An EXAFS study of zinc coordination in microbial cells

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Five microbes were isolated from metal amended enrichment cultures derived from the sediments of a lake contaminated by a zinc smelter. Each of these organisms was grown in pure culture in the presence of zinc. Quick Extended X-ray Absorption Fine Structure (QEXAFS) spectroscopy was used to investigate the average coordination environment of the zinc associated with the microbial biomass. Fitting of the first coordination shell of zinc shows that significant differences exist for each microbial species examined. The coordination environment of zinc varies between sulfurs to six-fold nitrogen/oxygen, with two microbial strains showing mixed coordination shells. Further study is required in order to characterize these sites and their locations within the cell.

Keywords: EXAFS; **Metal coordination by microbes.**

1. Introduction

As a result of industrialization, the modern world has left a legacy of heavy metal contamination throughout the environment. These industrial activities, such as smelting, foundries, and finishing, have introduced large concentrations of metals into many different environments, including aquatic systems (Nriagu, 1984). The design of appropriate clean-up actions rests on the detailed characterization of the fate of metals in the aqueous environment, so that educated choices can be made, *e.g.*, dredging and disposal or "intrinsic remediation". For this purpose, one needs to assess the mobility, reactivity, and toxicity of the metals in the impacted environment. Despite the importance of sediments as a sink for metals, what processes control the fate of such contaminants remains unclear. Consequently, the determination of the chemical speciation of metals becomes a key issue.

Of particular importance, it has been shown that viable, active, microbial communities thrive in many types of heavy metal contaminated environments (Aceves et al., 1999; Kelly and Tate, 1998; Kolensnikov et al., 1999; Roane and Kellogg, 1996). However, there is little information available for how these organisms cope with metal stress, and in turn, alter their physiology or environment to reduce toxicity. There is little understanding of the influences of metal stress on bacterial populations in freshwater sediments that are the driving force of early diagenesis. In particular, although response to metal stress has been studied thoroughly in aerobic systems (*i.e.*, in bacteria and algae), there is relatively little information for anaerobic bacteria. To address these concerns, this work examines the interactions of metals with anaerobes isolated from heavily contaminated environments.

The speciation of zinc in the enviromental system studied - Lake DePue (IL), a lake contaminated by Zn smelting operations - has been well-characterized in previous works using analytical electron microscopy (AEM) and X-ray absorption spectroscopy (Webb *et. al.*, 2000; Webb & Gaillard, 2000). These works show that zinc in lake sediments exists primarily in the form of carbonates and phosphates in the contaminated area of the lake, and predominately as sulfides in the less contaminated regions. In addition, zinc is intimately associated with biological structures in both the water column and sediments (Webb *et. al.*, 2000).

Five obligate anaerobes were isolated from enrichment cultures derived from the sediments of Lake DePue which have been contaminated through industrial zinc smelting. Each of the organisms was isolated and grown in the presence of zinc. Quick Extended X-ray Absorption Fine Structure (QEXAFS) spectroscopy was used to investigate the average coordination of the zinc associated with microbial biomass. Previous works have shown that several difficulties exist in determining the binding sites of zinc within protein structures (Clark-Baldwin *et. al.*, 1998; Hubbard *et. al.*, 1991). These problems arise from the difficulty of differentiating nitrogen and oxygen scatterers such that the determination of the number of mixed sulfur and nitrogen ligands in peptide systems is challenging.

The EXAFS spectrum of each sample was analyzed following a protocol developed by Clark-Baldwin *et. al.* (1998) to reliably determine peptide coordination in biological systems. Fitting of the first coordination shell of zinc shows that significant differences exist for each microbial species examined. The coordination environment of zinc varies between sulfurs to six-fold nitrogen/oxygen, with two microbial strains showing mixed coordination shells.

2. Methods

Bacteria were isolated from zinc contaminated lake sediments by using the roll tube method for cultivation of strict anaerobes as described by Hungate (1969). All the microbes that were isolated are obligate anaerobes, and exhibit tolerance to high zinc concentrations, *i.e.*, show growth up to 10 mM total zinc. Substrates include unsaturated organic acids, amino acids, hydrogen, and simple sugars. Preliminary sequence analyses of small ribosomal RNAs subunit (16S-rRNA) established a phylogenetic relation to members of the low %G+C division within the domain *Bacteria*. The bacteria were allowed to grow in media containing 500 μM zinc at circumneutral pH, and were then separated from the media by filtration through a 0.2 μm Nucleopore filter. The filter was encapsulated in between two stripes of Kapton tape, and placed in liquid N_2 until analysis to preserve the chemical properties of the sample.

X-ray absorption measurements were performed in Quick-XAFS mode at the Advanced Photon Source, Argonne National Laboratory on the DND-CAT bending magnet beamline. A detailed description of the QXAFS experimental setup can be found in Quintana (2000). A Si(111) double crystal monochromator was used to vary the X-ray energy from 200 eV below to 800 eV above the absorption K edge of Zn (9659 eV). The monochromator was detuned to approximately 70% of the maximum intensity to avoid harmonic interference. The incident intensity, I_0, and transmitted intensity, I_T, were measured by appropriately positioned ionization chambers. The fluorescence signal, I_F, was measured with a Lytle detector with 100% Ar gas sealed in the ion chamber. The output of the Lytle detector and ionization chambers were used as input to Stanford Research System SRS 570 Current Amplifiers. The output of these amplifiers was continuously sampled at a 12.5 kHz rate with a sixteen-bit analog to digital converter. Data were collected while the monochromator was continuously slewed between the beginning and ending energies. Ten successive scans were recorded for each sample at a rate of 120 seconds per scan

and averaged together to increase the signal to noise ratio. Comparison of the first and last scans of each sample showed no evidence of radiation damage. In addition, the utilization of the QXAFS method allowed the quantification of experimental errors. The raw k^3 weighted EXAFS for the isolates and the associated error bars are shown in Figure 1.

The first coordination shell was isolated from the EXAFS spectra by a Fourier transform over the region $k = 2.5 - 12$ Å^{-1} followed by a back-transformation over $R = 1.2 - 2.75$ Å. Fitting was performed using a procedure similar to that used by Clark-Baldwin, *et. al.* (1998) in order to differentiate and quantify the number of low Z (N or O) and high Z (S) ligands. Ab initio parameters, calculated from FEFF8 (Ankudinov & Rehr, 1997; Rehr *et. al.*, 1992), were used to fit the data.

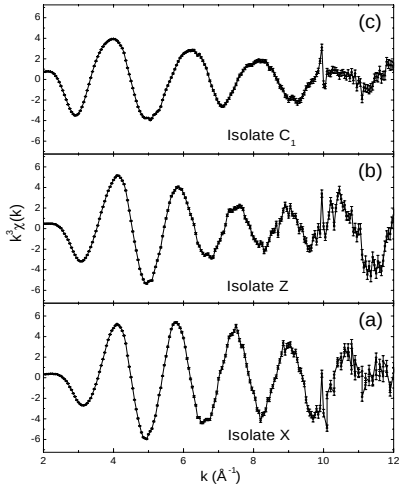

Figure 1
Zn K-edge EXAFS spectrum of the isolates including error bars obtained from experimental error.

3. Results

Qualitatively, the differences in the zinc coordination of the microbes can be seen in the Fourier transforms of the biomass removed from the cultures grown in zinc containing media (Figure 2). From top to bottom, a change in coordination from single scatterers at a pseudo-radial distance of 1.95 Å to single scatterers at 1.5 Å, with a mixed shell in between can be seen. Since sulfur has a larger radius than either oxygen or nitrogen, the qualitative nature of these spectra suggest that the coordination varies from dominantly sulfur to dominantly oxygen/nitrogen neighbors, with some species of microbes having a mixed coordination shell.

To quantify the coordination shells of these samples, a fitting procedure was used that successively varied the number of sulfur and oxygen/nitrogen ligands present in a two shell fit (Clark-Baldwin *et. al.*, 1998). Thus, each fit to the EXAFS equation uses the same number and type of variable parameters. The coordination number of zinc is fixed at four, and the number of oxygen/nitrogen scatterers is varied in increments of 0.2. The S_0^2 and e_0 parameters are fitted from standard compounds (ZnS, $ZnCO_3$, and $Zn_3(PO_4)_2$) and held constant throughout the fitting procedure. Variables which are adjusted at each iteration are the bond distances and the debye-waller parameter. Fitting was performed on the k^3 weighted EXAFS data over $k = 2.5 - 12$ Å^{-1}. The error of each fit was calculated by:

$$\varepsilon = \sqrt{\sum_{i=1}^{N} \frac{k^6(\chi_{obs}(k_i) - \chi_{calc}(k_i))^2}{N}}$$

where N is the number of data points in the spectrum. The number and types of scatterers was determined by the maximal fractional improvement (P_i) in the ith fit given by:

$$P_i = \frac{\varepsilon_{2S+2S} - \varepsilon_i}{\varepsilon_{2S+2S}}$$

where ε_{2S+2S} and ε_i are the error of a fit with two di-sulfur shells and the error of the ith fit respectively. The ratio of the sulfur to oxygen/nitrogen ligands can then be determined by the maximum point on the P_i curve for each sample. Figure 3 shows the results of the fitting for the microbes studied and Table 1 gives the calculated coordination. This shows that the coordination environment in the microbes studied varies significantly, from nearly all sulfur containing to all oxygen/nitrogen with varying proportions in between. Since microbes Mt and C1 showed exclusively low Z scatterers, the coordination number was allowed to increase for these samples. The best fits were achieved for four to five neighbors in the first shell.

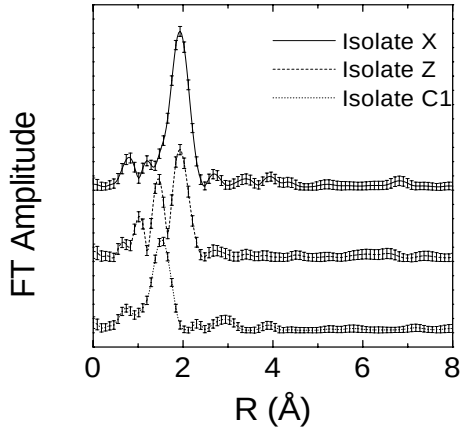

Figure 2
Fourier transform (not corrected for phase shift) of EXAFS data for three of the microbes studied.

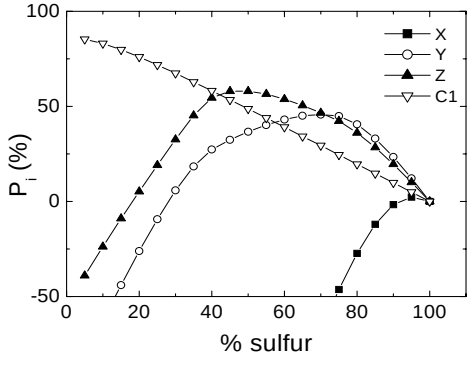

Figure 3
Percent improvement of fit, P_i, as a function of sulfur content in the fit.

Table 1
Fitting results of first shell Zn coordination numbers in microbial biomass

	Sulfur	Sulfur Distance	O/N ratio	O/N Distance
X	3.8	2.343 + 0.023 Å	0.2	1.971 + 0.021 Å
Y	3.2	2.342 + 0.020 Å	0.8	1.968 + 0.018 Å
Z	2.2	2.341 + 0.020 Å	1.8	1.964 + 0.010 Å
Mt	0		4.2	1.965 + 0.018 Å
C1	0		4.7	1.964 + 0.014 Å

The XANES spectra also provide some further details on the type of zinc coordination present in these microbes. The lack of an intense first peak and early XANES resonances of the microbial four-fold sulfur coordination compared to the inorganic ZnS (Figure 4a) shows that the zinc-sulfur in the isolates is not an inorganic ZnS precipitate. The XANES features suggest that the sulfur complex with zinc is an asymetric cellular product, most likely coordinated with four thiol groups. Further spectroscopic studies need to be performed in order to characterize the second coordination shell of the complex.

Additionally, the isolates dominated by oxygen/nitrogen coordination show XANES features (Figure 4b) are very nearly identical to zinc-phosphate. The XANES features of zinc-carbonate are also given to show that similar oxygen coordinated species have significantly different XANES resonances. This suggests that the zinc ligation in these microbes may be dominated by phosphoryl groups. Although there is not a strong signature for a phosphorus second shell signature in the C1 EXAFS, there is a small peak in the RDF which is significant over the errors (Figure 2). The examination of the imaginary part of the Fourier transform shows that this second shell present in Isolate C1 coincides with the phosphorus shell in zinc-phosphate, and that it is out of phase with the Zn-C shell of zinc-carbonate. This further supports phosphoryl type binding in this isolate.

The phosphoryl binding sites in isolates C1 and Mt are most likely ligating Zn outside the cell , *i.e.*, at the level of the cell membrane. Zn has been shown previously to have a high affinity for binding to phosphoryl groups (Sarret, *et. al.*, 1998). Zinc adsorption to cell walls should occur for all the isolates. Since we performed whole cell XAFS experiments, the average local environment of Zn reflects both the internal and the external fraction of the metal. This suggests that the isolates that are characterized dominantly by a Zn-S coordination environment sequester zinc inside the cell since the signal of the phosphoryl binding is swamped out. The presence of such a small amount of outer-cell binding is given by the small fraction of oxygen coordination in isolate X. Further study is required in order to characterize the nature of the sulfur containing ligands, the phosphoryl binding sites and the locations of each of these sites within the cell.

This work was funded by the NSF, Grant MCB: #9807697 and the DOE-NABIR project. This work was performed at the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) Synchrotron Research Center located at Sector 5 of the Advanced Photon Source. DND-CAT is supported by the E.I. DuPont de Nemours & Co., The Dow Chemical Company, the U.S. National Science Foundation through Grant DMR-9304725 and the State of Illinois through the Department of Commerce and the Board of Higher Education Grant IBHE HECA NWU 96. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Energy Research under Contract No. W-31-102-Eng-38.

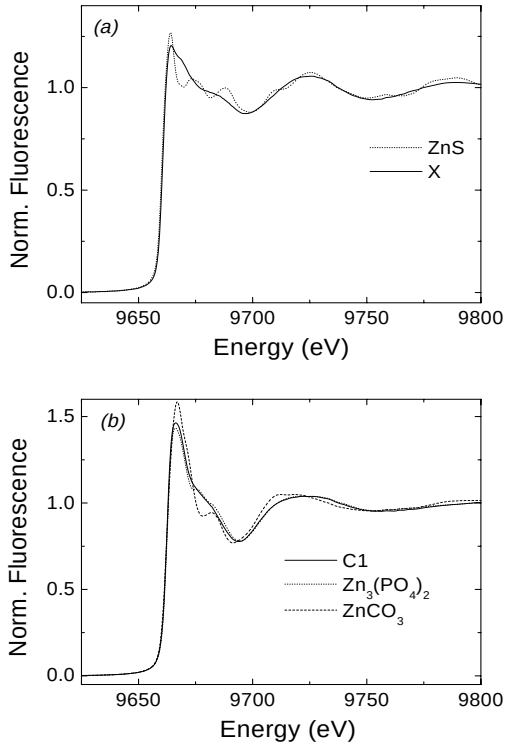

Figure 4
XANES spectra of inorganic standards compared to microbial samples. (*a*) ZnS and the 4-sulfur coordinated microbial sample. (*b*) Zinc phosphate and the 6-coordinate oxygen/nitrogen microbial sample.

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 Received 31 July 2000 • Accepted 21 December 2000