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Applications of “Tender” Energy (1-5 keV) X-ray Absorption Spectroscopy in Life Sciences

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Abstract

The “tender” energy range of 1 to 5 keV, between the energy ranges of most “hard” (>5 keV) and “soft” (<1 keV) synchrotron X-ray facilities, offers some unique opportunities for synchrotron-based X-ray absorption fine structure spectroscopy in life sciences. In particular the K absorption edges of Na through Ca offer opportunities to study local structure, speciation, and chemistry of many important biological compounds, structures and processes. This is an area of largely untapped science, in part due to a scarcity of optimized facilities. Such measurements also entail unique experimental challenges. This brief review describes the technique, its experimental challenges, recent progress in development of microbeam measurement capabilities, and several highlights illustrating applications in life sciences.

Introduction

X-ray Absorption Fine Structure (XAFS) Spectroscopy is an important synchrotron-based tool for study of molecular-scale chemistry and structure in the life sciences and beyond. Within its experimental limitations, XAFS provides unique insights into a variety of systems. XAFS is well established as an element-specific non-invasive probe of local physical and electronic structures and states in crystalline and non-crystalline materials. Fundamentally, when an X-ray photon has sufficient energy, exceeding the binding energy of a core electron of a specific element (i.e. at or above the absorption edge energy), it will be absorbed through a process that ejects a core electron. XAFS is generally classified into two subsets. X-ray absorption near edge structure (XANES) is useful as a probe of oxidation state and local electronic structure, and as a fingerprinting technique to identify and quantify chemical species. Spectral structure close to the absorption edge is dominated by a combination of a) small shifts in the absorption edge position depending on electronic (oxidation) state, and b) one or more ligand-sensitive electronic interactions such as electron transfer to unoccupied atomic or molecular orbitals. Extended X-ray absorption fine structure (EXAFS) results from backscattering of the photoelectron by neighboring atoms, and provides more detailed information about neighbor identities, coordination numbers and interatomic distances within 5-10 Å of the absorbing atom. This is a more demanding measurement of subtle spectral structure extending 1000 eV or more above the absorption edge. Classic bulk XAFS measurements require homogeneous samples, or average over a large area of a heterogeneous sample, to accommodate beam drift during scanning. In contrast, *spatially resolved* XAFS utilizes a controlled beam size and position to obtain data from selected components in samples that are heterogeneous or structured on a scale comparable to beam spot size. This can be realized now, with limitations, down to the sub- μm scale, and is more routine at a few μm resolution.

XAS measurements are typically carried out in one of two modes. Transmission measurements directly quantify the attenuation of beam passing through a sample as the incident beam energy is scanned across an element's absorption edge. Fluorescence measurements quantify the characteristic X-ray fluorescence emitted as a consequence of absorption, which is directly proportional to absorption. Either measurement requires minimal sample preparation; XAFS is a non-destructive technique, as an element is measured in its native state within a sample. However, some limitations and exceptions apply. First, it must be possible to get photons of the correct energy into and out of the sample, taking into account sample geometry, absorption, mounting, and containment. Second, the resulting spectra will represent an average of all atoms of the target element within the illuminated beam path and visible to the detector, requiring care in sample and mounting media selection. And third, care must be taken to avoid, minimize, or at least accommodate, photon-induced changes to the sample, which are more prevalent at modern high-brightness sources.

XAFS at hard X-ray energies, above ~5 keV, is well established in life sciences, primarily for the study of metalloproteins, metal complexes, and transport/storage mechanisms. For example, Riggs-Gelasco *et al.* in 1995 [1] presented an excellent early description of XAFS at dinuclear sites in proteins. This included theoretical discussions, application of the technique, and an in-depth discussion of the limitations and requirements of data collection for successful results. Ten years later, Penner-Hahn [2] presented a review of XAFS of metals in proteins. This included EXAFS and XANES, as well as exploring time-resolved and *in-situ* XAFS. In 2009, Ascone and Strange [3] reviewed the role of BioXAS in metalloproteomics. Those authors highlighted the value of XAFS for metalloprotein studies, as well as potential for single-crystal, microbeam, and *in-vivo* applications.

As pointed out in the above reviews, it became clear from the earliest applications that EXAFS, applied at any energy range, can be very informative and provide accurate details of local structure, but may also be susceptible to misinterpretation and error -- especially if not used carefully. As a fitting technique, EXAFS alone does not provide a unique solution and may successfully fit data to an incorrect model. Therefore it is necessary to use supporting information, and to apply complementary techniques, to constrain the initial EXAFS model. The

evolution of EXAFS data analysis and modeling has also significantly improved the application of EXAFS in the hard X-ray range, but more improvements are needed.

Despite the growth of XAFS at >5 keV in life sciences, the application of this technique at lower energies, between 1 and 5 keV, is much more limited. This is due to a combination of the challenges of conducting measurements in this energy range, and the lack of optimized facilities for tender-energy XAFS in biological materials. In 2005, Akabayov *et al.* [4] reviewed applications and potential for biological XAFS at 1-5 keV. Those authors presented an excellent description of measurements in this energy range, as well as a survey of possibly applicable facilities.

The scope of this paper is not to recreate the work of Akabayov *et al.* [4], but to present an overview of the technique, update the state of the science, and highlight some new directions and recent developments. We will also explore the experimental challenges specific to this energy range, and how these may be addressed.

Opportunities in tender energy (1-5 keV) X-ray absorption spectroscopy

The *tender* energy range is so named because it lies between the typical “hard” (above ~ 5 keV) and “soft” (below 1-2 keV) X-ray ranges. These ranges are loosely defined, in practice by synchrotron beamline and experimental limitations. Typical soft X-ray beamlines operate with sample in vacuum and utilize grating monochromators, which limit their performance above 1 or 2 keV. Most hard X-ray facilities, on the other hand, utilize silicon crystal monochromators with low-energy limits between 2 and 5 keV, and operate with the sample in air; absorption by air and by the thick beryllium beam-exit windows typically limits their performance below about 5 keV. This leaves a gap, within which neither dedicated soft nor hard X-ray facilities operate optimally. However, this tender energy range offers access to unique absorption edges that are of particular relevance to life sciences. These include the K edges of Na, Mg, Al, Si, P, S, Cl, K, and Ca [5]. In addition, many heavier elements, whose K or L absorption edges are readily accessed by hard X-ray facilities, have L or M edges in the tender-energy range that provide unique information about electronic state and ligand identity. These include As, Sb, Cd, Ag, Pt, and U, among others.

The primary focus of tender-energy XAFS measurements in biological systems remains sulfur. It is typically the lightest element that can be reached, even if not optimally, by a subset of hard X-ray facilities. Sulfur is also a versatile and useful element in many biochemical systems, and its XANES spectrum is highly diagnostic of its range of organic species and oxidation states (see Fig. 1 and, for example, [1, 6-10]). In biological systems, S is present as a structural constituent of amino acids and proteins, plays a role in many metalloproteins and enzyme active sites, and is involved in a number of metal transport processes. These can be studied by XAFS *in-situ* either spatially resolved or bulk (averaging), in isolated proteins or complexes, or in model reaction systems. One of the challenges of S XAFS in complex biological systems is its simultaneous presence in a variety of roles. Thus the spectra collected represent an average of all species present and it may be challenging to isolate the target species.

Figure 1. Spatially resolved in-situ XAFS. Sulfur XANES of selected microbeam spots in a leaf of *Alyssum murale* indicates locally variable S speciation. Shown in blue, one spot contains (distinct peaks from left to right) sulfide, sulfoxide, and dominant sulfonate; a contrasting spot on the same leaf (shown in red) contains much less sulfide and sulfoxide and is dominated by sulfate, the peak for which is at higher energy than sulfonate. Structures at higher energy than sulfate are the beginnings of EXAFS oscillations.

In addition to sulfur, lighter elements accessible in this energy range have a wide variety of important metabolic roles such as nutrient transport, storage and redox chemistry, energy pathways, and cellular controls, as well as structural roles in membranes. These elements may act as free ions or be bound in compounds. Yet another important role for light elements such as P, Ca, and Si, is in biomineralization: production of crystalline or amorphous inorganic material for structural, defensive or utilitarian applications. All of these processes and biochemical roles may be studied using XAFS.

Microbeam XAS

There have been continued advances in microbeam XAFS, applying what was initially a bulk technique to the μm , or even sub- μm scale. Beamlines designed for this utilize achromatic optics, such as mirrors in the Kirkpatrick-Baez geometry, to focus beam onto the sample. This allows spatially resolved measurement of a homogeneous region of any size larger than the beam size, or alternatively averages over the entire beam footprint on a material that is heterogeneous on a scale smaller than the beam size. The challenge with microbeam XAFS is

to deliver a beam that is positionally stable during energy scanning, so that chemical heterogeneities and sample topography are not sampled differently at different energies. Most hard X-ray microprobe beamlines currently offer microbeam XANES measurements. However, these facilities are generally optimized first for X-ray fluorescence (XRF) imaging, i.e. spatial resolution; their XAFS capabilities vary significantly and typically become increasingly limited as beam size decreases. Since high-quality EXAFS measurements are the most demanding, requiring positional and intensity stability over a long scanning range, far fewer facilities are optimized for microbeam EXAFS. One of note is Beamline 10.3.2 at the Advanced Light Source [11], with an energy range that can reach somewhat into the tender energy range. Two new beamlines under construction at the National Synchrotron Light Source II are specifically optimized for high-performance EXAFS at the microbeam scale: the TES Beamline (8-BM) in the tender energy range, 1-8 keV [12], and the XFM Beamline (4-BM), at higher energies from 2.3 to 23 keV [12]. In addition to the opportunity for spatially-resolved XAFS in heterogeneous materials, microbeam capabilities permit measurement of particularly small samples, which is key for many biological materials that are very difficult to synthesize or isolate in quantity. Several highlights of microbeam tender-energy XAFS in life sciences are included below.

Highlight: Natural Organochlorine

Chlorine (Cl) was long believed to exist in nature as inorganic chloride (Cl^-), an ion presumed inert under environmental conditions due to its electron affinity, the highest of any element. Consideration of organochlorines in the environment focused on persistent, toxic manmade pollutants like DDT, immortalized in Rachel Carson's *Silent Spring* [13]. In the past few decades, research has shown that organochlorines are also produced naturally on a large scale [14-16], through both biological [17, 18] and abiological [19, 20] pathways. XAFS spectroscopy has been instrumental in revealing the existence of natural organochlorine, starting with Myneni's discovery of natural organochlorine in decaying plant material [16]. Subsequent XANES-based studies showed that inorganic chloride in plant material could be transformed to organochlorine by the enzyme chloroperoxidase [21] and that organochlorine production in forest ecosystems followed a seasonal pattern with increased production in warmer months [22]. In 2015, XANES spectroscopy was used to illuminate naturally produced organochlorine in marine particulates, the first evidence of incorporation of seawater chloride into marine sediments [23].

These studies relied on the ability of XANES spectroscopy to differentiate among Cl species in chemically heterogeneous environmental samples. Cl XANES spectral features vary according to the coordination environment of Cl, such that inorganic and organic forms of Cl can be readily distinguished [21]. Compared with inorganic chloride compounds like sodium chloride (NaCl), organochlorine compounds such as chloropropanol and chlorophenol have intense low-energy peaks corresponding to electronic transitions from the $1s$ orbital to π^* and σ^* molecular orbitals (Fig. 2, e). The C-Cl bond length determines the energy of this low-energy absorption maximum; Cl atoms bound to aromatic carbon, as in chlorophenol, have absorption maxima occurring ~ 0.6 eV higher in energy than those of aliphatic organochlorine such as chloropropanol (Fig. 2, e). In environmental sample spectra, the relative proportions of inorganic chloride and aliphatic/aromatic organochlorine can be determined by least-squares fitting sample spectra with spectra of representative model compounds, with fitting errors below 10% [24].

Cl XANES spectroscopy has been valuable for bulk Cl speciation analysis of environmental samples in the examples cited above. In addition, the use of X-ray spectromicroscopy has illuminated microscale heterogeneities in the distribution and speciation of Cl in environmental samples, notably in soil organic matter [25] and marine sedimentary material [23]. In the former example, microbeam XRF and XANES data revealed the existence of 1) aliphatic organochlorine in healthy leaves that persists through advanced decay stages of the plant material on the forest floor; and 2) aromatic organochlorine associated with fungi colonizing the surfaces of decayed leaves on the forest floor [25]. In this way, spectromicroscopic measurements definitively revealed two distinct fractions of organochlorine in soil organic matter.

Microbeam XRF and XANES data measured at NSLS Beamline X15B during testing and commissioning of the NSLS-II TES instrument played a key role in the recent discovery of organochlorine in marine particulates [23]. In particular, spectromicroscopy proved crucial in 1) clarifying speciation ambiguities arising from bulk-level XANES measurements; and 2) determining whether there were associations between Cl and other elements. A similar example is presented here in Figure 2. Marine particulates collected in sediment traps deployed in the Arabian Sea produced bulk XANES spectra with the energy of the absorption maximum falling in between the characteristic maxima of aliphatic and aromatic organochlorine (Fig. 2, e, bulk sediment spectrum). Imaging the particulates with microbeam XRF revealed a heterogeneous distribution of Cl, with concentrated Cl "hotspots" against a background of lower Cl concentration (Fig. 2, a). Micro-XANES spectra collected on the Cl hotspots displayed aromatic speciation (Fig. 2, e, colored spectra), with the absorption maximum aligned with that of the chlorophenol standard. Aliphatic Cl hotspots were scarcer but more concentrated, accounting for the mixed

aliphatic/aromatic speciation in the bulk XANES spectrum. This microscale heterogeneity in Cl speciation was consistent with different pathways of marine chlorination [23]. Furthermore, contrasting images of Cl distribution with those of other elements revealed that the organochlorine hotspots are generally not coincident with the mineral elements Ca (Fig. 2, b) or Si (Fig. 2, d) but do show some association with S (Fig. 2, c). This substantiates the association of Cl with the organic rather than the mineral fraction of the sediment sample.

There are two particular caveats to emphasize in the use of XAFS spectroscopy for Cl measurements. First, environmental samples, particularly marine samples, often contain high concentrations of inorganic chloride, which can obscure smaller organochlorine signals in the XANES spectra. To reveal organochlorine in the signal, it may be necessary to rinse samples thoroughly in deionized water prior to analysis. As chloride is a very soluble ion, rinsing has been shown to be highly effective in eliminating inorganic chloride from the XANES signal, even in marine samples with very high background chloride levels [23]. Another experimental obstacle in Cl XANES measurements, particularly microbeam analyses, is radiation damage -- exposure to the intense microfocused X-ray beam often causes reductive dechlorination, with rapid transformation of organochlorine to inorganic chloride. Faster scan times reduce microbeam exposure, helping to mitigate the beam damage phenomenon.

Figure 2. X-ray spectromicroscopic analysis of particulate sediment trap material from the Arabian Sea (1882 m depth). **a-e)** Micro-XRF images (1.7 mm x 2.2 mm) of elemental distributions: **a)** Cl K_{α} ; **b)** Ca K_{α} ; **c)** S K_{α} ; **d)** Si K_{α} . Lighter shade corresponds to greater fluorescence intensity, i.e., higher concentration. **e)** Black traces = bulk Cl XANES spectra of representative standards, including inorganic (NaCl), aliphatic (chloropropanol), and aromatic (chlorophenol) forms of Cl, along with the bulk XANES spectrum of the sediment trap sample. Colored traces = normalized Cl micro-XANES spectra (20 μ m x 10 μ m spot size) corresponding to the circled areas in **a-d**. Colors of circles in **a-d** match colors of associated spectra in **e**.

Highlight: Application of Calcium K-edge XAFS to Plant Biology

Calcium is an essential element for living organisms. It is a major component of bone, teeth and shells, and is important for cellular signaling where the movement of Ca ions across the cell membrane functions as a trigger for many cellular processes. In plants, Ca is required for various structural roles in the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol [26]. Many plants store excess calcium in a mineral form - calcium oxalate, CaOx - that is not readily metabolized by higher organisms. As much as 90% w/w of the total plant Ca may be bound as the oxalate salt [27]. A Ca oxalate-defective mutant of the model forage legume *Medicago truncatula*, *cod5*, was identified previously in a genetic screen based on its inability to accumulate CaOx in its leaflets [28]. Application of microbeam Ca K-edge XAFS and tender-energy X-ray fluorescence (XRF) imaging (4.1 keV) was helpful to characterize Ca localization and speciation in the *cod5* mutant and wild-type plants [29].

As reviewed below, there are a number of experimental challenges that are unique to measurements in the tender energy range. For instance, the effective sampling depth for low-Z elements is shallow compared to elements of higher atomic number. For this reason, it was necessary to image an intact leaflet both with its adaxial (top) surface and abaxial (bottom) surface facing the incident X-ray beam to verify the localization pattern was symmetrical; symmetry was also confirmed by imaging cross-sections of leaf tissue. In addition, it is more common to encounter self-absorption effects (also called over-absorption) in the tender energy range. In this experiment, self-absorption corrections were necessary for quantitative interpretation of the Ca K-edge XAFS from intact *Medicago* leaflets, but were not necessary for thin sections (20 μm) of leaf tissue. This was due to the sample preparation where the effective sampling depth of Ca K exceeded the sample thickness of the tissue sections. Further, XAFS spectra of the tissue sections were useful for applying the self-absorption correction method described by Sarret et al. [30] to the spectra of intact leaflets. Thus, tender-energy XAFS measurements of thick samples should aim to capture representative XAFS spectra that are free of self-absorption effects to assist with self-absorption corrections.

Secondary veins of wild-type *Medicago* plants contained CaOx as a major component. It was determined from polarized Ca K-edge XAFS (Fig 3) -- and later verified with X-ray microdiffraction -- that CaOx in veins of wild-type plants was X-ray crystalline. In contrast, veins of *cod5* mutants contained Ca bound with pectin and organic acid anions including oxalate, but these complexes lack the crystallinity observed in veins of wild-type plants [29]. In *Medicago*, CaOx crystals accumulate along the vascular strands in a defined pattern [27], and the crystallographic c-axis is perpendicular to the leaf normal. Due to the polarized nature of the synchrotron beam (horizontal plane on wiggler or bend magnet source), one can obtain polarized XAFS by rotating the leaf in plane on the sample stage (out-of-plane rotations are also possible and yield spectral variations) and collecting XAFS spectra at these different orientations. This polarized XAFS measurement was possible because the X-ray beam was smaller than the single crystals of CaOx thus beam does not average over a range of crystal orientations; it is worth mention that at the highest spatial resolutions, one encounters almost exclusively single crystals and will measure orientation-dependent XAFS spectra. In Fig. 3, images of Ca in leaflets of wildtype (WT) and *cod5* mutants (MUT) show a difference in Ca localization, and Ca K-edge XAFS spectra from different orientations of intact leaflets (secondary veins) show a difference for the WT plants but not for the MUT plants because *cod5* leaflets do not contain single crystals of CaOx in secondary veins. Overall results from Punshon et al. [29] show that deletion of *cod5* changes both Ca localization and the form of CaOx within leaflets of *Medicago*, and also demonstrate the utility of coupled tender energy XRF imaging and microspectroscopy to plant biology and life sciences.

Figure 3. Images of Ca in leaflets of wildtype (WT) and *cod5* mutant (MUT) plants show a difference in Ca localization, and Ca K-edge polarized XAFS spectra from different orientations of intact leaflets (secondary veins) show a difference for WT plants but not MUT plants because the *cod5* leaflets do not contain single crystals of CaOx in secondary veins.

Highlight: XAFS of a protein single crystal

The study of metalloproteins by macromolecular crystallography has benefitted significantly from the complementary application of transition-metal XAFS [3], and this benefit is expected to be extended by XAFS of lighter elements. The available samples of most proteins are very small quantities, either solutions, single crystals or aggregates, necessitating a micron-scale beam. Small-volume fluid cells, capillaries, and similar mounting methods are required to contain such samples, as well as to maintain their hydrated state or to exclude atmosphere if they are air-sensitive.

As a proof-of-concept example, Figure 4 shows S K-edge XRF images and XANES spectra of a protein single crystal mounted in a thin-walled S-free polyimide capillary. The crystal was adhered to the capillary wall

with a thin film of mineral oil, in which the crystal had been stored to maintain its hydrated state. The capillary and the mineral oil both significantly reduced S fluorescence signal, but not to the point of impacting the measurement. Images of the transmitted X-ray intensity and the S K α fluorescence clearly show the outline of the crystal. Single-spot microbeam XANES measurements indicate the disulfide configuration of S in this structure, characterized by a double peak. Energy scanning was performed in on-the-fly fast-scanning mode, approximately 25 s for each scan. Given the high photon flux density in the microbeam spot, and the fact that at this energy most of that flux is absorbed by the sample, radiation-induced chemical changes may occur quickly. Comparison of the first 10 scans (<5 minutes exposure) with the next 10 scans clearly shows the breaking of disulfide bonds in favor of monosulfide or thiol species. See below for further discussion of radiation-induced changes.

Figure 4. A single crystal of lysozyme. At left, microbeam images, field of view 180 μm wide, 10.85 μm pixels, $\sim 10 \times 10 \mu\text{m}$ beam size, estimated 2×10^{10} photons/s flux. Far left is transmission contrast at 2800 eV; to the right is sulfur K α fluorescence (lighter colors higher signal). At right is spot microbeam quick-XANES at the sulfur K edge. Shown in blue (lower trace) is the sum of the first ten 25-second scans; shown in red is the sum of the next ten scans, indicating beam-induced changes.

Unique Experimental Requirements

There are a number of challenges to successful XAFS measurements at 1-5 keV. These have been addressed in various ways at different facilities and for different experiments, but tender-energy XAFS will always be a significantly different experience compared to hard X-ray measurements. Historically, XAFS measurements above 5 keV have been among the simplest synchrotron-based techniques to apply. A simple sample holder, energy-tuned monochromatic beam, and either transmission or fluorescence detection are set up in ambient atmosphere. Tender-energy XAFS, in contrast, imposes unique requirements on experimental aspects including beamline design, sample environment, detectors, and data analysis tools, and must accommodate very different

behavior as far as sample configuration, sampling depth, fluorescence yield, and contamination. These challenges are reviewed below.

Beamline optimization: facilities for tender-energy XAFS need to be optimized from the source to the sample. Although the principles and general configuration are similar, there are important differences that must be built in from the start. A broad-spectrum source is needed for XAFS in general, and this should have a low critical energy so that unused high-energy photons do not cause unnecessary thermal loads or damage. Beamline optics (mirrors) used for this energy range will have much higher incidence angles, up to 20 mrad. Standard mirror coatings (Rh or Pt) have absorption edges in the tender range, so Ni is a good substitute. Since harmonic rejection is more important at lower energy, where harmonics are closer in energy, the ability to tune harmonic-rejection mirror pitch is essential. This results in large deflections in beam trajectory, creating configuration challenges. Mirrors must be kept exceptionally clean, so optics should be under ultra-high vacuum and “windowless” to the endstation. Typical beryllium windows (at least 0.25 mm thick) are too absorptive for <5 keV, so only an ultra-thin window (e.g. 12 μm) can be used as the final separation from vacuum.

Standard monochromator geometries and crystal materials are inadequate for 1-5 keV. The most common crystal material, Si(111), cannot diffract below 2 keV. Alternatives include InSb, quartz, beryl, and YB₆₆. InSb is heat-sensitive, and is most often used for Si K-edge XAFS only. Quartz suffers from radiation damage over time, and has a narrow bandpass (low flux), but is useful for Al XAFS. Use of beryl has historically been limited due to degradation from radiation damage, but this can be avoided by limiting the incident beam photon energy to less than 5 keV. Beryl can reach to very low energy (800 eV). YB₆₆ is expensive, not easily obtained, and is often non-uniform, but a good set is very useful. Regardless of crystal used, these energies require Bragg angles between 30 and 80 degrees, and energy scans may span more than 35 degrees for a single spectrum. To maintain constant exit beam position therefore requires large motions of the crystals. Stability for microbeam applications is therefore more challenging than for hard X-ray energies where crystal motions are smaller. Grating monochromators are typically very effective below 2 keV for imaging and bulk spectroscopy applications, but are more challenging to apply to energy scanning with a fixed beam position. An ideal arrangement may be to have interchangeable fixed-exit crystal and grating monochromators.

Sample environment and detectors: at 2 keV, photons are completely absorbed within just a few cm in air. Although soft X-ray beamlines operate with the sample in vacuum, this is undesirable for many biological samples. Helium at atmospheric pressure is an attractive alternative for tender-energy XAFS, as absorption by He is minimal while samples are not strongly affected. Figure 5 shows the NSLS-II TES beamline helium-atmosphere chamber, housing the sample position as well as microfocusing mirrors. This necessitates some accommodations, such as configuration of the sample environment as a glove box with sample-exchange port, and customizing detectors to be compatible with helium. Detectors themselves must have ultra-thin windows to access the low-energy fluorescence emissions, without suffering from helium infiltration.

Figure 5. The NSLS-II TES beamline helium-atmosphere chamber, housing the sample position as well as microfocusing mirrors. Beam direction is left to right. At the entrance to the chamber is a 12.5 μm thick beryllium window separating the chamber from beamline vacuum. Just inside is an ionization chamber for I-zero measurement (windowless and therefore He-filled), followed by KB microfocusing mirrors. The KB system,

designed by Instrument Design Technology, is customized for high angles, and has the unusual arrangement of horizontal-focusing mirror first so that it faces away from the detectors to minimize background scatter. Downstream of the mirrors there is an optical microscope viewing the sample, which is oriented at 45 degrees facing toward the detectors. Two fluorescence detectors (Ge and Si) insert through the back of the chamber, perpendicular to the beam direction. The xyz sample stage is mounted from the top of the chamber with the sample at 45 degrees to beam. At the end of the chamber is another ionization chamber for transmission measurements, and a beam stop.

Sample mounting, sampling depth and fluorescence yields: the challenge of sample mounting is that signal will be strongly attenuated by mounting materials. It is ideal to measure samples uncovered, but it is often necessary to contain a sample or protect it from drying in the He atmosphere. In that case, only very thin, non-absorbing covering material such as 5 μm polypropylene can be used. The effective sampling depth within the sample is likewise very limited. Table 1 shows the effective sampling depth, in representative matrices, for selected absorption edges. The limited sampling depth in solution is an important factor determining the minimum concentration that can be measured at a given facility. The small volume sampled at low energy is compounded by the reduced fluorescence yield for these elements [5].

Table 1. Effective sampling depths.

Element	Fluorescence energy (approximate, eV)	Sampling depth in water (μm at 90 degrees, divide by 1.414 for 45-degree geometry)	in cellulose having density of 1.0 (μm at 90 degrees)
Fe	6400	493	650
Ca	3690	95	125
K	3310	69	91
Cl	2622	35	46
S	2309	24	32
P	2010	16	21
Si	1740	11	14
Al	1486	7	9
Mg	1253	4.5	6

In the tender energy range, one must also be aware of ubiquitous contamination from the environment. Light elements, either the target of the measurement or a potential interference in the energy range of the measurement, are present in many common materials and mounting media. For example, clays, (Al, Si), soil (Ca, Si), chalk (Ca), and salt (Na, Cl) are common dusts. Thin films (mylar, polyimide), and tape adhesives, frequently contain S and Si. Epoxies and waxes usually contain S. Fingerprints contain S, P, Na and Cl. We have found that thin polypropylene is quite clean, as is petrolatum, which can be used as a binder. Chemical reagents for use should be of the highest purity available, as for example nitric acid often has S or P impurities. And lastly, a significant source for interfering S and P is from pH buffers, even indirectly introduced via pH meter electrodes.

Beam-induced sample alteration: as an intense dose of ionizing radiation, the incident beam used for XAFS measurements may cause radiation damage in a variety of ways including structural damage, chemical breakdown, oxidation-state changes, and thermal damage. This is most pronounced for microbeam measurements, where the photon flux density is very high. Such alteration must be identified, mitigated or avoided, but in some systems cannot be eliminated. Cryogenic cooling of samples to reduce radiation damage had a revolutionary impact for macromolecular crystallography. Applying such cooling for XAS and XAS imaging has had some success but in many cases oxidation state or local chemistry is much more sensitive to radiation-induced change than is crystal structure. In the tender energy range, a helium atmosphere sample environment makes cryogenic cooling a challenge, while not all samples are amenable to a vacuum environment. Making measurements as rapidly as possible reduces total radiation dose. Often the best approach is to collect several fast scans and monitor them for changes. This will show how long it takes for damage to occur in a given system, and identify the direction and mode of alteration, so an appropriate measurement strategy may be pursued. In the worst cases, this will at least permit extrapolation to what the likely starting state was prior to exposure.

Data analysis and interpretation: the limitations and challenges of data analysis and interpretation of hard X-ray absorption spectra have been well described. The tender energy range imposes several others, most resulting from the fact that at lower energy spectral features are much more compressed. Compared to higher energy edges, spectral features for low-energy edges are much closer in energy, elements are closer in energy to each other, L_2 and

L₁ edges are closer to the L₃ edge, and typical k-ranges for EXAFS are shorter. Ligands are usually at closer distances, and multiple scattering contributes significantly to the EXAFS. Available data-analysis software is optimized for higher energy edges, so typical default values for fitting ranges, etc. require adjustment.

A look ahead

In spite of some experimental challenges, there are bright prospects for tender-energy XAFS in life sciences, including microbeam XAFS, as new instrumentation comes online at NSLS-II and other facilities. Based on the still-growing value of transition-metal XAFS in biological materials, particularly as a complement to macromolecular crystallography and for materials that are not available in single-crystal form, there is substantial potential for lower-energy XAFS to elucidate the role of lighter elements in proteins and in biochemical processes. This is an area of largely untapped science. While Ca and S have been successfully exploited to some extent, there have been far fewer studies of P and Cl XAFS, and there are only a handful of measurements of edges between 1 and 2 keV. However, as next-generation facilities begin operations, and we have collectively addressed the challenges and unique needs of tender-energy XAFS measurements, more of this range will be readily accessible.

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References

- [1] Riggs-Gelasco, P., Stemmler, T., and Penner-Hahn, J., XAFS of dinuclear metal sites in proteins and model compounds, *Coord. Chem. Rev.* **1995**, 144, 245-286.
- [2] Penner-Hahn, J., Characterization of “spectroscopically quiet” metals in biology, *Coord. Chem. Rev.*, **2005**, 249 (1-2), 161-177.
- [3] Ascone, I. and Strange, R., Biological X-ray absorption spectroscopy and metalloproteomics, *J. Synchrotron Rad.* **2009**, 16, 413-421. doi:10.1107/S0909049509010425
- [4] Akabayov, B., Doonan, C. J., Pickering, I. J., George, G. N. and Sagi, I. Using softer X-ray absorption spectroscopy to probe biological systems. *J. Synchrotron Rad.* **2005**, 12, 392-401.
- [5] Bearden, J.A. and Burr, A.F. Reevaluation of X-ray atomic energy levels. *Rev. Mod. Phys.* **1967**, 39, 125.
- [6] Xia, K., Weesner, F., Bleam, W. F., Bloom, P. R., Skyllberg, U. L., and Helmke, P. XANES Studies of Oxidation States of Sulfur in Aquatic and Soil Humic Substances. *Soil Sci. Soc. Am. J.* **1998**, 62, 1240-1246
- [7] Huffman G. P., Mitra, S., Huggins, F. E., Shah, N., Vaidya, S. & Lu, F. Quantitative Analysis of All Major Forms of Sulfur in Coal by X-ray Absorption Fine Structure Spectroscopy, *Energy & Fuels*, **1991**, 5, 574-581.
- [8] Schäfer, T., Buckau, G., Artinger, R., Kim, J.I., Geyer, S., Wolf, M., Bleam, W.F., Wirick, S., Jacobsen, C. Origin and mobility of fulvic acids in the Gorleben aquifer system: implications from isotopic data and carbon/sulfur XANES. *Org. Geochem.* **2005**, 36, 567-582.
- [9] Prietzel, J., Thieme, J., Salome, M., and Knicker, H.. Sulfur K-edge XANES spectroscopy reveals differences in sulfur speciation of bulk soils, humic acid, fulvic acid, and particle size separates. *Soil Biology & Biochemistry*. **2007**, 39:877-890.
- [10] Riquelme, F., Northrup, P., Ruvalcaba-Sil, J., Stojanoff, V., Siddons, D., Alvarado-Ortega, J., Insights into Molecular Chemistry of Chiapas Amber Using Infrared-Light Microscopy, PIXE/RBS, and Sulfur K-edge XANES Spectroscopy, *Appl. Phys. A*. **2014**, 116, 97-109.
- [11] Marcus, M. A., MacDowell, A. A., Celestre, R., Manceau, A., Miller, T., Padmore, H. A. and Sublett, R. E.. Beamline 10.3.2 at ALS: a hard X-ray microprobe for environmental and materials sciences. *J. Synchrotron Rad.* **2004**, 11, 239-247
- [12] Current information available at URL <https://www.bnl.gov/ps/beamlines> as accessed September 2015.
- [13] Carson, Rachel. *Silent Spring*. **1962**. Houghton Mifflin, Boston.
- [14] Öberg, G., Chloride and organic chlorine in soil. *Acta Hydrochemica et Hydrobiologica* **1998**, 26, 137-144.
- [15] Asplund, G.; Grimvall, A., Organohalogens in nature, more widespread than previously assumed. *Environmental Science and Technology* **1991**, 25, 1346-1350.
- [16] Myneni, S. C. B., Formation of Stable Chlorinated Hydrocarbons in Weathering Plant Material. *Science* **2002**, 295, 1039-1041.

- [17] Ortiz-Bermúdez, P.; Hirth, K. C.; Srebotnik, E.; Hammel, K. E., Chlorination of lignin by ubiquitous fungi has a likely role in global organochlorine production. *Proceedings of the National Academy of Sciences* **2007**, 104, (10), 3895-3900.
- [18] Asplund, G.; Christiansen, J. V.; Grimvall, A., A chloroperoxidase-like catalyst in soil: detection and characterization of some properties. *Soil Biol. Biochem.* **1993**, 25, 41-46.
- [19] Keppler, F.; Eiden, R.; Niedan, V.; Pracht, J.; Schöler, H. F., Halocarbons produced by natural oxidation processes during degradation of organic matter. *Nature* **2000**, 403, 298-301.
- [20] Schoeler, H. F.; Keppler, F., Abiotic Formation of Organohalogenes During Early Diagenetic Processes. *Handbook of Environmental Chemistry* **2002**, 3, (P), 63-84.
- [21] Reina, R. G.; Leri, A. C.; Myneni, S. C. B., Cl K-edge x-ray spectroscopic investigation of enzymatic formation of organochlorines in weathering plant material. *Environ Sci Technol* **2004**, 38, (3), 783-789.
- [22] Leri, A. C.; Myneni, S. C. B., Organochlorine turnover in forest ecosystems: The missing link in the terrestrial chlorine cycle. *Global Biogeochem. Cycles* **2010**, 24, (4), GB4021.
- [23] Leri, A. C.; Mayer, L. M.; Thornton, K. R.; Northrup, P. A.; Dunigan, M. R.; Ness, K. J.; Gellis, A. B., A marine sink for chlorine in natural organic matter. *Nature Geosci* **2015**, 8, (8), 620-624.
- [24] Leri, A. C.; Hay, M. B.; Lanzirrotti, A.; Rao, W.; Myneni, S. C. B., Quantitative determination of absolute organohalogen concentrations in environmental samples by X-ray absorption spectroscopy. *Anal Chem* **2006**, 78, (16), 5711-5718.
- [25] Leri, A. C.; Marcus, M. A.; Myneni, S. C. B., X-ray spectromicroscopic investigation of natural organochlorine distribution in weathering plant material. *Geochimica et Cosmochimica Acta* **2007**, 71, 5834-5846.
- [26] Marschner, H. *Mineral nutrition of higher plants*, 2nd edn. New York, NY, USA: Academic Press. **1995**.
- [27] Nakata, P.A. and McConn, M.M. Calcium oxalate crystal formation is not essential for growth of *Medicago truncatula*. *Plant Physiol. Biochem.* **2003**, 41: 325-329.
- [28] Nakata, P.A. and McConn, M.M. Isolation of *Medicago truncatula* mutants defective in calcium oxalate crystal formation. *Plant Physiol.* **2000**, 124: 1097-1104.
- [29] Punshon, T., Tappero, R., Ricachenevsky, F.K., Hirschi, K., and Nakata, P.A.. Contrasting calcium localization and speciation in leaves of *Medicago truncatula* mutant COD5 analyzed via synchrotron X-ray techniques. *The Plant Journal.* **2013**, 76:627-633.
- [30] Sarret, G., Isaure, M.P., Marcus, M.A., Harada, E., Choi, Y.E., Fakra, S. and Manceau, A. Chemical forms of calcium in Ca,Zn- and Ca,Cd-containing grains excreted by tobacco trichomes. *Can. J. Chem.* **2007**, 85:738-746.