introduction

Despite a decade of experimental studies involving adsorption of metals to bacterial surfaces, the mechanistic basis of the adsorption reactions remains an open question. Early experimental studies relied almost exclusively on surface complexation modelling to postulate reaction stoichiometry and the identity of surface sites to which the metals were
adsorbed (Fein et al., 1997; Daughney et al., 1998; Fowle & Fein, 1999; Haas et al., 2001; Ngwenya et al., 2003; Yee et al., 2004). Central to this postulate was the assumption that surface functional groups must deprotonate to generate a negative surface site before positively charged metal ions could adsorb (Fein et al., 1997). Given that potentiometric titrations have tentatively identified surface functional groups with different $pK_a$ values (Fein et al., 1997; Small et al., 1999; Haas et al., 2001; Yee and Fein, 2001; Martinez et al., 2002; Phoenix et al., 2002; Ngwenya et al., 2003; Borrok et al., 2005; Dittrich & Sibler, 2006; Gélabert et al. 2006; Guiné et al., 2006; Guiné et al., 2007; Ojeda et al., 2008; Tourney et al., 2008, Lalonde et al., 2008; Pokrovsky et al., 2008), including acidic (carboxyl groups), neutral (phosphate groups) and basic (hydroxyl/amine groups), surface complexation models suggested that metal adsorption at acidic and circum-neutral pH occurred predominantly to carboxyl groups.

Three subsequent developments cast doubt on this assumption. (i) Fowle et al (2000) reported significant uranyl (UO$_2^{2+}$) adsorption onto Bacillus subtilis at very low pH, which could only be successfully modelled assuming adsorption to undeprotonated phosphate sites. This was later confirmed by X-ray adsorption spectroscopy (XAS) experiments by Kelly et al (2002). (ii) Through a rigorous mathematical description of ferrous iron adsorption to B. subtilis, Châtellier and Fortin (2004) showed that metal adsorption commences well before sites start to de-protonate, and that even at low pH, adsorption appeared to occur predominantly to neutral $pK_a$ sites. (iii) Further XAS experiments by Boyanov et al (2003) revealed that at low pH, Cd$^{2+}$ adsorption occurred to phosphate sites, as opposed to carboxyl sites postulated by Fein et al (1997). What emerged from these observations was that surface complexation models provided only circumstantial evidence of the adsorption stoichiometry but that a detailed understanding of the binding mechanism required spectroscopic confirmation (Kelly et al., 2002). Nevertheless, stability constants derived from surface
complexation models have been used to predict metal mobility in porous media (Yee & Fein, 2002; Turner & Fein, 2007) and biofilms (Phoenix & Holmes, 2008) with reasonable success.

In the last decade or so, the biogeochemical behaviour of lanthanides has received increasing attention. One reason for this emphasis is that lanthanides have been used as fertilisers for over 20 years, in East Asia at least (Tyler, 2004). Although their toxicity in such systems is unknown, they provide possible analogues for studying the physiological uptake mechanisms of similarly charged (trivalent) toxic metals such as Al (Bennet & Green, 1992; Ishikawa et al., 1996; Ding et al., 2005), which are often difficult to study because of their low solubility under natural pH conditions. Some lanthanides are also by-products of the nuclear fuel cycle, and the similarity in valence to some of the actinides makes them good analogues for understanding the behaviour of these more problematic elements (Markai et al., 2003). If studied as a suite, lanthanide fractionation patterns make them important indicators of geochemical processes (Henderson, 1984), and have recently been suggested as potential bio-signatures owing to unique fractionation patterns that develop in contact with biological surfaces (Takahashi et al., 2005; Takahashi et al., 2007).

Unlike the common trace metals, however, relatively fewer studies have examined the adsorption of lanthanides by microbes. Among the early reports on selected lanthanides, there was an overwhelming view that lanthanide interaction with bacteria occurred predominantly via surface adsorption, postulating adsorption to carboxyl sites as the main mechanism (e.g. Bayer & Bayer, 1991; Andres et al., 1993; Texier et al., 1999; Philip et al., 2000; Texier et al., 2000). More recently, Fein et al (2001) calculated a log K of 5.1±0.2 for monodentate Nd adsorption to carboxyl sites on Bacillus subtilis cells. By comparison, Markai et al (2003) used time-resolved laser-induced fluorescence spectroscopy to identify surface sites responsible for Eu adsorption to Bacillus subtilis. The spectroscopic measurements suggested carboxyl complexation at low pH, with minor contribution from phosphate sites at circum-


neutral pH. Similar techniques, applied to the adsorption of Eu to three different gram-
negative bacteria by Ozaki et al (2005), revealed differences in the coordination environment
of Eu among strains, suggesting that coordination may depend on fine scale differences in
cell surface chemistry. Lastly, using distribution coefficients for the simultaneous adsorption
of 15 lanthanides, Takahashi et al (2005) have postulated that adsorption is likely to occur
predominantly to phosphate groups at low pH, with carboxyl sites only coming into play at
low biomass concentrations. The selective adsorption of the heavy rare earth elements
(HREE) by phosphate sites was invoked to explain the extreme HREE-enrichment observed
at high biomass concentrations, based on pattern matching using phosphate-containing
ligands.

The objective of this study was to attempt a consistent model of lanthanide adsorption on
bacterial cell surfaces using selected elements representing light (Lanthanum and
Neodymium), middle (Samarium and Gadolinium) and heavy (Erbium and Ytterbium)
lanthanides. Macroscopic adsorption and surface complexation modelling of the adsorption
data is combined with X-ray absorption spectroscopic measurements in order to calculate
site-specific surface complexation constants for lanthanide adsorption, and to identify
adsorption sites on cell surfaces. Several studies have used time-resolved laser-induced
fluorescence spectroscopy (Texier et al., 2000; Markai et al., 2003; Ozaki et al., 2005)) to
study lanthanide adsorption to bacterial surfaces. However, to our knowledge, no previous
study has employed XAS to investigate adsorption of lanthanides to bacterial cells.

2 Experimental Methods

2.1 Biomass preparation

A copper-resistant strain of gram-negative Pantoea agglomerans (formerly identified to
genus level as belonging to Enterobacteriaceae, Ngwenya et al., 2003) was grown for 24
hours in 2L flasks containing 1L of media made with 30g/L tryptone soya broth and 0.5% yeast extract. The bacteria were harvested by centrifugation for 20 minutes at 23,420 x g and 4°C. The cells were re-suspended in 1L of de-ionised water and stirred at 4°C for about 20 minutes on a magnetic stirrer. This process was repeated three times, after which cells were frozen overnight and then freeze-dried to yield a dry powder that was used in the experiments. Although this approach is different from similar metal-bacteria adsorption studies which use fresh cells, our ultimate objective is to study the whole suite of lanthanides (plus Y), using the same batch of cells, in order to avoid inter-culture variability reported in these other studies (e.g. Heinrich et al., 2007). Viability tests using LIVE/DEAD BacLight molecular probes have shown most of the cells (>90%) to be viable after this treatment.

2.2 Adsorption Edge Experiments

Sorption experiments were conducted as a function of pH using suspensions of the bacteria in 0.01M NaClO₄ electrolyte in acid-cleaned, 50 ml polycarbonate centrifuge tubes. A stock suspension was made from the lyophilised cells by first re-hydrating the cells for 1 hour in 0.01M NaClO₄ at 4°C. Cells were then rinsed in the electrolyte three times, each followed by centrifugation at 17,210 x g for 10 minutes. After the final rinse, electrolyte was added to dilute the cell suspension to the desired concentration, followed by addition of metal from 1000 mg/L stock solutions in 1% HNO₃. The pH of this stock suspension was then adjusted upward in ~0.25 pH steps whilst continuously purging the headspace with N₂ to avoid dissolution of CO₂ and potential precipitation of carbonates. At each pH, from 2.5 upwards, 20 ml was transferred into a 50 ml polycarbonate centrifuge tube and equilibrated for 3 hours on a carousel rotating at 30 revolutions per minute. Two 5 ml sub-samples were transferred into pre-weighed glass vials and evaporated to constant weight in order to determine the exact biomass concentration, after correcting for a 5 ml electrolyte blank. Suspension pH was
measured at room temperature (23±1 °C) using a glass combination electrode connected to a
Hanna Instrument HI 9025 pH/Eh meter after a 3-point calibration using Merck buffers of
4.00, 7.00 and 9.22. Although the background electrolyte (0.01 M) is within the range of
NBS buffers (∼0.1 M), we tested the response of the pH probe by checking the calibration
against a pH 4.00 buffer made in 0.01M NaClO4 instead of ultrapure water, and recorded a
pH of 3.99±0.01. Furthermore a 10⁻³ M HCl solution diluted from a 1 M volumetric standard
gave a pH of 3.01±0.02.

The above equilibration time was chosen based on preliminary kinetic experiments which
showed attainment of constant adsorption and suspension pH between 1 and 3 hours.
Furthermore, the upward-pH adjustment was adopted because previous experiments with this
strain have shown that it can produce soluble organics around circum-neutral pH values
(Ngwenya et al., 2003; Ngwenya, 2007), which are likely to decrease sorption density, as also
observed for *Bacillus subtilis* by Takahashi et al (2005). Nevertheless, a reversibility test was
performed based on a modification of the method of Fowle and Fein (1999), in which a
parent suspension spiked with Er was split into equal volumes to ensure the biomass and
initial metal concentrations were the same. One half was equilibrated at pH 2.5 for 3 hours,
then adjusted upwards in roughly 0.25 pH steps, with sub-sampling (20 ml) followed by
further equilibration for 3 hours. The second half was initially equilibrated at pH 6.5, then
adjusted downwards and re-equilibrated for a further 3 hours.

For four of the six lanthanides (La, Nd, Sm and Yb), two experiments, each with a
different biomass and initial metal concentration, were carried out. We present data for
suspending using nominally ~0.2 g/L biomass with both 2 mg/L and 4 mg/L initial
lanthanide concentrations. Details of individual experiments are given in Table 1.
Calculations using MINTEQA2 (Allison et al., 1991) and first hydrolysis constants from
Klungness and Byrne (2004) and from Smith & Martell (1976) showed that at these
concentrations, metal hydroxides do not precipitate out at the target pH values. However, controls (metal without biomass) showed that as much as 20% of each metal was potentially adsorbed to containers around pH 7. No adsorption to containers was observed at pH values below 5.5 but we often detected around 3% adsorption around pH 6, increasing to about 7% by pH 6.5. We tested these observations using Teflon centrifuge tubes and measured similar adsorption. Thus our experiments were restricted to pH ≤ 6.5, where speciation calculations showed that between 97% (Yb) and 99% (La) of the lanthanide was in the form of the hydrated trivalent ion and the rest as LnOH$^{2+}$. Nevertheless, we are confident that the error introduced by this artefact on the experimental data is small given the stronger adsorption to cells, especially as initial addition of the lanthanide was done at low pH. Sampling involved pelleting (17,210 x g) the cells and filtering 10 ml of the supernatant into an acid-cleaned bottle. These solutions were acidified to 2% v/v HNO$_3$ and stored at 4°C before metal analysis by ICP-MS using matrix-matched standards. The use of freeze-dried cells can affect total metal adsorbed, as demonstrated recently by Gabr et al. (2008). Thus, a further adsorption edge experiment was carried out to compare fresh and freeze-dried cells, using the element Nd, and similar biomass concentrations (0.21±0.01 g/L).

For the analysis, our sample solutions were diluted 1000 fold with 5% HNO$_3$, and metal concentration was determined using a VG Elemental PlasmaQuad II+ Quadrupole mass spectrometer at the Scottish Universities Environmental Research Centre (SUERC). The metal concentration in the solution was obtained by reference to a calibration line produced by the analysis of standard solutions containing known concentrations of the element. Each sample value was corrected for procedural blank containing ultrapure water and 5% HNO$_3$. Indium, Rhenium, and Ruthenium were selected as internal standards to monitor the condition of the VG PQII+ within each session. The accuracy of the procedure was measured by including an international environmental reference material BCR-1 (Govindaraju, 1984).
Although BCR-1 is not representative of the sample matrix, its light REE enrichment is ideal for assessing the stability of the ICP on the day of the analysis to minimise interferences and monitor changes during the analysis. Isotope peaks were determined in peak jumping mode with 3 points per peak using three 60s integrations. When available, multiple isotopes were selected for each element and the values averaged. Thus we used the following isotopes for each element: La ($^{139}$La), Nd ($^{145}$Nd and $^{146}$Nd), Sm ($^{147}$Sm, $^{149}$Sm, and $^{152}$Sm), Gd ($^{155}$Gd and $^{157}$Gd), Er ($^{166}$Er and $^{168}$Er) and Yb ($^{172}$Yb, $^{173}$Yb and $^{174}$Yb). Isotopes free from interference (oxide or isobaric) were selected. Such care was necessary because some samples were analysed alongside solutions containing mixtures of lanthanides, results of which will be published elsewhere (Ngwenya et al., 2009). As a precaution against high Ba blanks, we also routinely check for Ba oxide interference even during individual lanthanide analysis. The average value of 3 biomass free controls over the range pH 2-4 (to ensure no adsorption to containers walls) was used to determine the true starting concentration, which is given in Table 1. Precision of sample preparation was monitored by analysing 3 duplicate pH values and differences were smaller than 10%.

2.3 Data Analysis

Metal adsorption was calculated by mass balance from the difference between initial concentration and the amount in solution after equilibration. The resulting adsorption edges were modelled using the FITEQL 4 optimisation routine (Herbelin & Westall, 1999) to determine intrinsic metal-site stability constants, using the weighted sum of squares normalised by the number of degrees of freedom ($WSOS/DF$) to select the best-fitting model. Values between 0.1 and 20 are normally considered good fits (Herbelin and Westall, 1999). A constant capacitance electric field model with activity correction was used, with the same surface area (140m$^2$/g), capacitance (8F/m$^2$), deprotonation constants and surface site
densities as in Ngwenya et al (2003). Despite its limitations, the constant capacitance model was preferred over more recent, non-electrostatic approaches (e.g. Fein et al., 2005; Borrok et al., 2005) because of the high lanthanide valence (Marmier & Fromage, 1999), and because attempts with non-electrostatic models did not always produce consistent results between different lanthanide to biomass ratios. Acid-base equilibria for the electrolyte and water were included in the equilibrium problem, including lanthanide hydrolysis reactions whose stability constants were taken from Klungness and Byrne (2000).

2.4 Samples and standards for X-Ray Absorption Spectroscopy

Based on the fact that adsorption density of cations increases with pH and previous studies have shown that the coordination environment can vary depending on adsorption density (e.g. Kelly et al., 2002; Boyanov et al., 2003; Guiné et al., 2006), it was necessary to analyse biomass samples at low and high (circum-neutral) pH in order to examine speciation at low and high adsorption densities. Our experiments were focussed on four of the six lanthanides (Nd, Sm, Er and Yb), again chosen to represent light, middle and heavy lanthanides, and with biomass samples (0.2g/L and metal concentrations of 4 mg/L) adjusted to pH 3.5 and 6. After equilibration for 3 hours, the suspension was centrifuged at 23,420 x g for 20 minutes, followed by a quick rinse in pH-adjusted 0.01M NaClO4 electrolyte and further centrifugation to remove un-adsorbed metal. The resulting biomass paste was loaded onto slots in Al plates to a thickness of 1mm, covered with Kapton tape and stored at -80°C until analysis. For Sm and Yb, we also tested their coordination environment at a higher biomass (1g/L) with 10 mg/L initial metal concentration to examine if the coordination changed when each of the different surface sites were slightly in excess to probe possible site selectivity. In order to validate our analysis methods reference solution standards of perchlorate, acetate,
citrate and glycerol-2-phosphate were analysed, and these gave broadly similar results to biomass samples in terms of bond distances.

2.5 X-Ray Absorption Spectroscopy measurements and data analysis

X-ray absorption spectra were collected in fluorescence mode on Stations 7.1 and 16.5 of the SRS, Daresbury Laboratory. Both beamlines were operating with sagittally focussing double crystal monochromators. Station 7.1 had a Si (111) set of crystals and a 9 element monolithic Ge solid state detector. Station 16.5 had a Si (220) set of crystals and a 30 element Ge detector. Data from the Nd and Sm (4 mg/L) samples were collected on station 7.1, whilst the Yb, Er and the rest of the Sm data was collected on station 16.5. All the spectra were of the L3 edge except for Er, where the L2 edge was used. The biomass samples were at 80K when the data was collected to minimise any possible beam damage. There was no noticeable difference in the XANES between the first and last scan of each biomass sample, showing that the samples were unaffected by any short-lived beam damage. Up to 32 scans were recorded and averaged for each biomass sample. The monochromator was calibrated using appropriate metal foils, Ti, Mn, Fe and Cu.

The spectra were reduced using the programs EXCALIB, EXBROOK and EXSPLINE (Ellis, 1995). The EXAFS was analysed in the program DL-EXCURVE (Tomic et al., 2005). Data were fitted using ab initio phaseshifts calculated using Hedin-Lundqvist exchange and Von Barth ground state potentials and single scattering using rapid curved wave theory. The data were minimised using the fit index R, defined as follows:

\[ R = \Sigma_i [(1/\sigma_i)(|\text{experiment}(i)-\text{theory}(i)|/\sigma_i)] \cdot 100\% \] (1)

where:
In each case ab initio modelling of the data began with fitting the first coordination sphere with oxygen and then attempts were made to fit further coordination shells of either phosphorus or carbon. The fits for carbon and phosphorus were compared. Further to this a fit was attempted using a second coordination sphere of both carbon and phosphorus. In order to reduce the number of refined variables in modelling the second coordination sphere, initially the shell occupancy number was fixed at half the value of the shell occupancy number for the oxygen shell; once a bonding mode had been established this number was refined to the nearest half integral value. The second sphere data is only considered valid where a 10% improvement in the fit index is seen on addition of this shell. In differentiating between C and P in this second sphere, we believe where the C or P model has a 5% lower fit index than the other, this model is definitely the preferred one according to the EXAFS, and is consequently the model shown in the results table. The data quality and number of free parameters mean that a model with a mixed second coordination sphere of C and P is not justified statistically, though the EXAFS cannot rule out a component of carbonate or phosphate bonding being present in samples where the other is the dominant mode.

3 Results and Discussion

3.1 Kinetics, reversibility and biomass preparation

Our time course experiments, conducted using Gd at pH around 4, showed that lanthanide adsorption to the biomass was rapid, with approximately 99% of the 4 mg/L Gd adsorbed within 5 minutes of contact. As shown in Figure 1a, however, the pH of the suspension rose gradually during the first 100 minutes or so and only attained constancy thereafter. It was for this reason that we chose (a) an equilibration time of 3 hours for our adsorption edge experiments and (b) present our adsorption edge data as a function of final pH below.
Figure 1b shows that after this 3-hour equilibration, the adsorption reaction is also completely reversible, as exemplified by the adsorption of 4 mg/L Er. Although individual suspensions equilibrate to slightly differing final pH values, the two curves overlie each other, clearly demonstrating equilibrium thermodynamic attainment. Figure 1c shows further that by combining this data with that obtained on a different, independent suspension yields excellent reproducibility. Finally, a recent study by Gabr et al (2008) has shown that the amount of Pb and Ni adsorbed by a strain of Pseudomonas aeruginosa was slightly higher when freeze-dried cells were used instead of freshly prepared cells. We tested this with our Pantoea agglomerans strain using 2 mg/L Nd and found no differences in adsorption density between fresh and freeze-dried cells (Fig. 1d), both in terms of adsorption edges and modelled stability constants.

3.2 Macroscopic adsorption and surface complexation modelling

Macroscopic adsorption experiments were designed to provide information on what type of sites are involved in lanthanide uptake under the limited range of acidic pH conditions tested. The experiments were focussed on pH-dependent adsorption because it has been established that such experiments provide ideal data to quantify the stability constants between each metal and the sites involved, although adsorption isotherm experiments are also useful confirmatory tools. Results from these experiments are shown in Figure 2, where the percentage of metal adsorbed is plotted against final suspension pH, and curves represent different model fits to the data. In all cases, the amount of lanthanide adsorbed increases with increasing pH, consistent with the expected behaviour for cationic adsorbents.

Considering that kinetic and reversibility experiments indicated attainment of equilibrium, mass balance constraints on the total concentration of each metal should show an increase in
percent lanthanide adsorbed with decreasing initial metal (4 mg/L to 2 mg/L) to biomass ratio. This behaviour is clearly evident in the Sm and Yb adsorption edges. In contrast, the La and Nd edges are practically identical. We found that the biomass concentration in the 2 mg/L La experiment was much lower than the nominal 0.2 g/L, being about 0.12 g (due to losses during washing), resulting in a higher metal to biomass ratio for this suspension. Equally, the measured starting biomass concentration for 4 mg/L Nd was 0.25 g/L explaining the higher percentage adsorption in this experiment. To illustrate this further, we calculated relative metal to biomass concentrations between the 4 mg/L and the 2 mg/L suspensions in our study, by dividing the lanthanide to biomass ratio for the 4 mg/L experiment by the corresponding ratio in the 2 mg/L experiment. This revealed that by using similar mg/L instead of equimolar concentrations, the relative metal to biomass ratio increased with increasing atomic number. What emerges from this inadvertent approach is that relative ratios below about 1.5 are not able to resolve the two adsorption edges, being within the error scatter of the data.

Similarly, comparison amongst the elements using adsorption at 50% does not show systematic variation with atomic number. For the 4 mg/L suspensions, where the biomass concentrations are closer to each other, we find that 50% adsorption occurs above pH 4 for both La and Nd and below pH 4 for Sm, Gd, Er and Yb, suggesting that middle and heavy lanthanides sorb more strongly to the biomass. Surface complexation modelling is therefore critical to confirm that the mass balance constraints on biomass are applicable, as well as to confirm the relative adsorptive strength of the 6 elements.

Modelling was set with the following generic reaction stoichiometry:

\[
ROH_n^{n-1} + \text{Ln}^{3+} \leftrightarrow \left(\text{LnROH}_n\right)^{(n+2)^+}
\]  (3)
where $ROH_n$ represents a protonated surface functional group, $Ln^{3+}$ is the lanthanide cation and $n$ represents the number of protons attached to the surface functional group. For the constant capacitance electrostatic model, we define an intrinsic stability constant ($K_{int}$), thus:

$$K_{int} = \frac{(LnROH_n)^{(n+2)}}{ROH_n^{n-1}Ln^{3+}} e^{\left(\frac{(F\psi / RT)(n+2)}{nRT}\right)}$$  

(4)

where $F$ is the Faraday constant, $\psi$ is the potential at the cell surface, $R$ is the universal gas constant and $T$ is temperature. The following stoichiometries were tested:

$$ROH + Ln^{3+} \Leftrightarrow Ln(ROH)^{3+}, \quad n=1.$$  

(5)

$$RO^- + Ln^{3+} \Leftrightarrow Ln(RO)^{2+}, \quad n = 0.$$  

(6)

Reactions 5 and 6 are essentially regarded as representing outer-sphere and inner-sphere complexation respectively (Langmuir, 1997). Deprotonation constants and site densities determined from acid-base titrations by Ngwenya et al (2003) were used as input in metal adsorption models. Other thermodynamic parameters were obtained from Smith & Martell, (1976) and from Klungness & Byrne (2000). The WSOS/DF calculated by FITEQL was used to select the best-fitting model, in conjunction with visual adherence of the model curves to the experimental data. Reaction (5) was tested, either alone or in combination with reaction (6), because of previous findings suggesting the involvement of neutral surface sites for high valance cations such as UO$_2^{2+}$ (Fowle et al., 2000, Haas et al., 2001). However, both approaches invariably resulted in much higher WSOS/DF values. Other stoichiometries (e.g. bidentate) were tested with similar negative outcomes. Thus, the modelling results collated in Table 2 represent best-fitting stability constants based on reaction (6).

For La, lower WSOS/DF values were associated with deprotonation constants tentatively ascribed to phosphate sites at both metal to biomass ratios. The difference in WSOS/DF values between adsorption to carboxyl and phosphate sites is higher for the 4 mg/L dataset than the 2 mg/L dataset, where these values are practically indistinguishable. Nevertheless, a
forward modelling of the adsorption isotherm using the mean pK values shows clearly that both datasets are optimally fit with the phosphate surface complex (Fig. 2a), although carboxyl complexation appears to be equally reasonable at low pH. Attempts to reproduce the adsorption data with a forward model involving simultaneous adsorption to carboxyl and phosphate sites overestimated the adsorption density across the whole range of pH.

Modelling of the Nd data was consistent between the two metal/biomass ratios in yielding a lower WSOS/DF value for carboxyl adsorption. However, the WSOS/DF value for carboxyl complexation in the 2 mg/L dataset was well below the acceptable value of 0.1, apparently suggesting that the model contains too many adjustable parameters or that the error estimates are too large (Herbelin & Westall, 1999). By comparison, the WSOS/DF value for phosphate adsorption was more reasonable at 0.32. As can be seen in Figure 2b, mean phosphate complexation constants perform marginally better in predicting the measured adsorption edges, particularly at the lower pH end. Interestingly, Fein et al (2001) calculated a Log K of $5.1 \pm 0.2$ for monodentate Nd adsorption to carboxyl sites on Bacillus subtilis. Our Nd value of $5.06 \pm 0.1$ is therefore practically identical to their value. No comparison can be made for phosphate complexation as Fein et al (2001) did not report a stability constant for this reaction.

Sm models yielded lower WSOS/DF values whenever carboxyl surface parameters were used. However, differences in WSOS/DF values were very small and the phosphate model parameters were just as tightly constrained. As shown in Figure 2c, mean carboxyl and phosphate stability constants provide reasonable reproduction of the adsorption edges. Subtle differences are evident at low pH where carboxyl models perform better but the phosphate site performs marginally better at pH values above 4.5. The single metal to biomass data that we have for Gd suggests adsorption to phosphate sites performs slightly better (Fig. 2d).
Carboxyl models appear to provide better fits to all the 4ppm Er data than phosphate models (Fig. 2e). Similarly, lower WSOS/DF values were associated with Yb adsorption to carboxyl sites for both the 2 mg/L and 4 mg/L data (Fig. 2f). This is also reflected in the slightly better fit to the adsorption data using carboxyl models, although the phosphate site predicts the data slightly better at low pH in the 2 mg/L data.

In summary, it appears that lanthanide adsorption edges below pH 6.5 are consistent with adsorption to phosphate groups for both of the light lanthanides examined in this study. By contrast, the two middle lanthanides (Sm and Gd) are not consistent, with Sm apparently preferring carboxyl sites whereas Gd is best fit with phosphate sites, although the Gd adsorption is less well constrained based on one metal to biomass dataset. In contrast, both of the heavy lanthanides are best fit with carboxyl adsorption, although phosphate fits to the data are also generally good. The phosphate predictions are indistinguishable from carboxyl predictions within the error bounds found in most experiments of metal adsorption to bacteria where biomass and/or metal concentration is varied (Fein et al., 1997; Daughney et al., 2001; Haas et al., 2001; Martinez & Ferris., 2001; Ngwenya et al., 2003; Châtellier & Fortin, 2004; Yee et al., 2004; Borrok et al., 2005; Toner et al., 2005; Pokrovsky et al., 2005; Gélabert et al., 2006; Burnett et al., 2006). We conclude that surface complexation modelling of the macroscopic adsorption data either points to the involvement of both sites or that it is simply not able to reveal selectivity in surface speciation. One possible reason for this is that in all our experiments, the concentration of each site in the suspension is in excess of the initial total lanthanide concentration. Equally, the pH range of the data may be too narrow to resolve between the different possible surface complexes. However, the measured adsorption does not change above pH 6.5 so data beyond this pH does not yield additional information. Attempts to use higher initial lanthanide concentrations (15ppm) were not useful as there was some evidence of surface precipitation above pH 5 (data not shown).
3.3 Lanthanide coordination from EXAFS measurements

Attempts were made to obtain X-ray absorption spectra for Nd, Sm, Yb and Er samples with 4 mg/L initial metal concentration for both pH’s (3.5 and 6). However, in the case of Nd, the concentration of adsorbed metal in the pH 3.5 sample was not sufficient to give analysable data. Data was collected out to 12 k (Å⁻¹), but in most cases could only be analysed to ca.10 k as beyond this point the signal-to-noise ratio is poor. This accounts for the different data ranges in Figures 3 and 4. In one Sm sample and Yb sample the analysis is restricted to about 8 k because of Fe contamination in the cryosystem (Sm), and low data quality for the Yb sample. The EXAFS analysis results are shown in Table 3.

In a previous study of Nd co-ordinated to alfalfa biomass, Parsons et al (2005) showed two Nd-O first coordination sphere distances of around 2.38 and 2.56 Å. They attributed these to water bound O (2.38Å) and surface bound O (2.56Å). Acetate or similar carboxylate groups displayed an Nd-C distance of about 3.6 Å. In our modelling, attempts to split the oxygen coordination shell led to very high correlations between the distances and Debye-Waller factors, thus the shell has been left unsplit. At 3.92 Å, the Nd(-O-)P distance is too long for bidentate phosphate coordination, but reasonable for monodentate phosphate bonding. This is because in Nd-Monazite (NdPO₄), the Nd-P distances are 3.15 and 3.25 Å for bidentate phosphate and 3.47 and 3.73 Å for monodentate phosphate, with the Nd –O distances ranging between 2.42 and 2.79 Å, with a mean of 2.52 Å (Ni et al, 1995). Our distance of 3.92 Å for Nd-P is similar to that found in ultraphosphate and metaphosphate glasses (3.87 Å) by Karabulut et al (2005). No Nd-C coordination was evident in the pH6, 4ppm biomass data.

We saw the most significant pH-dependant changes in the EXAFS for Samarium (Figure 3). At low pH (3.5) there are indications of monodentate phosphate binding at 3.86 Å, comparable to the monodentate Sm-O-P of 3.63 -3.78 Å in KSmHP₃O₁₀ (Zouari et al., 2000).
The pH 6 sample shows carbon in the second coordination sphere. In the 10 mg/L sample this shell is quite distinct at 3.48 Å, similar to a monodentate Sm(-O-)C of 3.47 Å in Samarium Carbonate Hydroxide (Xu et al., 2006). In the 4 mg/L pH 6 sample the carbon shell does not fit the Fourier Transform well (Figure 3), though it improves the EXAFS fit. The amplitude of this oscillation is also quite low compared to the other Sm second shell EXAFS (Figure 3). Further the Sm-C distance is quite different and a little longer than expected (3.64 Å), thus this may be indicative of mixed speciation in this sample, where more than one bonding mode exists in substantial fraction and thus the bare two shell fit is unrepresentative - the EXAFS being washed out by destructive interference. Thus we believe the binding mode may actually be a mixture of phosphate and carboxyl in this sample. It seems therefore that when the biomass to Sm ratio is increased (1g/L, 10ppm) the carboxyl mode predominates. These observations are entirely consistent and may suggest carboxyl site preference by Sm.

For the higher lanthanides, Er and Yb, the EXAFS results (Figure 4) are similar with both showing P in the second shell at distances 3.81-3.83 Å (Er) and 3.75-3.80 Å (Yb) similar to monodentate phosphate bonding (Er –O–P 3.75 Å, Yb–O–P 3.72 Å in LnPO4 (Milligan and Mullica, 1983). This distance would need to be ca. 0.4 Å less for bidentate coordination. No C in the second coordination sphere could be fitted convincingly for any of these datasets, however examination of the individual shell EXAFS contributions (Figure 4) and Fourier transforms (Figure 4) reveals again that for the pH 6 samples the P shell amplitude is smaller, and also the shell occupancy numbers for the Yb pH 6 P shells are lower than for the pH 3.5 samples. These related observations are both indicative that, in the higher pH bonding, monodentate phosphate coordination may not be the whole story; however, our analysis of the EXAFS data for the Er and Yb does not allow us to say whether this is due to some carboxyl bonding or other coordination mode.
In the EXAFS the lanthanide contraction is noticeable with the Ln – O distance gradually decreasing from 2.48 – 2.45 – 2.33 – 2.28 from Nd to Sm to Er to Yb. Also the Er appears to have slightly fewer oxygen atoms around it than the other lanthanides. The coordination number of oxygen atoms in the first shell is similar for all the atoms, refining to values between 7.5 and 10.5.

Our spectroscopic findings are consistent with other EXAFS studies in two respects. Firstly, they show the predominance of phosphate binding at low pH, as previously reported for uranyl and Cd adsorption to *Bacillus subtilis* by Kelly et al (2002) and Boyanov et al. (2003) respectively. Secondly, they show that as the pH increases at a constant biomass concentration, the carboxyl group starts to get involved in lanthanide bonding. Thus, in summary the EXAFS analysis is strongly indicative of monodentate phosphate coordination at low pH for the 4 lanthanides studied by XAFS here, whereas at higher pH, phosphate coordination dominates for Nd, Er and Yb, whereas carboxyl coordination dominates for Sm. However the data indicates that for the heavy lanthanides, there may well be more than one bonding mode present.

### 3.4 General synthesis

This study combined surface complexation modelling of macroscopic adsorption data with X-ray spectroscopic measurements to identify lanthanide sorption sites on the bacterial surface. The experiments were limited to the acidic region of the pH spectrum because nearly 100% adsorption was attained above pH 5 for the range of metal to biomass ratios used. As a result, surface complexation modelling was focussed on sites that deprotonate in this pH range, and suggested that there may be variations in the dominant sorption sites across the lanthanide series. Specifically, the adsorption of both of the light lanthanides was best modelled assuming adsorption to phosphate sites. However, the rest of the lanthanides could
be modelled equally well with carboxyl or phosphate sites, although Samarium was better modelled with carboxyl relative to phosphate sites. Nevertheless, the differences in performance between the two models were generally small, suggesting that surface complexation modelling does not adequately discriminate between the two models. Lastly, we found that for all the lanthanides, inner-sphere (proton exchange) complexation was the most likely reaction stoichiometry, although this needs to be confirmed by conducting ionic strength-dependent metal adsorption experiments. By comparison, X-ray spectroscopic analyses are more consistent with adsorption of most lanthanides to phosphate sites, at least at low adsorption densities (at low pH), with secondary involvement of carboxyl sites at high adsorption density (high pH). Furthermore, spectroscopic analysis suggests that the coordination to phosphate sites is monodentate. Some indication of carboxyl dominance was inferred for Sm in the high biomass sample.

Thus, the first conclusion that arises from this study is that surface complexation modelling and spectroscopic analysis are broadly consistent in their information content with regard to surface site identity and lanthanide coordination environment. Coordination of light lanthanides to phosphate groups, as implied by both techniques, is consistent with the findings of Merroun et al (2003) for La adsorption to \textit{M. xanthus}. Such a model was also suggested by Takahashi et al (2005) for all lanthanides adsorbed onto \textit{B. subtilis}, although their experiments were conducted only at low pH values, where recent spectroscopic studies (Kelly et al., 2002; Boyanov et al., 2003) seem to indicate that metal coordination to phosphate site is a common phenomenon. These studies have also indicated that with increasing pH, carboxyl sites become more involved in the adsorption reaction, because carboxyl sites start to deprotonate (Fowle et al., 2000; Kelly et al., 2002). Although we do not report Eu adsorption in this study, the pH-dependent behaviour contrasts with the findings of Markai et al (2003), that low pH adsorption of Eu was due to carboxyl complexation, based
on time resolved laser-induced fluorescence spectroscopy (TRLFS) measurements, with phosphate groups only coming into play at high pH and/or adsorption density. Notably, the coordination environment of lanthanides has also been found to vary between bacterial species (Ozaki et al., 2005). Thus it is not possible categorically to generalise our observations, indicating that further work is required to develop a better understanding of the lanthanide coordination environment in biological materials. More importantly, our study demonstrates clearly that neither technique is capable of providing unambiguous coordination information for lanthanide adsorption. This clearly justifies the use of complimentary techniques in metal adsorption studies.

Finally, we note that the best fitting model for adsorption to phosphate sites is consistent with inner-sphere (reaction 6) complexation, with adsorption to undeprotonated phosphate sites (reaction 5) yielding WSOS/DF values around 50, and is therefore unlike the coordination environment of the uranyl ion (Fowle et al., 2000; Kelly et al., 2002). Within the gram-negative cell wall, the only structural components containing phosphate groups in the outer membrane are phospholipids and N-acetylglucosamine phosphate, a component of Lipid A in the lipopolysaccharide membrane (Beveridge & Fyfe, 1985; Madigan et al., 2003; Guiné et al., 2006). As shown in Figure 5, both molecules contain phosphoester bonds, with a monophosphoester bond in N-acetylglucosamine-6-phosphate (Nishitani et al., 2006), and a phosphodiester bond in phospholipids, typified here by phosphatidylethanolamine (Mayes, 1985).

Experimental studies of protonation reactions for phosphodiesters in aqueous solutions are consistent with a $pK_a$ of about -0.7 for the single hydroxylated functional group (Azema et al., 2005). Thus, this functional group is likely to be deprotonated both at physiological conditions and across our experimental pH spectrum. As such, it may be responsible for the observed low pH adsorption of the light lanthanides in this study, and could also explain the
apparent pH-independent adsorption of uranyl ions reported by Fowle et al (2000) at low pH. However, our model outcomes were realised with a $p$Ka of 6.9 for phosphate groups (Ngwenya et al., 2003), which is closer to the deprotonation constant for the second hydroxyl group on phosphoric acid, ($p$Ka $\sim$7). Attempts to model the data with a non-electrostatic model, which yielded a phosphodiester $p$Ka around 3.9 (based on 4 variable biomass titrations) for *Pantoea agglomerans*, did not produce consistent results across different metal to biomass ratios and the resulting WSOS/DF values were always higher. This leads us to speculate that the second hydroxyl group on N-acetylg glucosamine phosphate makes this a more likely candidate for lanthanide binding on a gram-negative bacterium. It may be considered analogous to methylphosphoric acid, which has a second $p$Ka around 6.3 (Saha et al., 1996). Such a conjecture need not conflict with phosphate binding of cations on gram-positive cell walls, where phosphate groups are dominated by phosphodiester linkages in teichoic acids (Heinrich et al., 2007), because gram-positive cell walls also contain other phosphate groups in addition to phosphodiester linkages.

4 Conclusions

The objective of this study was to combine surface complexation modelling of macroscopic adsorption data with X-ray spectroscopic measurements to identify lanthanide sorption sites on the bacterial surface. We have shown that surface complexation modelling and spectroscopic analysis yield complimentary information on the coordination environment of the light lanthanides. Surface complexation modelling was consistent with the light lanthanides adsorbing to phosphate sites, whereas the adsorption of middle and heavy lanthanides could be modelled equally well by carboxyl and phosphate sites. Moreover, proton exchange is the most likely reaction stoichiometry. The existence of such mixed mode coordination was also confirmed by EXAFS analyses, which was consistent with adsorption
to phosphate sites at low pH, with secondary involvement of carboxyl sites at high pH.

Importantly, however, neither surface complexation modelling nor EXAFS analysis gave the whole picture alone, emphasising the importance of using complimentary techniques in understanding sorption mechanisms. Apparently, coordination to phosphate sites is monodentate, and occurs to phosphate sites around pKa ~7. Based on these observations, we conjecture that the phosphate sites are located on N-acetylglucosamine phosphate, the most likely polymer with potential phosphate sites that deprotonate around neutral pH.

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References


Figure 1. Graphs showing (a) adsorption of Gd and suspension pH as a function of time, (b) adsorption reversibility as exemplified by Er, (c) reproducibility of 3 suspensions of ~0.2g/L biomass and 4 mg/L Er, and (d) comparison of fresh (FS) and freeze-dried (FZ) cells of the same dry biomass concentration, showing that the use of freeze-dried does not have an effect on the Nd adsorption edge. Note also that equilibration of pH and adsorption occurs after 100 minutes or so.

Figure 2. Experimental adsorption data (symbols) and FITEQL model fits (curves) for the adsorption of different lanthanides to *Pantoea agglomerans* cells. Model curves represent adsorption to carboxyl (dotted line) and phosphate (solid line) sites respectively. Thus the legend label “La2P” refers to a model curve predicted for the adsorption of 2mg/l lanthanum assuming adsorption to a phosphate site whereas “La2C” refers to the same model assuming adsorption to a carboxyl site etc.

Figure 3: $k^3$-weighted EXAFS, each shell's contribution to the EXAFS fit (shell 1 is above shell 2 for each sample), and phase shifted Fourier transforms for (a) pH 6 4 mg/l Nd sample, (b) pH6 10 mg/l Sm, (c) pH6 4 mg/l Sm and (d) pH 3.5 4 mg/l Sm samples. Spectra have been offset for clarity. Experimental data is solid line and fit is dotted line.

Figure 4. $k^3$-weighted EXAFS, each shell's contribution to the EXAFS fit (shell 1 is above shell 2 for each sample), and phase shifted Fourier transforms for (a) pH3.5, 4 mg/l Yb, (b) pH3.5, 10 mg/l Yb (c) pH6 4 mg/l Yb and (d) pH6, 10 mg/l Yb samples, as well as (e) pH3.5 4 mg/l Er and (f) pH 6 4 mg/l Er. Spectra have been offset for clarity. Experimental 514 data is solid line and fit is dotted line.
Figure 5. Molecular structures of N-acetylglucosamine-6-phosphate (redrawn from Nishitani et al., 2006) and phosphatidylethanolamine (redrawn from Mayes, 1985), showing possible phosphate groups that may be involved in lanthanide coordination on a gram-negative bacterial cell surface. In practice, the cell surface composition is likely more complex but the deprotonation constants for the different protons attached to the phosphate group appear to have a relatively narrow range.
Table Captions.

Table 1. Compilation of experiments reported in this study, summarising the initial biomass and lanthanide concentrations. Column 6 represents the relative metal to biomass ratio between the 4 mg/L and the 2 mg/L suspensions, calculated by dividing the lanthanide to biomass ratio for the 4 mg/L experiment by the corresponding ratio in the 2 mg/L experiment.

Table 2. Results from FITEQL optimisation of stability constants for the adsorption of the studied lanthanides, with errors on the mean representing one times the standard deviation. Models were realised using bacterial deprotonation constants and surface site densities determined by Ngwenya et al (2003). For carboxyl sites, the values are pKa = 4.3±0.2 and site density = 5.0±0.7 mol/g cells, whereas the corresponding values for phosphate sites are pKa= 6.9±0.5 and site density = 2.2±0.6 mol/g cells. The abbreviations “La2” represent an experiment using 2 mg/L lanthanum, etc whereas ErUP and ErDN refer to reversibility experiments in which the pH of the initial suspension was adjusted upwards or downwards respectively.

Table 3. EXAFS analysis results for the lanthanide L3 edge collected in Fluorescence mode.
### Table 1.

<table>
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<th>Element</th>
<th>Experiment</th>
<th>Biomass (g/L)</th>
<th>Log Molarity Initial [Ln^{3+}]</th>
<th>Lanthanide/Biomass (mol/g) x 10^{-4}</th>
<th>La4/ La2 metal/biomass ratio</th>
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### Table 2.

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Table 3.

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(a): ±15%; (b) ± 0.5 %
Figures

Figure 1, Ngwenya et al
Figure 2, Ngwenya et al.

(a) Lanthanum

(b) Neodymium
Figure 2 cont'd, Ngwenya et al.

(d) Gadolinium

(c) Samarium

% adsorbed (%)

pH

Gd Adsorbed (%)

2ppm

4ppm

Gd4C

Gd4P

Sm Adsorbed (%)

2ppm

4ppm

Sm2P

Sm4P

Sm4C

Sm2C
Figure 2 cont’d, Ngwenya et al.

(e) Erbium

(f) Ytterbium
Figure 3, Ngwenya et al.
Figure 4, Ngwenya et al.
Figure 5, Ngwenya et al

(a) N-acetylglucosamine-6-phosphate

(b) Phosphatidylethanolamine

\[
\begin{align*}
\text{O}^- & \quad \text{P} \quad \text{O}^- \\
\text{O}^- & \quad \text{P} \quad \text{O}^- \\
\text{C}=\text{O} \quad \text{CH}_3 & \\
\text{O}^- & \quad \text{P} \quad \text{O}^- \\
\end{align*}
\]