A microchannel confocal examination of arsenic speciation and distribution in *Bufo americanus*

Michelle M. Nearing¹, Iris Koch¹, Robert A. Gordon², Kenneth J. Reimer¹

¹Environmental Sciences Group, Royal Military College, Kingston, ON, Canada
²Moyie Institute and Dept. of Physics, Simon Fraser University, Port Coquitlam, BC, Canada

E-mail: ragordon@alumni.sfu.ca

Abstract. We have used confocal methods to examine the distribution and speciation of arsenic within amphibian tissue (*Bufo americanus*) from a contaminated mine site. The use of new microchannel technology permits a confocal, and energy-independent, examination of a given voxel within the amphibian tissue without the need for sectioning. We observe differences in arsenic concentration and speciation depending on tissue type, with the Eberth-Katschenko layer in particular containing Mn, Fe, Cu and Zn in addition to Ca and pentavalent arsenic.

1. Introduction

Amphibians such as *Bufo americanus*, whose life-cycles comprise both aquatic and terrestrial environments, can be exposed to arsenic through soil, food or water sources, allowing these species to serve as indicators of overall environmental quality. Understanding the potential uptake and biotransformation pathways for arsenic in these indicator species is important for assessing risk to ecological systems. A previous study has examined arsenic speciation in frog and toad species from an arsenic contaminated site using high-performance-liquid-chromatography – inductively-coupled-plasma-mass-spectrometry (HPLC-ICPMS) and complementary X-ray absorption spectroscopy (XAS) [1]. Although the XAS techniques used in this study allowed for the speciation of all arsenic in these samples, the spatial resolution of the arsenic in the skin and legs tissues was not determined due to difficulties in obtaining suitable tissue sections.

Confocal methods can provide an alternate means of obtaining distribution and speciation through fluorescence imaging and XAS without obtaining a thin section. A confocal set-up is comprised of both a focusing element and solid-angle-limiting detection element, and where these two elements overlap focus and detection, there is a defined volume (voxel) of investigation [2-4]. Polycapillary glass optics are commonly used in such x-ray fluorescence imaging set-ups, and, when used as the detection optic, the solid-angle defining the detection region is dependent on the critical angle of the glass, which leads to an energy-dependence [4] of the voxel size, with lower energy fluorescence being detectable from a larger volume than higher energies. This is problematic when one is seeking to correlate the presence of lighter and heavier atoms.

Here we make use of a germanium lithographically-fabricated microchannel optic for the detection aspect of the measurement [5]. While the polycapillary optic functions like a two-dimensional spatial filter [6], albeit energy-dependent, the microchannel optic functions similarly to Soller slits [7], constraining the detection of emitted x-rays along the beam, while the focusing...
constrains the other two dimensions to give a defined voxel without the energy-dependence of the glass polycapillary. By using this method, we have determined elemental distributions and measured arsenic speciation at select locations in a bulk piece of *Bufo americanus* skin.

2. Experimental

A patch of tissue from the back of an eastern American toad (*Bufo americanus*) was obtained from a freeze-dried specimen collected from a contaminated mine site near Upper Seal Harbour, Nova Scotia, as per Moriarty *et al.*[1]. The irregularly-textured patch, approximately 20 mm² in area, was sealed in kapton tape for measurement at room temperature. The region of examination is indicated in Figure 1.

![Figure 1](https://example.com/figure1.png)

*Figure 1.* (Colour online) Patch of skin tissue indicating region of examination (green, middle), approximate location on a representative (not actual before freeze-drying, left) toad back and overhead view (right) of measurement showing 1mm working distance of optic to sample.

X-ray measurements were carried out using the microprobe end-station of beamline 20ID at the Advanced Photon Source [8]. The undulator beam through the LN2-cooled Si(111) monochromator was focused to a cross-section of 2 × 2 μm² using Kirkpatrick-Baez mirrors, with the beam incident on the sample stage at a 38° angle. Samples were affixed by tape to a plastic holder mounted on an xy-stage (Melles-Griot) capable of submicron stepping, further mounted on an xyz stage (ADC) with approximately 0.5 μm resolution as per the typical operating set-up at 20ID [8]. For finer resolution depth-scanning, a high-resolution stage (Newport) was added. Incident beam intensity was monitored using a He-filled transmission ion chamber (incident intensity ~ 9 × 10¹⁰ ph/s at 12 keV). A germanium microchannel optic [5], lithographically fabricated at Cornell University, was mounted in front of a single-element Vortex silicon drift detector and optimized, using similar motor stages, at 1mm working distance from the focal spot of the beam with nominal 2 μm selectivity. The tissue sample was scanned either in horizontal position and depth or in horizontal and vertical positions with the full fluorescence energy spectrum collected at each voxel at an excitation energy of 12 keV. X-ray Absorption Near Edge Spectra (XANES) at the arsenic K-edge were collected at select voxels after mapping. Three to five XANES scans (10 minute duration) were averaged before background removal and normalization to edge jump. While some changes in amplitude were evident, possibly due to some positional drift (not observed optically, but within limits of instrumentation and thermal stability of the experimental set-up and a sample heterogeneous in three dimensions) during or between scans, no change in white line energy was observed from first to last scan, indicating the beam was not changing the arsenic speciation during the XANES measurements.

3. Results and Discussion

To locate the irregular surface, an initial coarse (10 μm step) scan of the horizontal position and depth (xz) was conducted. Upon doing so, the existence of a calcium-rich sub-surface layer, some 10 – 30 μm thick, became apparent, as shown in Figure 2. For *Bufo americanus*, this likely corresponds to the Eberth-Katschenko (EK) layer [9] observed in *Bufo itericus*. The EK layer helps the amphibian...
maintain hydric balance on land, but can also regulate or inhibit transport of other ionic species between the external environment and the interior of the toad. Arsenic is present in the EK layer (Figure 2) as are Mn, Fe, and Zn, with a trace of copper as well. A comparison of x-ray fluorescence spectra confirms these metals are substantially less evident below the EK layer. Note, with the beam incident from lower right and fluorescence detection to lower left in Figure 2, no correction for matrix effects have been applied to these spectra – i.e. at the EK position, the incident beam must pass through more tissue (∼500 µm of 1g/ml low density carbon, or a 6 – 7% additional incident attenuation), while at the sub-EK position, fluorescence must pass through an extra ∼80 µm of tissue, making the Ca signal about 1/3 less than if the escape depths were equivalent. Such a correction here would be small in comparison to the apparent differences. The presence of arsenic on the surface may indicate some residual contamination from the mine site, where the majority species of arsenic is in arsenate form [10].

Figure 2. (colour online) X-ray fluorescence maps for calcium, arsenic and scattered intensity (left) and sample XRF spectra (right) for two regions: one in the Eberth-Katschenko layer (blue) and one below the E-K layer (green) illustrating the change in metal content. Spectra were obtained from binning 12 pixel-spectra in the image as indicated, with 0.4s collection time per pixel.

Figure 3. (colour online) Arsenic cross-section maps and XANES data from select locations showing different species of arsenic present.

A higher-resolution examination in cross-section (horizontal – vertical, xy) was conducted at three different depths of the protruding portion in Figure 2 (not centered on the x-depth section map).
While presenting a different view, such xy mapping does not permit one to follow the path of the beam through the tissue as the xz does. The arsenic maps at these different depths and XANES spectra from select points in them are presented in Figure 3. Spot b, near the surface of the tissue, exhibits two distinct valence (3,5+) white line features. Spot a, from within the EK layer, only exhibits pentavalent arsenic, while spot c, further within the tissue (away from epidermis), is dominated by trivalent species, closer to As^{3+}-O than As^{3+}-S. Both As^{3+}-O and As^{3+}-S were identified in bulk analysis of the dried toad leg [1]. Since spot b is bordering the surface, and though the locale favoured As^{3+}-O species [10], if the 3+ species observed interior is part of the glandular secretion used to coat the skin (which is the function of the glands on the skin examined), then it is possible that some of the trivalent arsenic is due to remnant surface coating, but further tests would be needed to confirm this.

There are two possible routes for arsenic into the body of the toad – ingestion and absorption. The presence of As^{5+} within the EK layer suggests that the absorption route is occurring, however, further within the tissue As^{3+} is observed. Additional control studies would be needed to determine if the EK layer is an effective barrier against arsenic migration further into the toad, or if some reduction occurs on transiting through the EK layer.

4. Conclusions
We have used novel microchannel confocal methods to examine skin tissue from the back of a Bufo americanus (eastern American toad). The Eberth-Katschenko layer within the dermal tissue, in addition to a strong calcium signature, contains arsenic and additional metals, likely obtained from absorption. These metals are less evident in lower tissue. Evidence for reduced arsenic species was found within the skin tissue, but the EK layer was dominated by pentavalent arsenic. Further control studies are needed to identify the EK layer barrier function.

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