

High-Resolution X-Ray Imaging for Microbiology at the Advanced Photon Source

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Abstract. Exciting new applications of high-resolution x-ray imaging have emerged recently due to major advances in high-brilliance synchrotron sources and high-performance zone plate optics. Imaging with submicron resolution is now routine with hard x-rays: we have demonstrated 150 nm in the 6-10 keV range with x-ray microscopes at the Advanced Photon Source (APS), a third-generation synchrotron radiation facility. This has fueled interest in using x-ray imaging in applications ranging from the biomedical, environmental, and materials science fields to the microelectronics industry.

One important application we have pursued at the APS is a study of the microbiology of bacteria and their associated extracellular material (biofilms) using fluorescence microanalysis. No microscopy techniques were previously available with sufficient resolution to study live bacteria ($\approx 1 \mu\text{m} \times 4 \mu\text{m}$ in size) and biofilms in their natural hydrated state with better than part-per-million elemental sensitivity and the capability of determining chemical speciation. *In vivo* x-ray imaging minimizes artifacts due to sample fixation, drying, and staining. This provides key insights into the transport of metal contaminants by bacteria in the environment and potential new designs for remediation and sequestration strategies.

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INTRODUCTION

X-ray microscopy and imaging in the hard x-ray regime has emerged as one of the most important applications of high-brilliance third-generation synchrotron sources such as the Advanced Photon Source (APS). With the advent of high-resolution microfocusing optics, such as Kirkpatrick-Baez mirrors (1) and Fresnel zone plates (2), it is now possible to focus 8-keV x-rays to a spot size of only 150 nanometers with a gain of > 30,000 in the flux density (3). This means 10^9 - 10^{10} photon/sec can be delivered into a tight submicron spot. With the improved performance in both x-ray sources and optics, it is now possible to build practical x-ray microprobes, which open up many new applications never previously considered. One such applications is the environmental study of the interaction between bacteria and heavy-metal contaminants described here.

Understanding the fate of heavy-metal contaminants in the environment (4) is of fundamental importance in the development and evaluation of effective remediation and sequestration strategies. Among the factors influencing the transport of these contaminants are their chemical speciation and the chemical and physical attributes of the surrounding medium. Bacteria and the extracellular material associated with them are thought to play a key role in determining a contaminant's speciation and thus its mobility in the environment. In addition, the microenvironment at and adjacent to actively metabolizing cell surfaces can be significantly different from the bulk environment. Thus, the spatial distribution and chemical speciation of contaminants and elements that are key to biological processes must be characterized at micron and submicron resolution in order to understand the microscopic physical, geological, chemical, and biological interfaces that determine a contaminant's macroscopic fate. Hard x-ray microimaging is a powerful technique for the element-specific investigation of complex environmental samples at the needed micron and submicron resolution. This paper presents results of studies of the spatial distribution of naturally occurring metals and a heavy-metal contaminant (Cr) in and near hydrated bacteria (*Pseudomonas fluorescens*) in the early stages of biofilm development. The experiments were performed at the Advanced Photon Source 2-ID-D x-ray microscopy beamline.

X-RAY MICROPROBE STUDY

The objectives of our studies are 1) to determine the spatial distribution and chemical speciation of metals near bacterial-geosurface interfaces and 2) to use this information to identify the interactions occurring near these interfaces among the metals, mineral surfaces, and bacterially produced extracellular materials under a

variety of conditions. The microprobe used an APS undulator source, which supplies a very high-brilliance x-ray beam above 3 keV. The broad spectrum x-rays were then monochromatized by a pair of Si(111) crystals. A phase zone plate, located at 71 m from the source, was used to focus the x-rays to a cross section of 0.15 μm . The zone plate used in these microscopy experiments had an effective focal length of 12.5 cm at 10.0 keV (3).

We used hard x-ray phase zone plates to investigate the spatial distribution of 3d elements in single hydrated *Pseudomonas fluorescens* bacteria adhered to a Kapton film. Another layer of Kapton film was used to enclose the bacteria and maintain them in a hydrated condition. The samples were mounted on a computer-controlled XY piezo-stage at 10 degrees to the incident beam, thus negligibly affecting the x-ray footprint on the sample in the horizontal dimension. The intensity of the fluorescence radiation from the sample was monitored by a single-element solid-state detector that enables efficient detection of fluorescent x-rays with energies greater than 1.5 keV. Spatial maps of several elements were obtained by scanning the sample in 0.15- μm steps through the focused monochromatic x-ray beam and integrating the selected $K\alpha$ fluorescence for 5 sec/pt. The total data collection time was approximately 6 hours.

Figure 1 shows results of the x-ray microprobe measurements, qualitatively indicating the spatial distributions of Cr, K, and Ca in and near a hydrated *Pseudomonas fluorescens* bacterium adhered to a Kapton film at ambient temperature that was exposed to 1000 ppm Cr in solution for 6 hours. Observation of these images indicates that monitoring the spatial distribution of the K and Ca $K\alpha$ fluorescent radiation coming from the sample enables identification of the rod-shaped *Pseudomonas fluorescens* as well as the extracellular exudes associated with it. Additionally, comparison of the distribution of Cr, relative to that of K or Ca, indicate that the majority of the Cr in this sample is associated extracellularly. These results indicate that the majority of the Cr(VI) that was introduced to the sample was probably not actively metabolized. Finally, although these results demonstrate the utility of imaging hydrated bacteria at ambient temperature, in the future, a cryostat may be required to quick-freeze the samples in order to reduce the effects of radiation damage when performing spectromicroscopy studies.

This study illustrates several unique capabilities of a x-ray microprobe compared to conventional charged particle microprobes. For instance, fluorescence cross sections for excitation by x-rays are typically 10 to 10^3 times larger than those by charged particles. Thus, the elemental sensitivity of the x-ray microprobe can reach the tens of part-per-billion (ppb) level with substantially reduced energy deposition on the sample. In particular, the fluorescence yield is very high for third-row and heavier elements, many of which are important nutrients, micronutrients, and environmental

contaminants. For this reason, we are pursuing x-ray microprobe studies of the subcellular distribution of drugs and trace elements in different biological cells. Another advantage of x-rays is the minimal sample preparation needed, with no special staining nor fixing procedures required. In fact, biological samples can be examined in their natural hydrated state because there is no vacuum requirement and because of the high penetrating power of x-rays in water. For instance, hydrated plant roots and fungi have also been studied with the x-ray microprobe (5), in a way similar to the bacteria study presented here.

SUMMARY

We have demonstrated the utility of x-ray microbeams, particularly those produced by hard x-ray phase zone plates, for investigating biological and environmental systems. Specifically, we have illustrated the use of submicron hard x-ray beams ($0.15\ \mu\text{m}$) for determining the spatial distribution of metals in a hydrated bacterium that was exposed to 1000 ppm Cr for six hours. The further development of these techniques for such applications promises to provide unique opportunities in the fields of microbiology and environmental research.

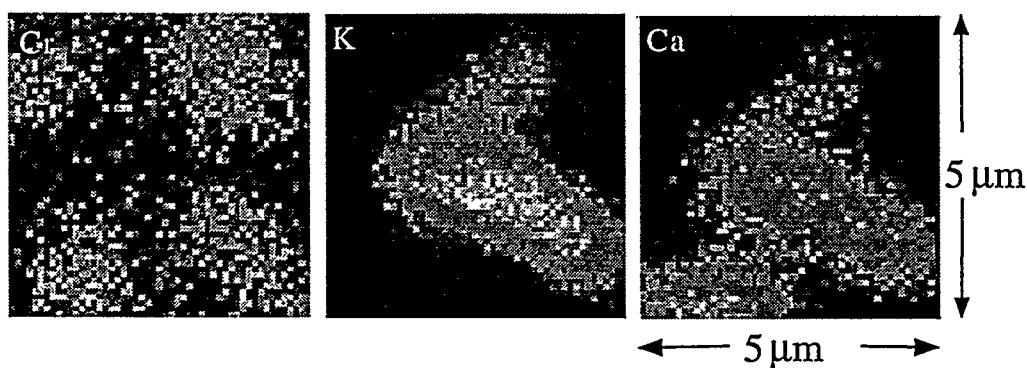


FIGURE 1. Grey-scale elemental maps of hydrated *Pseudomonas fluorescens* bacterium treated with Cr(VI) solution. See text for further details.

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