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| ESRF | **Experiment title:**<br>Zn(II) coordination in common soil and surface water bacteria | **Experiment number**:<br>ES-702 |
| **Beamline**:<br>BM16 | **Date of experiment**:<br>from: April 18, 2018 to: April 24, 2018 | **Date of report**:<br>May 29, 2018 |
| **Shifts:**<br>18 | **Local contact(s)**:<br>Olivier Proux | *Received at ESRF:* |

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**Report:**

Zinc (Zn) is an essential metal for all organisms. The chemical speciation of Zn determines its bioavailability and mobility in the environment. Bacteria are ubiquitous in the environment and contain different functional groups that can bind Zn(II), ultimately affecting environmental Zn(II) speciation. Previous studies have shown with EXAFS various Zn(II) coordination environments that can exist in bacteria (e.g., coordinating atoms and coordination number) but the nature of the coordinating ligands (e.g., Zn-phosphate, Zn-carboxyl, Zn-amine etc.) are difficult to identify with EXAFS, which typically only captures first coordination shells in biological samples (i.e., amorphous material). To gain a better understanding of the ligands in bacteria that coordinate Zn(II) at the cell surface and in the cytoplasm (proposal reference number 87781), we employed high energy resolution XANES (HR-XANES) on 2 species of bacteria: *Bacillus subtilis*, a common gram-positive soil bacterium, and *Pseudomonas putida*, a common gram-negative soil bacterium. In addition, we measured a variety of known Zn(II) standards, including biologically-relevant peptides, to compare results obtained from separate analyses of conventional XANES, HR-XANES, and EXAFS.

***Experiment:*** *B. subtilis* and *P. putida* were grown in defined media and harvested at both exponential and stationary growth phase. The bacterial cells were washed in a defined Zn(II) exposure medium and resuspended to an $OD_{600}$ of ~0.2. Bacterial cells were exposed to 0, 10, and 100 µM of added Zn(II) as $Zn(NO_3)_2$ for 2 hours. After Zn exposure, the cells were washed with 0.1 M $NaClO_4$ and the cell pellet was collected, sandwiched between pieces of Kapton tape, plunged in $LN_2$, and kept frozen until analysis. The samples were shipped to the ESRF on dry ice. Zn powdered references were diluted in boron nitride and pressed into 5 mm diameter pellets. Zn liquid references were prepared in Milli-Q water (≤ 50 mM total Zn), pipetted into copper sample holders, and immediately flash frozen into $LN_2$. All samples were measured at ~10 K in HERFD mode with 5 spherically bent Si crystal analyzers (bending radius = 1 m, crystal diameter = 0.1 m). The Zn $K_{\alpha 1}$ fluoresence line was selected using the 642

reflection, and the diffracted fluorescence was measured with a silicon drift detector (SDD, Vortex EX-90). Transmission data was also collected in order to compare the conventional and HR-XANES. Data normalization and processing were performed with Athena.[1]

***Results:*** In total, we collected HR-XANES and EXAFS spectra of 15 powdered and liquid Zn(II) references and 6 bacterial samples that contained Zn(II). In addition, we have conventional XANES for references/samples with higher Zn(II) concentrations that were simultaneuosly scanned in transmission mode. We observe an improvement in energy resolution with HR-XANES compared to conventional XANES at the Zn K-edge, although the improvement is not as drastic as what has been observed for heavier metals at $L_{III}$ edges (e.g., Pb and Hg).

Surprisingly, the *B. subtilis* and *P. putida* samples that were exposed to Zn(II) in exponential phase coordinate Zn(II) identically and Zn(II) coordination does not change with added Zn(II) (see Figure 1 for *B. subtilis*). In stationary phase samples of *P. putida*, the Zn(II) coordination environment also did not change with increasing added Zn(II) and was also identical to the exponential phase samples. These spectra were best modeled by linear combination fits (LCFs) to the XANES region with $Zn_3(PO_4)_{2(s)}$, $Zn(histidine)_{4(aq)}$, and $Hg(cysteine)_{4(aq)}$ references at 56.0%, 35.4%, and 8.6% respectively. In contrast, *B. subtilis* in stationary phase did coordinate Zn(II) differently with increasing added Zn concentration. The *B. subtilis* sample in stationary phase with no added Zn(II) was best fit by LCF with $Hg(cysteine)_{4(aq)}$, $Zn(histidine)_{4(aq)}$, $Zn_3(PO_4)_{2(s)}$, and $Zn(acetate)_{4(s)}$ at 34.0%, 31.1%, 21.4%, and 13.5%, respectively. When 10 µM Zn(II) was added to stationary phase *B. subtilis*, the spectrum was best fit with $Zn_3(PO_4)_{2(s)}$, $Zn(histidine)_{4(aq)}$, $Hg(cysteine)_{4(aq)}$, $Zn(acetate)_{4(s)}$ at 37.9%, 38.6%, 18.9%, and 4.6%. *B. subtilis* is known to form spores when it reaches nutrient limited conditions, entering a latent state to increase chances of survival. The changes observed in Zn(II) coordination in *B. subtilis* reflect a shifting metabolism (e.g., possibly for Zn(II) storage or structural purposes for spore formation).

An analysis of the EXAFS by LCF confirms the results that we obtained from LCFs performed on the HR-XANES. It is important to note that many solutions were possible with the EXAFS LCFs, while HR-XANES provided a unique solution from our reference library. Thus, HR-XANES is preferred for determining the nature of Zn(II) coordinating ligands in biological samples.

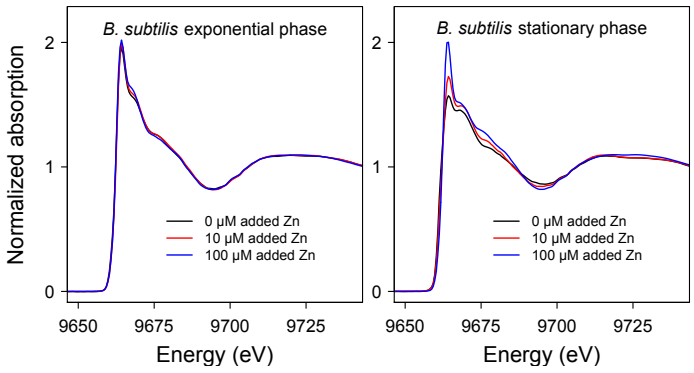


Figure 1: HR-XANES of Zn(II) in cell pellets of *B. subtilis* measured at the Zn K-edge. The cells were harvested in exponential and stationary phase and exposed to 0, 10, and 100 µM added Zn. The Zn in the 0 µM added Zn samples was absorbed by the cells from the growth medium.

## *References:*

1. Ravel, B.; Newville, M., Athena, artemis, hephaestus: Data analysis for x-ray absorption spectroscopy using ifeffit. *J Synchrotron Radiat* **2005,** *12*, 537-541.