Using X-ray microprobes for environmental research

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ABSTRACT

Understanding the fate of environmental contaminants is of fundamental importance in the development and evaluation of effective remediation strategies. Among the factors influencing the transport of these contaminants are the chemical speciation of the sample and the chemical and physical attributes of the surrounding medium. Characterization of the spatial distribution and chemical speciation at micron and submicron resolution is essential for studying the microscopic physical, geological, chemical, and biological interfaces that play a crucial role in determining contaminant fate and mobility. Hard X-ray spectroscopy and imaging are powerful techniques for the element-specific investigation of complex environmental samples at the needed micron and submicron resolution. An important advantage of these techniques results from the large penetration depth of hard X-rays in water. This minimizes the requirements for sample preparation and allows the detailed study of hydrated samples. This paper discusses some current problems in environmental science that can be addressed by using synchrotron-based X-ray imaging and spectroscopy. These concepts are illustrated by the results of recent X-ray microscopy studies at the Advanced Photon Source.

Keywords: zone plates, environmental science, soil science, botany, X-ray microbeams, X-ray imaging, synchrotron, biogeochemistry

1. ENVIRONMENTAL RESEARCH

Chemical contamination of soil and groundwater is a universal problem of immense complexity and great global concern. Sources of contamination include past and present agricultural and industrial activities, operations at national defense sites, and mining and manufacturing processes. Assessment of thousands of hazardous waste sites in the United States alone (including over 1,200 on the National Priority List) has identified the presence of an array of toxic substances. These include heavy metals (such as Pb, Cr, As, Zn, Cu, Cd, Ba, Ni, and Hg), radionuclides (including U, Pu, Sr, Cs, Co, and Tc), and potentially hazardous anions such as selenate.[1] Organic compounds such as halogenated hydrocarbons, pesticides, and herbicides are also toxic contaminants. The restoration of soil and groundwater that has been contaminated by combinations of these stable and radioactive substances (e.g., mixed wastes) presents significant scientific and engineering challenges because the interactions that occur among the contaminants are unknown.

Development of the cost-effective remediation technologies needed to restore contaminated soil and groundwater requires a multidisciplinary approach. Scientists and engineers from the fields of chemistry, hydrology, biology, soil science, physics, geology, and materials science are needed to tackle this complex problem. The first step in the remediation process is to determine the concentration and nature of the hazardous wastes at a particular site. Next, methods to separate and recover the contaminants must be developed and implemented. The final step involves packaging the separation products in appropriate waste forms and safely isolating them from the rest of the biosphere. In some situations, applying current remediation techniques may change the chemical mobility of the contaminant. This could result in a greater health risk than before the remediation was attempted. In these situations, sequestration technologies designed to immobilize the contaminant in situ may be a more viable and practical option.

An understanding of contaminant mobility is essential in the development, application, evaluation, and choice of remediation and sequestration technologies. This understanding requires molecular-level information about the speciation of the contaminant. Speciation refers to the composition, oxidation state, molecular-level structure, and phase in which a contaminant occurs.

2. THE NEED FOR AND USE OF MICRON AND SUBMICRON HIGH-ENERGY X-RAY MICROPROBES IN ENVIRONMENTAL MOLECULAR RESEARCH

A current focal point of molecular environmental science involves the pathways, products, and kinetics of chemical reactions of contaminant species with inorganic and organic compounds, plants, and organisms in the environment.[1] These reactions often occur at aqueous solution-solid interfaces and can have many different results. The contaminant can be precipitated from the solution to the solid interface, transformed into a different species, incorporated into a solid phase, or released from the solid surface into the solution. Such interfacial reactions play a very important role in the transport and dispersal of toxic species in soils and natural waters. Therefore, discovering what is occurring at these interfacial surfaces is key in understanding the bioavailability of many contaminants. Despite this importance, these surfaces and their associated chemical reactions are not well understood. Consequently, little is known about the mechanisms by which plants, fungi, and microorganisms determine the speciation, forms, reaction rates, and distribution of contaminants in soils and groundwater.

Interfaces of environmental importance may involve microbes, plants and their roots, fungi, groundwater, and soil constituents such as minerals and organic debris. Unfortunately, the heterogeneity of these interfaces makes their study very difficult. One approach to studying the problem involves using a simplified system with a single interface. Although this can be a good starting point, it can produce unrealistic results. To avoid this limitation, the simplified systems need to include the essential interactions found in the environment. In particular, because environmental samples are almost always hydrated, high-energy X-rays having the ability to penetrate water are very useful. In addition, it can be valuable to probe both sides of the interface to elucidate transformations that result in the movement of the contaminant across the interface. Thus, it is imperative that the smallest possible probe is used so that the homogeneous regions on either side of the interface can be analyzed selectively. These requirements make the use of micron and submicron X-ray beams advantageous.

X-ray fluorescence (XRF) microscopy offers significant advantages over other techniques for determining the spatial distribution of trace elements in environmental samples. For example, fluorescence signal-to-background ratios are $10\text{-}10^5$ times larger for excitation by X-rays than by charged particles. Thus, although charged-particle microprobes may provide significantly better spatial resolution than X-rays, the elemental sensitivity of the former techniques (10-100 ppm) is much worse than that achieved by X-ray microprobes.[2] Another advantage is that, for a given sensitivity, the radiation dose to a sample from an X-ray microprobe is typically 10^{-3} to 10^{-5} times less than the dose from a charged-particle microbeam.[3] In addition, X-ray absorption microspectroscopy makes it far easier to obtain chemical state information about a particular substance. Finally, although soft X-ray microprobes have considerable utility for studying biological samples,[4] hard X-ray microprobes provide improved fluorescence yields, better penetration of hydrated samples, and access to the K edges of third-row and heavier elements. Many of these elements are important nutrients, micronutrients, and environmental contaminants.

3. THE USE OF X-RAY MICROPROBES FOR INVESTIGATING ENVIRONMENTAL SYSTEMS

Third-generation X-ray sources such as the Advanced Photon Source (APS), where our experiments were performed, provide an increase in brilliance of approximately three orders of magnitude compared to second-generation synchrotron X-ray sources. In addition, advances in microfabrication technologies have resulted in X-ray phase zone plates[5] with spatial resolution better than 0.5 μm and focusing efficiency better than 33%. The combination of the increased brilliance of X-ray beams provided by the APS and improved zone plate fabrication technology provides unique capabilities in X-ray microscopy and spectromicroscopy.

3.1 The role of mycorrhizal fungi in contaminant transport

Photosynthetic organisms are one of the principal entry points for heavy metals and radionuclides into the food chain.[6] The way plants respond to high concentrations of heavy metals and radionuclides has a significant effect on the transport dynamics of these contaminants through the topsoil and the root zone and plays a large role in determining the ultimate ecological effects of the contamination. For example, if a contaminant is taken up rapidly by plants, a substantial fraction of the contaminant is retained in the soil horizon, reducing the potential for contamination of groundwater. Thus, an understanding of the mechanisms for uptake and regulation of metal concentrations in plants is essential for an accurate assessment of the risk associated with a given contaminated site and for the development of the most effective remediation and restoration schemes.

Many metals are micronutrients essential for plant survival; at high concentrations, however, most of these same metals become toxic to the plant. Thus, plants with internal mechanisms for regulating metal concentrations have a significant advantage over unregulated plants. In natural environments there is generally a deficiency, rather than an excess, in the soil concentration of one or more essential micronutrients. One of the most effective strategies plants have developed to address such deficiencies is to form symbiotic relationships with mycorrhizal fungi that increase the ability of the plants to access essential nutrients.[7-9] So effective is this strategy that approximately 90% of the world's vascular plants are estimated to be mycorrhizal, and even the earliest plant fossils show evidence of this symbiosis. Thus, an understanding of the plant-fungus relationship is essential for any discussion of the plant's uptake and transport mechanisms for heavy metals and radionuclides in the environment.

The basis of the plant-fungus relationship is that the fungus increases the plant's access to vital nutrients, while the plant supplies the fungus with a source of hydrocarbons.[7-9] Perhaps the best known example of the benefits of this relationship to the plant is the mycorrhizal enhancement of plant growth in low-phosphorus environments. The fungus is thought to increase the ability of the plant to extract phosphorus from the soil, primarily by increasing the effective volume of soil that can be accessed by the high density of fungal hyphae; however, the hyphae can also contain or release biochemicals that increase the availability of metals in the soil for uptake.[7-9] Similar considerations apply for micronutrients such as Mn, Zn, Cu, and Fe.

Evidence suggests that a fundamental role for one type of mycorrhizal fungi, arbuscular mycorrhizal fungi, is that their hyphae bridge the annular space within soil, producing a physical connection between the root surface and surrounding soil particles.[9] In creating such bridges, the hyphae increase the effective surface area of the root and decrease resistance to water flow to the root surface by allowing closer contact with the soil particles. This physical relationship between the root surface, hyphae, and soil matrix could be especially important to plants growing in soils of high conductive resistance or where drought is commonplace, and would also allow continued uptake of nutrients from the soil solution during a drought cycle.

The enhancement of metal uptake by mycorrhizal fungi might be expected to be highly disadvantageous in environments with potentially toxic concentrations of metals.[10] Surprisingly, however, numerous examples have been reported of exactly the opposite behavior; that is, the mycorrhizal fungi enable the plant to tolerate high metal concentrations, allowing the plant to survive in soils that are toxic to nonmycorrhizal plants.[11-13] Although numerous studies have addressed this mycorrhizal-mediated tolerance or resistance, no detailed understanding of the mechanism involved has been developed. Indeed, early studies indicate that a variety of mechanisms are operative.[6,13] One possible mechanism involves the sequestration of the metal within the cell walls of the hyphae. Another suggested mechanism is the adsorption of the metal to polyphosphate granules within the cytoplasm. Other metals may be associated with sulfur-containing amino acids. All of these mechanisms may be occurring, each associated with a particular metal. Hence, an improved understanding of the plant-fungus relationship, particularly with respect to the uptake and retention mechanisms for metals, is expected to have significant implications in both agriculture and the remediation and restoration of contaminated soils.

Many of the heavy metals and radionuclide contaminants are not essential to the metabolism of plants and fungi; thus, mechanisms for uptake of, tolerance to, or resistance to these metals may not be developed or expressed in uncontaminated environments. In some cases, when such plants and fungi are exposed to new contaminants, the mechanisms associated with chemically similar elements come into play. For example, the uptake mechanisms for Cd, a nonessential heavy metal, might follow those for Zn, an essential micronutrient. In some cases, the response to contaminants might

involve nonspecific mechanisms. Perhaps the most interesting situations involve plants and fungi in either naturally occurring or polluted environments containing potentially toxic levels of one or more heavy metals. In several such environments, mycorrhizal plants have good viability but nonmycorrhizal plants have severely reduced viability.[7,11,12] Furthermore, plants infected by a fungus of the same species, isolated from an uncontaminated environment, are less viable than mycorrhizal plants that have adapted to the contaminated site. An understanding of the biochemical and physiological basis of this tolerance/resistance, its associated mechanisms, and its genetic stability clearly would have significant implications for the remediation and restoration of contaminated sites and for the assessment of risk associated with such sites. A particularly important issue is whether this adaptation results from tolerance, whereby the fungus either sequesters or restricts the uptake of the metal, or from resistance, whereby the plant/fungus transforms the contaminant into a nontoxic species. In general, the relative importance of these mechanisms is expected to depend on the specific system of interest.

We used hard X-ray phase zone plates to investigate the fungus-root symbiotic relationship, specifically studying the spatial distribution of many of the *3d* transition metals in a fungal-infected plant root. The samples were prepared as follows. *Plantago lanceolota* seedlings were transplanted in flint sand and inoculated with approximately 40 spores of *Glomus moseae*. The plants were watered daily, alternating each day between deionized water and 10% Hoagland's solution. After 45 days, the plants were harvested by washing the sand from the roots and the fungal hyphae, then rinsing with deionized water. The zone plate used in these microscopy experiments produced a focused beam of cross section 1 μm × 3 μm and 4×10^{10} photons/s/0.02% bandwidth. The zone plate had an effective focal length of 52.5 cm at 10.5 KeV and an effective spot size of 1 μm (vertical) × 3 μm (horizontal). The X-ray beam passed through a 20-mm order-sorting aperture, and the focus was adjusted to be on the sample. The samples were mounted on a computer-controlled XYZ stage at 20 or 45 degrees to the incident beam, producing a footprint up to 4.2 μm on the sample in the horizontal dimension of the sample. The X-ray fluorescence radiation intensities were monitored by using an energy-dispersive, single-element, solid-state detector.

Figure 1 shows the spatial distributions of Fe and Zn in (a) a wet root-fungus sample and (b) a fungal hyphae sample. The maps of the elements in Figures 1a and 1b were obtained by scanning the sample in 5-μm steps through the focused monochromatic X-ray beam (10.5 KeV) and integrating the selected Kα fluorescence for 3 sec/pt. The total data collection time was approximately 4 hr, and the elemental sensitivity was approximately 500 ppb. Scale bars of (a) 50 μm and (b) 10 μm are included in the figure. Comparison of Figure 1 with cross sectional XRF images indicates that the Fe tends to be most concentrated on the edge of the root, perhaps reflecting the precipitation of Fe in this location. In contrast, Zn typically seems most concentrated in the fungal hyphae and in the center of the root, most likely in the inner cortex, where the proliferation of the fungus is usually greatest.[8] This observation suggests that Zn would be useful as a surrogate measure of mycorrhizal fungi in roots. Additional work is under way to confirm this correlation.

3.2 The role of bacteria in contaminant transport

Although microorganisms are known to participate in a wide range of metal oxidation-reduction reactions, the effect of microbiological activity on geochemical speciation is not well understood. A true understanding of bacterially catalyzed molecular transformation of inorganic contaminants is fundamental to risk assessment and the evaluation of bioremediation processes.[1] The focus of our work in this area is the interactions among trace metal contaminants, mineral surfaces, and bacterially produced extracellular material at the microbe-metal contaminant-geosurface interface.

The objectives of our studies are (1) to determine the spatial distribution and chemical speciation of metals near bacteria-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. To accomplish these objectives, we have begun using X-ray microbeams to investigate the spatial distribution of metals in single hydrated bacteria adhered to Kapton film, with the goal of progressing to spectromicroscopy studies of metals at the bacteria-geosurface interface.

The rate at which metal contaminants move through soil is highly dependent on the chemical interactions between the hydrated contaminant metal and the mineral surface. Sorption of an aqueous metal cation contaminant to a geosurface typically results in the loss of one or more of the metal contaminant's waters of hydration and the development of one or more strong chemical bonds between the contaminant and the geosurface. The entity formed is often referred to as an inner-sphere complex and is generally rather stable. Typically, for metal (hydr)oxide surfaces, the contaminant forms an oxide or

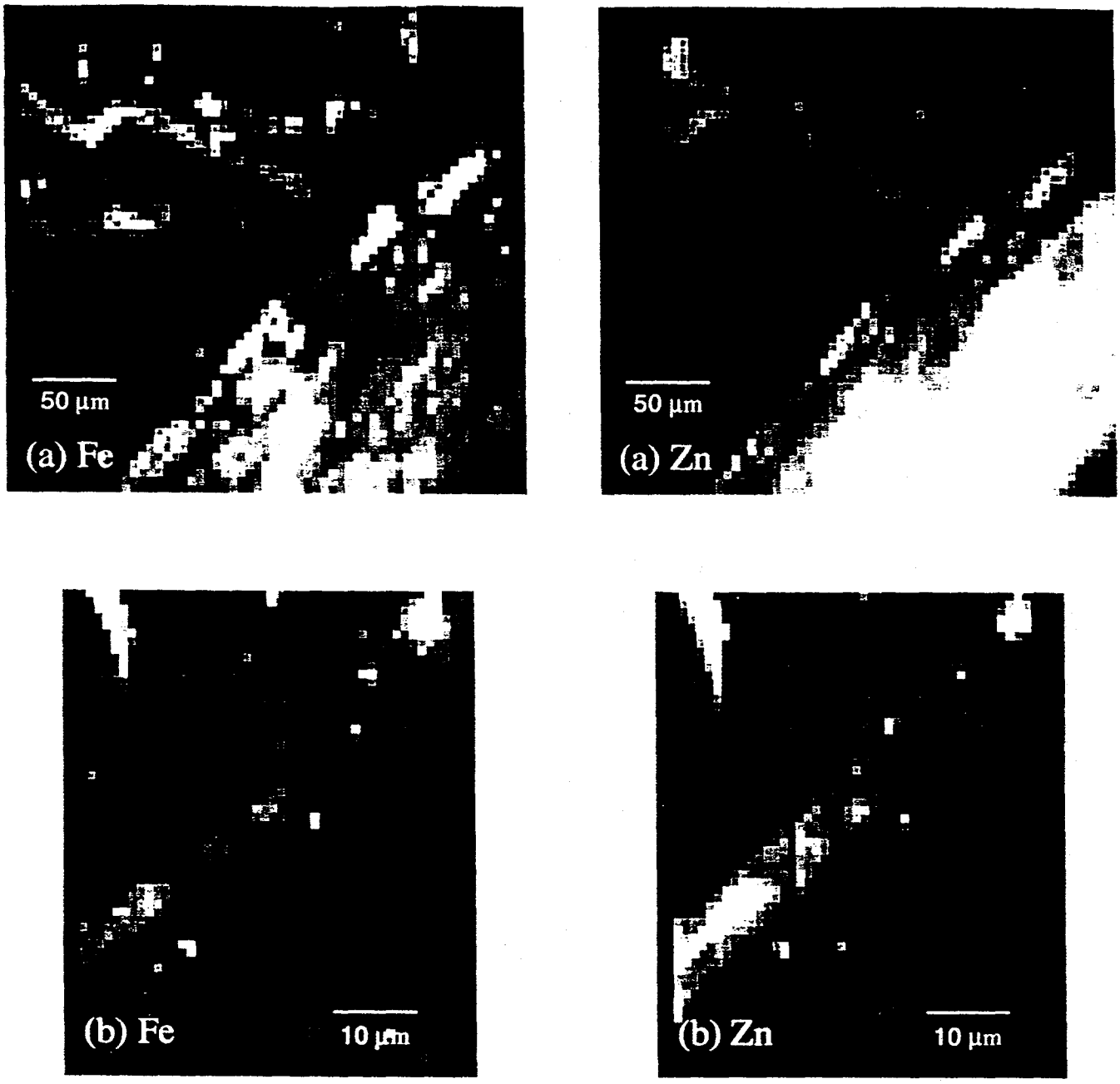

Figure 1. Spatial distributions of Fe and Zn for (a) a hydrated *Plantago* plant root infected by the mycorrhizal fungus *G. mossae* and (b) a single hydrated *G. mossae* hypha. Elemental distributions were determined from the characteristic Kα fluorescence intensities produced by a focused 10.5-KeV X-ray beam.

hydroxide bond to the geosurface, with one to three oxygen atoms forming a bridge between the contaminant metal and the metal (hydr)oxide geosurface. In other instances, however, the contaminant metal does not shed its hydration sphere, and a weaker coulombic attraction exists between the hydrated metal ion and the geosurface. This type of sorption, thought to be rather nonspecific, is often referred to as an outer-sphere complex. Because outer-sphere interactions are much weaker than inner-sphere interactions, the probability of remobilization and transport of the contaminant is much higher. Thus, contaminants found only as outer-sphere complexes tend to be more mobile and can pose greater environmental risk. Much work has been done to investigate the effect of concentration and pH on the local bonding configuration of trace contaminant metals to the metal oxides typically found in Earth's outer crust (oxides of Fe, Mn, and Al, for example).[14-17]

Microorganisms produce many different types of extracellular macromolecular exudates. Because of their net negative charge, these macromolecules can travel relatively unimpeded through porous media, dissolve mineral surfaces, complex cations, and reincorporate the ions into solution. These processes affect the chemistry of elements that are constituents of the solid phase, as well as those that are sorbed to that solid phase. Ultimately, these reactions can affect the mobility and bioavailability of metals. Depending on the macromolecule, metals are thought to bind to hydroxyl, carboxyl, or phosphoryl functional groups. The microbially produced macromolecules with metal-binding properties include the cyclodextrins, exopolysaccharides, and amphipathic molecules (often referred to as biosurfactants). The stability constants for Cr^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, and Hg^{2+} with these biopolymers in aqueous solution are about $10^{0.5}$ to 10^{4}. In addition, because the local pH at the interface between these organic macromolecules and metal (hydr)oxides can be very acidic, bioexudates can play a major role in surface dissolution and precipitation of new mineral phases.

As with the mycorrhizal fungus-plant root system, one difficulty encountered in investigating the contaminant-microbe-geosurface interface is sample heterogeneity. Typically, a soil contaminated with a metal has many different organic and inorganic components. One obvious way to decrease sample heterogeneity is to limit the constituents in the system to be studied. More specifically, a single geosurface with a single contaminant and a single microbe must be studied before more complicated systems. However, even under these controlled circumstances, sample heterogeneity can still be an issue. For instance, in such a system, the metal contaminant may be bound in a variety of ways: (1) in solution, (2) to the geosurface, (3) to extracellular material, (4) within cell membrane regions, (5) within the bacteria, or (6) between the extracellular material and the geosurface, forming a ternary complex. To study the spatial distribution and chemical speciation of a contaminant metal at the microbe-metal-geosurface interface and thus to elucidate the interactions occurring at this interface, the dimension of the X-ray probe must allow the vast majority of the X-rays to be positioned at the contaminant-microbe-mineral interface. The size of most bacteria is approximately 1 μm. Therefore, to investigate the speciation and spatial distribution of elements associated with bacteria, the dimensions of the X-ray probe must be smaller than 1 μm.

We have used hard X-ray phase zone plates to investigate the spatial distribution of 3*d* elements in a single hydrated *Pseudomonas fluorescens* bacterium adhered to a Kapton film. The zone plate used in these microscopy experiments produced a focused beam of cross section 0.15 μm^2 and had an effective focal length of 12.5 cm at 10.0 KeV. The X-ray beam passed through a 10-μm order-sorting aperture, and the focus was adjusted to be on the sample. The samples were mounted on a piezo that in turn was mounted on a computer-controlled XYZ stage at 5 degrees to the incident beam, thus negligibly affecting the X-ray footprint on the sample in the horizontal dimension. Figure 2 shows the spatial distributions of S, Co, and Zn in a hydrated *Pseudomonas fluorescens* bacterium adhered to a Kapton film at ambient temperature. Figure 2d shows an optical micrograph of the area of the sample probed by the X-ray beam, visually identifying the bacterium and the track marks created by the interaction of the X-ray beam with the adhesive of the Kapton tape adhered to the back side of the sample cell. The map of the elements was obtained by scanning the sample in 0.15-μm steps through the focused monochromatic X-ray beam (10.0 KeV) and integrating the selected Kα fluorescence for 5 sec/pt. The total data collection time was approximately 8 hr. Although these results demonstrate the utility of imaging hydrated bacteria at ambient temperature, a cryostat will be required to freeze the samples in order to reduce the effects of radiation damage for spectromicroscopy studies.

3.3 The role of soil porosity in contaminant transport

The three-dimensional spatial distributions of organic and mineral substrates and heavy metal contaminants in the environment can have a large effect on microbial activity and contaminant mobility. These spatial distributions can control fluid flow rates and can constrain the rates of growth and substrate transformation by limiting nutrient delivery rates and

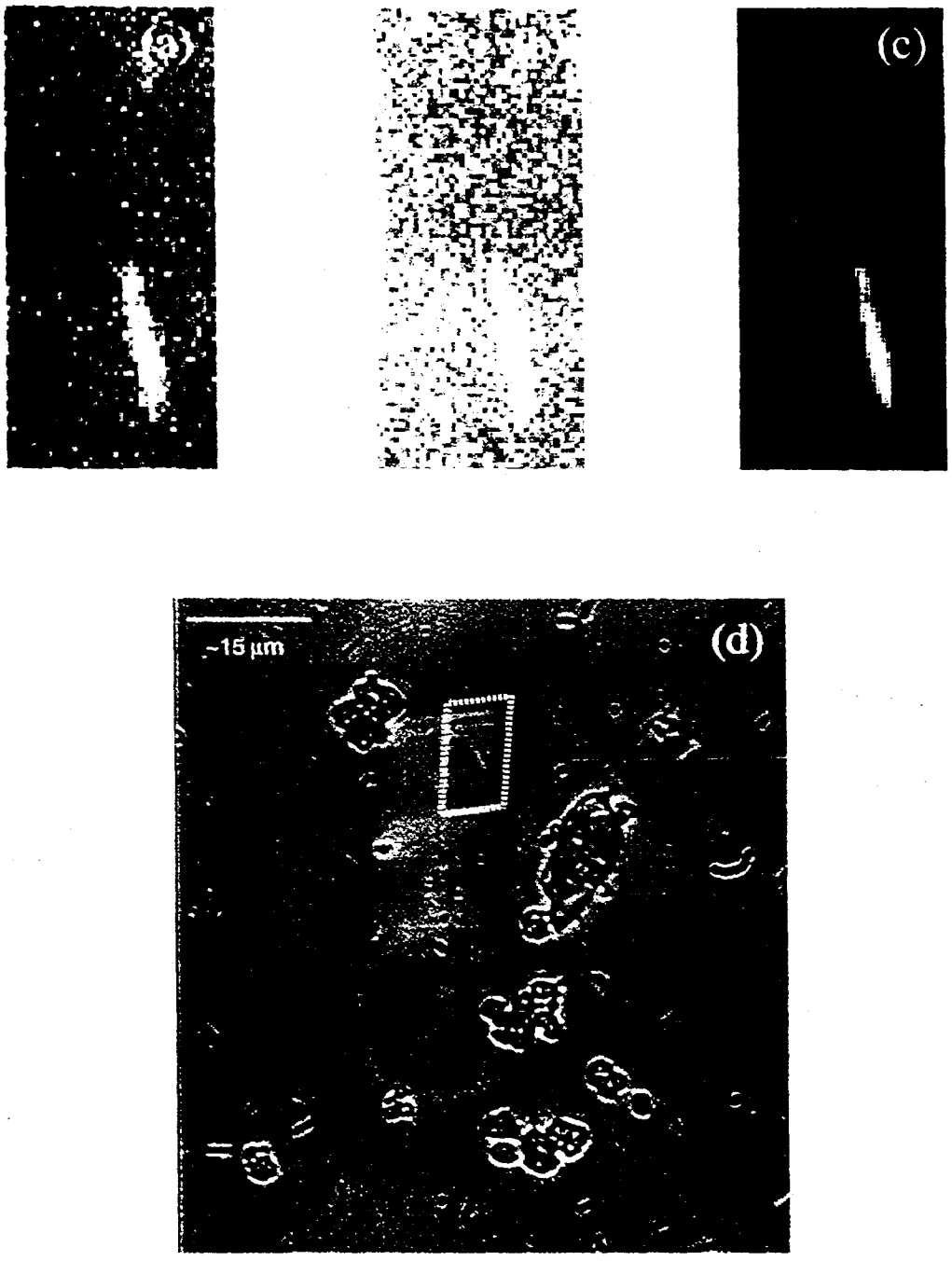

Figure 2. Spatial distributions of elements for a hydrated *Pseudomonas fluorescens* bacterium adhered to Kapton film, as determined from the Kα fluorescence intensities produced by a 0.14-µm-diameter, 11.9-KeV X-ray beam: (a) S, (b) Co, (c) Zn. The lower image (d) shows an optical micrograph of the area of the sample probed by the X-ray beam, visually identifying the bacterium. The dashed box indicates the area corresponding to the X-ray fluorescence images.

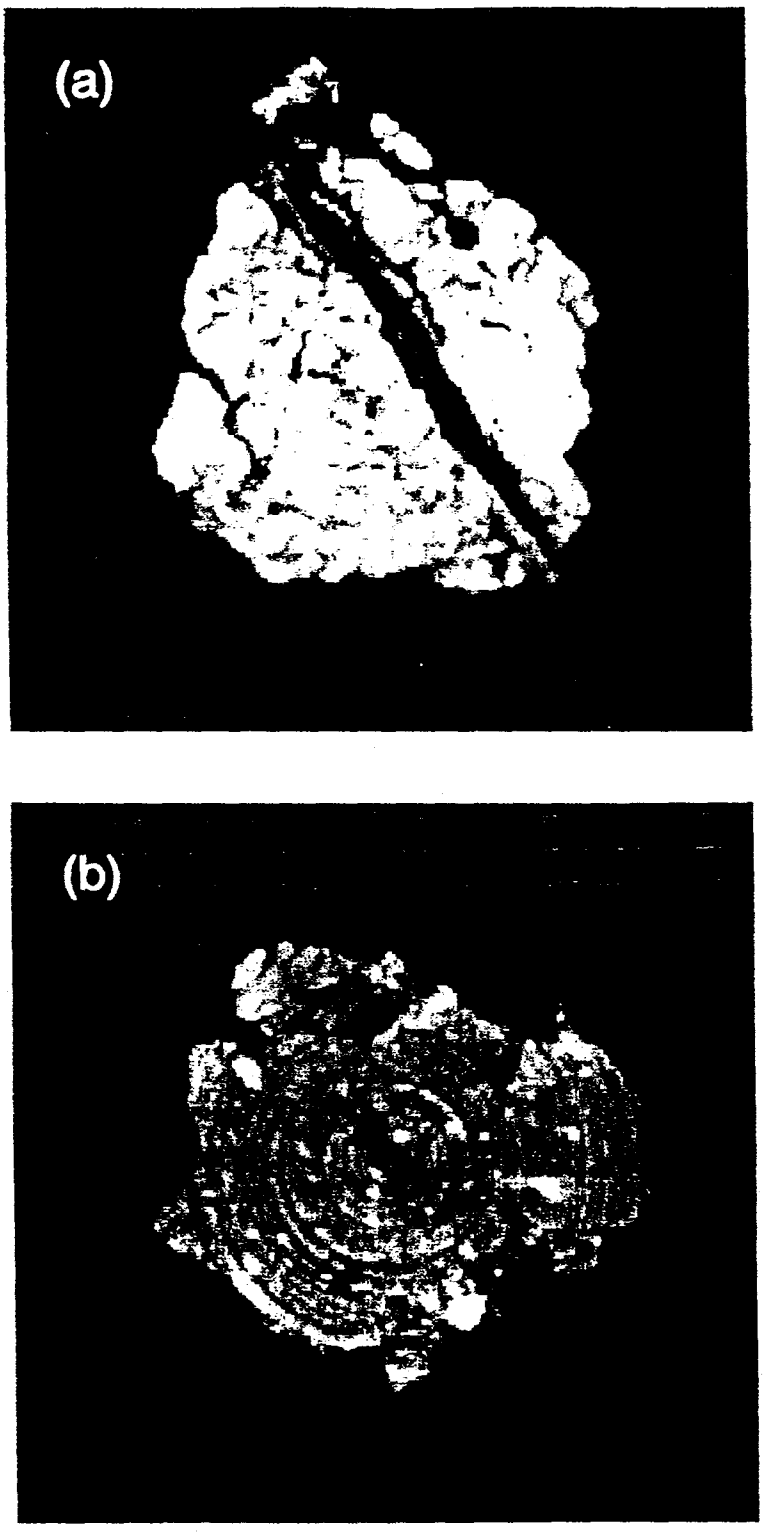

Figure 3. Individual [illegible] particulates taken from (a) an undisturbed [illegible] were recorded by using a 12-bit charge-coupled [illegible] cell size, coupled with a 20X [illegible]

contaminant bioavailability. The importance of these spatial effects makes the development of X-ray microtomography and spectrotomography very important.

Our recent X-ray microtomography experiments at Sector II of the APS indicated that determination of the three-dimensional distribution of organic and inorganic materials and pore space in a dried natural soil sample is possible. Presently, microtomographic spatial resolutions of 4-5 μm have been achieved, but 1-μm spatial resolution should be possible.[18] In these studies, we are investigating two different kinds of soil aggregates (each approximately 1 mm in diameter); one is from a undisturbed prairie site, and the other is from a recently cultivated cornfield site. To analyze the mass density distribution of the soil specimens quantitatively, pieces of single-crystal Si and Plexiglas (each approximately 50 μm thick) were mounted near the soils as references of known density.

Figure 3 shows individual slices from microtomographic reconstructions of the two soil aggregates. The dark band extending from the top left to the bottom right of the undisturbed prairie soil sample (Figure 3a) is a dehydrated plant root that can be seen, upon visual inspection, entering and exiting the soil aggregate at its surface. Comparison of Figure 3a to Figure 3b indicates that the undisturbed prairie soil sample is more homogeneous and denser than the cultivated soil. Additional studies to determine whether these differences in microstructure are statistically significant or are unique to the specific tomographic slices illustrated here are presently under way. High-density microconcretions (appearing as white objects in Figure 3) are seen in both reconstructions. Initial XRF studies of microconcretions (of similar dimensions) extracted from other soil samples indicated these to be predominantly iron oxide or some type of clay. X-ray absorption fine structure (XAFS) studies at the Fe K edge and X-ray diffraction studies are planned to resolve this issue. Evaluations of the natural positioning of these small microconcretions within aggregates, along with XAFS studies to determine the oxidation state of the Fe within the concretions, may provide a very powerful tool for determining how soil aggregates of different sizes and porosity are developed. Finally, the three-dimensional information resulting from these measurements will provide a clearer understanding of the interplay among microbial activity, soil porosity, organic and inorganic concentration, and fluid flow and their effect on contaminant transport in soils and soil components.

4. SUMMARY

We have demonstrated the utility of X-ray microbeams, particularly those produced by hard X-ray phase zone plates, for investigating a variety of environmental systems. Specifically, we have illustrated (1) the use of 1- to 5-μm hard X-ray beams for determining the spatial distribution of metals in fungal-infected plant roots, (2) the use of submicron hard X-ray beams (0.15 μm) for determining the spatial distribution of metals in a hydrated bacterium adhered to Kapton film and (3) the utility of microtomography for visualizing the internal structures of soil particulates. The further development of these techniques for such applications promises to provide unique opportunities in the field of environmental research.

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