Dual detection X-ray fluorescence cryotomography and mapping on the model organism *Daphnia magna*

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(Received 25 March 2010; accepted 27 March 2010)

Micro-X-ray fluorescence (μ-XRF) is a rapidly evolving analytical technique which allows visualising the trace level metal distributions within a specimen in an essentially nondestructive manner. At second generation synchrotron radiation sources, detection limits at the sub-parts per million level can be obtained with micrometer resolution, while at third generation sources the spatial resolution can be better than 100 nm. Consequently, the analysis of metals within biological systems using micro- and nano-X-ray fluorescence imaging is a quickly developing field of research. Since X-ray fluorescence is a scanning technique, the elemental distribution within the sample should not change during analysis. Biological samples pose challenges in this context due to their high water content. A dehydration procedure is commonly used for sample preparation enabling an analysis of the sample under ambient temperature conditions. Unfortunately, a potential change in elemental redistribution during the sample preparation is difficult to verify experimentally and therefore cannot be excluded completely. Creating a cryogenic sample environment allowing an analysis of the sample under cryogenic condition is an attractive alternative but not available on a routine basis. In this article, we make a comparison between the elemental distributions obtained by micro-SR-XRF within a chemically fixed and a cryogenically frozen *Daphnia magna*, a model organism to study the environmental impact of metals. In what follows, we explore the potential of a dual detector setup for investigating a full ecotoxicological experiment. Next to conventional 2D analysis, dual detector X-ray fluorescence cryotomography is illustrated and the potential of its coupling with laboratory absorption micro-CT for investigating the tissue-specific elemental distributions within this model organism is highlighted. [DOI: 10.1154/1.3397114]

Key words: micro-XRF, cryostream, ecotoxicology, sample preparation

I. INTRODUCTION

The freshwater crustacean *Daphnia magna* is a frequently used model organism to investigate the mechanisms of the toxicity of metals, e.g., Cu, Zn, Ni, and Co (Heijerick *et al.*, 2005). Historically, it has often been difficult to link the bioaccumulation of metals to toxic effects in daphnids because the total organism digestion cannot distinguish the accumulation in critical tissues from whole body accumulation. Also, the small size of these organisms (~3 mm) does not allow a straightforward determination of the metal contents of tissues of interest by conventional techniques such as dissection, acid digestion, and subsequent atomic spectrometry (Balcaen *et al.*, 2008). In previous studies, we compared the potential of micro-XRF and LA-ICPMS to "virtually dissect" *Daphnia magna* and study the tissue-specific Zn accumulation nondestructively (Gholap *et al.*, 2010). The use of combined X-ray techniques including 2D-micro-XRF, XRF micro-CT, confocal micro-XRF, and absorption microtomography under conventional sample environments was also demonstrated (De Samber *et al.*, 2008; De Samber *et al.*, 2010).

However, when biological samples with high water content are analyzed under conventional sample environments, the usual approach of sample preparation is based on sample dehydration using chemicals and subsequent embedding using paraffin or resin, or based on drying procedures such as freeze-drying or critical point drying. With respect to a spatially resolved investigation of the chemical composition, the drawback of these methods is the risk of altering the chemical composition due to dislocation and/or contamination. In contrast, an X-ray analysis of frozen water-rich samples is an interesting alternative which should not impose major modifications on the metal distributions (Kanngießer *et al.*, 2007). In our previous micro-XRF studies on *Daphnia magna*, a HMDS drying procedure for SEM investigations was adapted (Laforsch and Tollrian, 2000). Unlike critical point drying, which causes major shrinkage of the carapace in the region of the neck and the brood pouch, this procedure features an excellent morphological preservation. In order to study the degree of alteration of chemical composition, pos-

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sible changes between the elemental distributions within a frozen hydrated and a dehydrated chemically fixed *Daphnia magna* were used, which required the implementation of a cryostream in the experimental setup.

The use of a cryostream in protein crystallography in which the sample is shock frozen and maintained at a temperature of about 80 K during the measurement is very common at synchrotron sources. Surprisingly, even though such cryostream equipment is mobile, it has rarely been coupled with beamlines aiming at micro-XRF analysis on biological samples. Since frozen hydrated samples show higher absorption of low-energy fluorescent lines (e.g., K, Ca, and Fe), the potential of a dual detector setup was explored to provide reliable elemental maps for an ecotoxicological experiment with different exposure levels. The dual detector setup also allows the accelerated measurement of XRF cryotomography cross sections. Thus, a next aim was to verify the feasibility of dual detector XRF cryotomography and its correlation with laboratory absorption micro-CT.

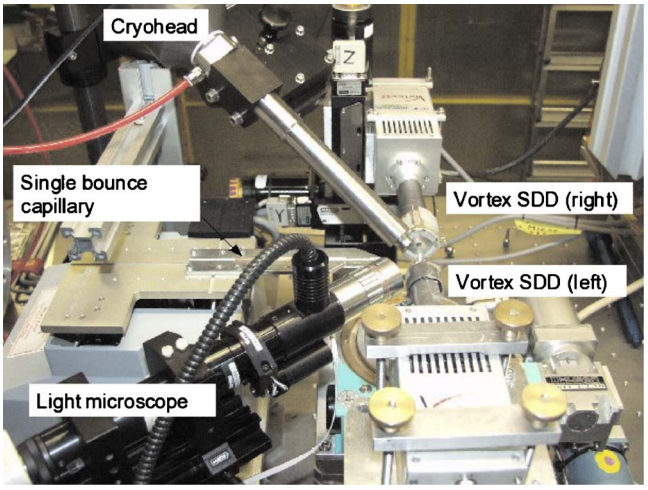

Figure 1. (Color online) Overview of the experimental setup.

II. EXPERIMENTAL

The synchrotron radiation micro-XRF experiments were performed at Beamline L of the DORIS-III storage ring, HASYLAB (Hamburg, Germany). This beamline is dedicated to micro-XRF experiments using either white or monochromatic bending magnet excitation and monocapillary or polycapillary focusing, with routinely available beam sizes of 10 to 30 μm. The primary X-ray beam is generated by a 1.2 T bending magnet source, which, given the machine energy of 4.465 GeV, provides a polychromatic spectral distribution with a critical energy of 16.6 keV. After initial low-energy filtering and collimation, the beam was monochromatized by a W/Ni multilayer monochromator within the 15 to 20 keV energy range to excite the metals of biological relevance (Falkenberg *et al.*, 2001). The SR-XRF spectra are measured under a 90° detection angle relative to the incident beam using two Radiant Vortex-EX silicon drift detectors (SDD) having an active area of 50 mm^2 each and a nominal crystal thickness of 350 μm. These energy-dispersive detectors were coupled with an XIA pulse processor for recording the single XRF point spectra. Silver detector collimators of sufficient solid angle were used in order to detect the fluorescent radiation emerging from the full beam path within the sample. Both detectors were covered by a thin ultralene foil to protect them from ice deposition originating from the cryostream nozzle. Both detectors were gain optimized and their sample distance was adjusted to provide an essentially identical elemental spectrum using the SRM 1577B (bovine liver) from NIST.

Figure 1 shows the experimental setup. The 700 series cryostream cooler from Oxford Cryosystems draws up liquid nitrogen by the action of a small pump from an unpressurised Dewar, through a flexible vacuum insulated transfer line, into a coldhead. Once inside the coldhead, the liquid nitrogen passes through a heater, which evaporates most of the liquid into vapour. This vapour then flows outward along one path of the heat exchanger. The flow rate of the gas from the pump is then regulated by a variable flow controller. This gas flows back into the cryostream coldhead where it is recooled along the second path of the heat exchanger. The gas temperature is then regulated before entering the nozzle of the cryostream. The gas then flows along the isothermal nozzle and out over the sample. A laminar gas flow of 5 L/min insures a temperature of 100 K at an obligatory 8 mm distance from the cryostream nozzle. Since icing can occur on objects around the path of the laminar flow, a long working distance (5 cm) single bounce capillary, designed and manufactured at Cornell University, was used for focusing the monochromatic hard X-rays (Falkenberg *et al.*, 2003). The low focused X-ray beam divergence produced by the single bounce capillary (<4 mrad) also provides sharper images on thicker samples in comparison with polycapillary optics. The beam size at 20.6 keV incident energy was determined to be approximately 15 μm full width at half maximum (FWHM). Additionally, a Kapton foil was inserted between the collimator head and the beryllium window of the detector to prevent damage from the cryostream.

Sum spectra were used to identify the elements which needed to be included in the fitting model of the spectrum evaluation software AXIL (Vekemans *et al.*, 1994). The software package MICROXRF2 was used to generate the elemental maps based on a recursive fitting procedure of the individual point spectra (Vekemans *et al.*, 1997). All elemental maps were normalised to a live time (LT) of 1 s and a DORIS-III current of 100 mA and were corrected for detector dead time in order to be able to compare the elemental distributions between the cryogenic and chemically fixed sample.

For comparison between chemical fixation based sample preparation techniques and cryogenic analysis, adult living *Daphnia magna* were harvested from the culture medium (Muyssen *et al.*, 2006), rinsed with deionised water, and glued to the tip of a glass capillary. This was subsequently attached to a goniometer head and immediately inserted into the cryostream, which induced a fast freezing of the sample. In order to make comparison possible between chemical fixation and cryofixation, another *Daphnia magna* was harvested immediately after the first sample was inserted into the cryostream. Following the HMDS (1,1,1,3,3,3-hexamethyldisilazane) drying procedure, the sample was dehydrated in graded acetone solutions and immersed in HMDS (Laforsch and Tollrian, 2000). After 30 min, the sample vials were immediately transferred to a desiccator.

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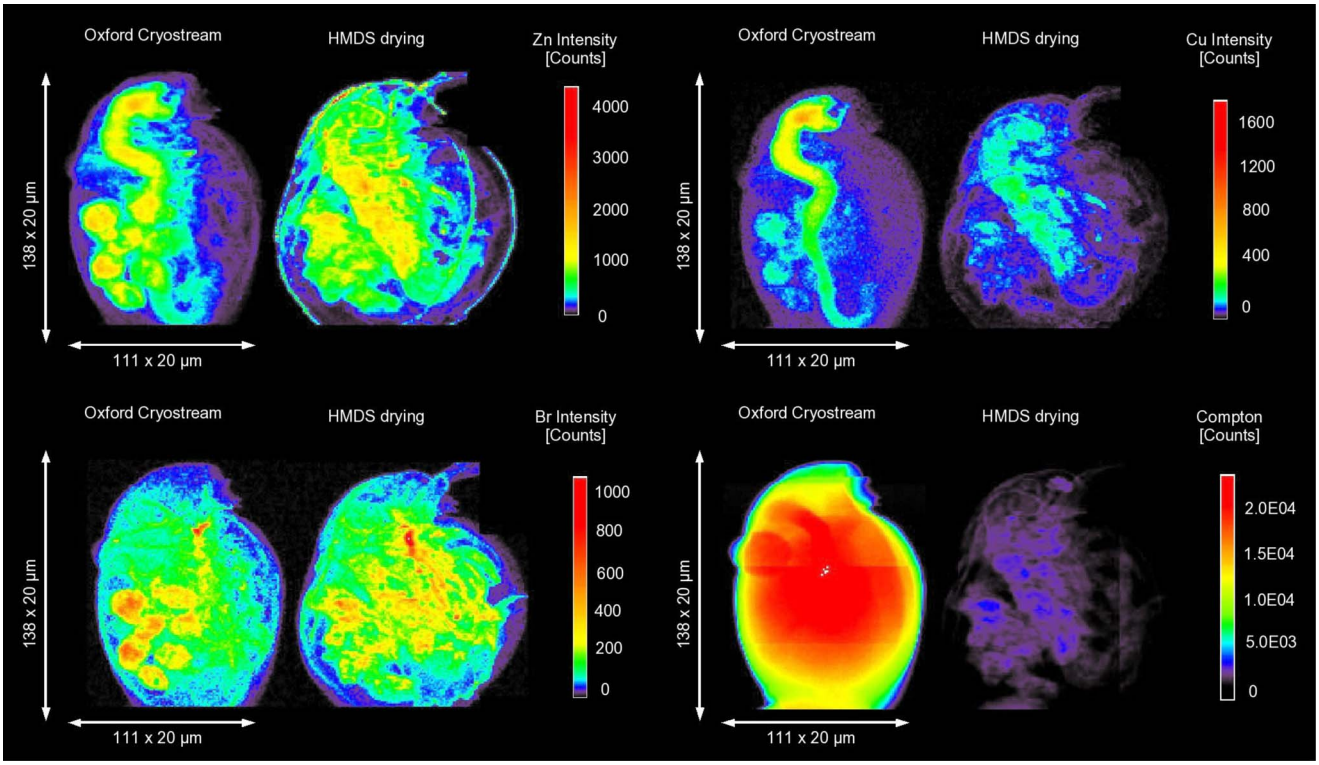

Figure 2. (Color online) Comparison between a chemically fixed and cryogenically frozen *Daphnia magna*.

The bottom of the desiccator was covered by silica gel beads and the desiccator itself was evacuated to avoid water contamination which would cause shrinkage in the specimens. The remaining HMDS was allowed to evaporate overnight under anhydrous conditions during the cryomapping of the first sample. After drying, the sample was inspected using an optical (reflection) microscope and then glued onto another glass capillary for analysis. After the cryomapping was finished, the cryostream could easily be turned off keeping the analytical characteristics of the setup unchanged, and the dried sample could be mounted onto the rotation/scanning stage of the synchrotron radiation micro-XRF setup. For our ecotoxicological experiment, two different metal tolerant genotypes were harvested from locations featuring a higher and lower level of metal pollution. Ecotoxicity tests proved that genotype O22 (high metal pollution site) revealed a higher metal sensitivity than genotype M27 (low metal pollution site). Both types were put in a control medium and in a medium with an elevated Zn concentration of 200 μg/L.

III. SAMPLE PREPARATION COMPARISON

After insertion of *Daphnia magna* in the cryostream, the sample freezes immediately. Subsequently, a 2D micro-XRF "cryomapping" was started with a 20 μm step size and 1.5 s dwell time/point resulting in a total scanning time of approximately 6 h. A so-called dynamic scanning mode was used, in which the sample is moved continuously across the beam (instead of the stepwise motion corresponding to the conventional mode), with periodic readout of the XRF spectra from the data acquisition system. The multichannel analyser (MCA) integrates the spectra within a predefined time interval (sampling time) while the sample is being dynamically scanned. This provides a fast scanning capability in the subsecond per pixel regime with minimum time loss due to overhead (Falkenberg *et al.*, 2005). During this time, no icing on the sample due to air moisture was observed and vibrations of the sample due to the cryostream were not detectable on the scale comparable to our resolution level of ~20 μm. Previous research has been performed with X-ray fluorescence under cryogenic conditions by Kanngießer *et al.* (2007). Unlike in their experiment, the goniometer head which was carrying the sample did not require heating to prevent the formation of ice. Also, the gas flow rate was 5 L/min, so by using a 60 L Dewar cryogenic X-ray fluorescence scans could be performed over a continuous time frame of several days on the very same or different biological specimens.

Figure 2 shows a comparison between elemental distributions of Zn, Cu, and Br of a cryofrozen (left) and a chemically fixed (right) *Daphnia magna*. A color bar shows the net peak intensities in counts. It can immediately be observed that deformations of the tissue structure in the chemically fixed sample are present such as more opened exoskeleton and less clear resolved tissue structures. However, the Zn concentration is very similar for both samples, with the exception of the upper gut and exoskeleton, which confirms the validity of our previous work in which the Zn distribution in differently exposed *Daphnia magna* was investigated using 2D, CT, and confocal micro-XRF (De Samber *et al.*, 2008; De Samber *et al.*, 2010). Cu, however, seems to show a more significant leaching from the gut. The Br distribution features very similar count rates in the regions of the antennae and eggs, although a higher intensity can be noted for the gill tissue of the dried sample. A higher Compton scattering in the frozen sample can be observed due to the fact that the

https://doi.org/10.1154/1.3397114 Published online by Cambridge University Press

cryofrozen sample contains mainly water, having a higher density in comparison to the dried sample. An interesting finding is that the Compton map corresponding to the cryogenic experiment shows a very uniform distribution suggesting that the metals are distributed in a mainly water containing matrix with a very uniform density. In the Compton map of the dried sample, tissues become identifiable and are also revealing a very similar density, whereas areas within the sample which were originally mainly liquid are replaced by air resulting in their low Compton scattering. Metals present in this liquid phase could indeed be relocalized and/or removed during the fixation procedure.

IV. DUAL DETECTOR XRF CRYOMAPPING

Figure 3 shows the results when performing dual detection scanning micro-XRF under cryogenic conditions. The left column shows the elemental maps built from the spectra collected from the SDD detector looking at the left of the sample with respect the optical beam path, while the middle column shows the elemental maps built from the spectra collected from the SDD detector looking at the right of the sample. The elemental maps are composed of 158 $\times 130$ pixels featuring a step size of 20 μm and a real measuring time of 0.6 s. Using a dynamic scanning mode in which the sample is continuously moving across the beam results in a decreased scanning time. It can clearly be observed that the Ca-$K\alpha$ fluorescence is unable to escape from the entire sample towards a single detector. Even the Fe-$K\alpha$ fluorescence is mildly absorbed, e.g., in the region of the eggs for the left detector and in the region of the gut for the right detector. For elements such as Cu, Zn, Rb, and Sr no significant self-absorption could be observed. However, these elements benefit from a dual detector setup since twice as much fluorescence signal is collected, which allows a reduction in the scanning time per sample. The elemental distribution originating from the left and right SDD detectors can be summed to provide an elemental map for metals with low fluorescent energies (e.g., K and Ca), which is to a high extent corrected from self-absorption effects within the sample.

Figure 4 shows the elemental distribution of Zn from several *Daphnia magna* samples corresponding to different genotypes and different exposure levels. All individual element maps were scaled with respect to the same scale bar indicating the Zn intensity in counts/s. A histogram of the data set was used to remove hot spots from the element maps which prevented an adequate scaling and comparison of the samples. Per exposure condition and genotype, two replicates were measured (replicate A and replicate B). The variation in Zn distributions between control and exposed samples is higher than the variation between replicates within the same exposure condition. A higher Zn concentration can be observed in the region of the gut, eggs, gill tissue, and digestive gland of the Zn exposed *Daphnia* compared to those from the control medium. Interestingly, the less Zn tolerant clone "O22" showed a higher Zn accumulation in these tissues when exposed to 250 μg/L of Zn as compared to the more Zn tolerant clone "M22." This is a first indication that genetically determined differences in Zn tolerance may be related to differences in Zn accumulation (e.g., through higher Zn uptake rates or lower Zn elimination rates in less Zn tolerant genotypes).

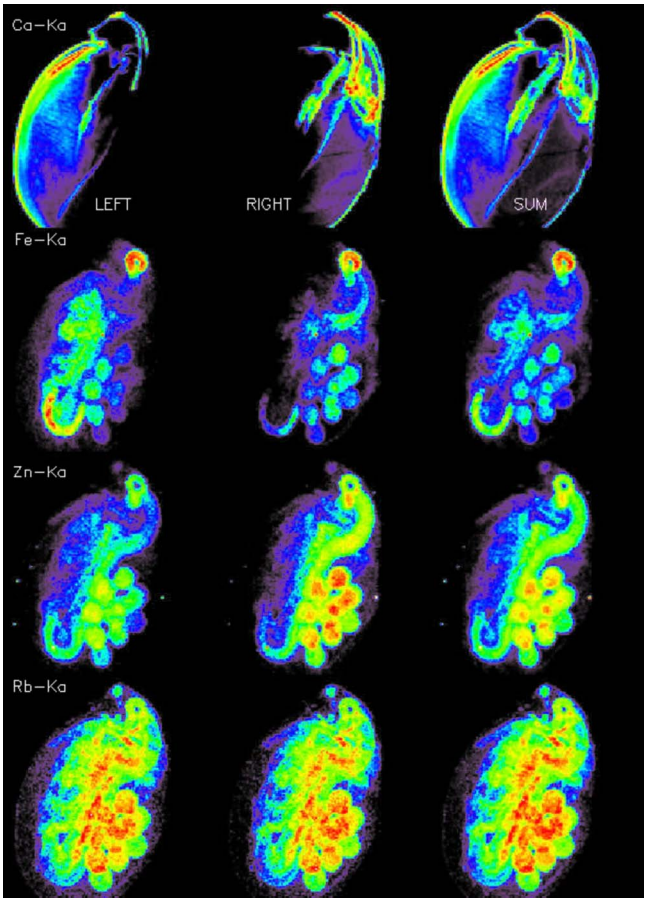

Figure 3. (Color online) Separate and summed elemental distributions within a cryogenic frozen *Daphnia magna* as collected by both silicon drift detectors. For higher energies, less self-absorption can be observed.

V. DUAL DETECTOR XRF CRYOTOMOGRAPHY AND ABSORPTION MICRO-CT

Figure 5 illustrates the elemental sinograms obtained when performing dual detector cryotomography on *Daphnia magna.* When only a single detector is available, the rotation of the sample is necessary over the full 360°. However, for the dual arrangement, a rotation scan of 0° to 180° is sufficient. A 120 steps line scan of 20 μm was repeated by 180 times with a rotation step size of 1°, decreasing the measurement time by a factor of 2 compared with the 360° full rotation scan. The first row shows the sinogram obtained from the left detector, whereas the second row shows the sinogram obtained from the right detector. Similar to the 2D cryomappings performed on *Daphnia magna,* Ca-$K\alpha$ fluorescence cannot reach the detector when the line scan is performed further than halfway of the sample with respect to the considered detector. However, both elemental sinograms can be summed, thus, providing an elemental sinogram which is to a large extent corrected for self-absorption effects, as can be seen in the last row of the figure. An RGB representation is very useful to reveal the colocalisation of elements of interest such as Ca, Cu, and Rb within a single glance. After applying a conventional backprojection algorithm to the el-

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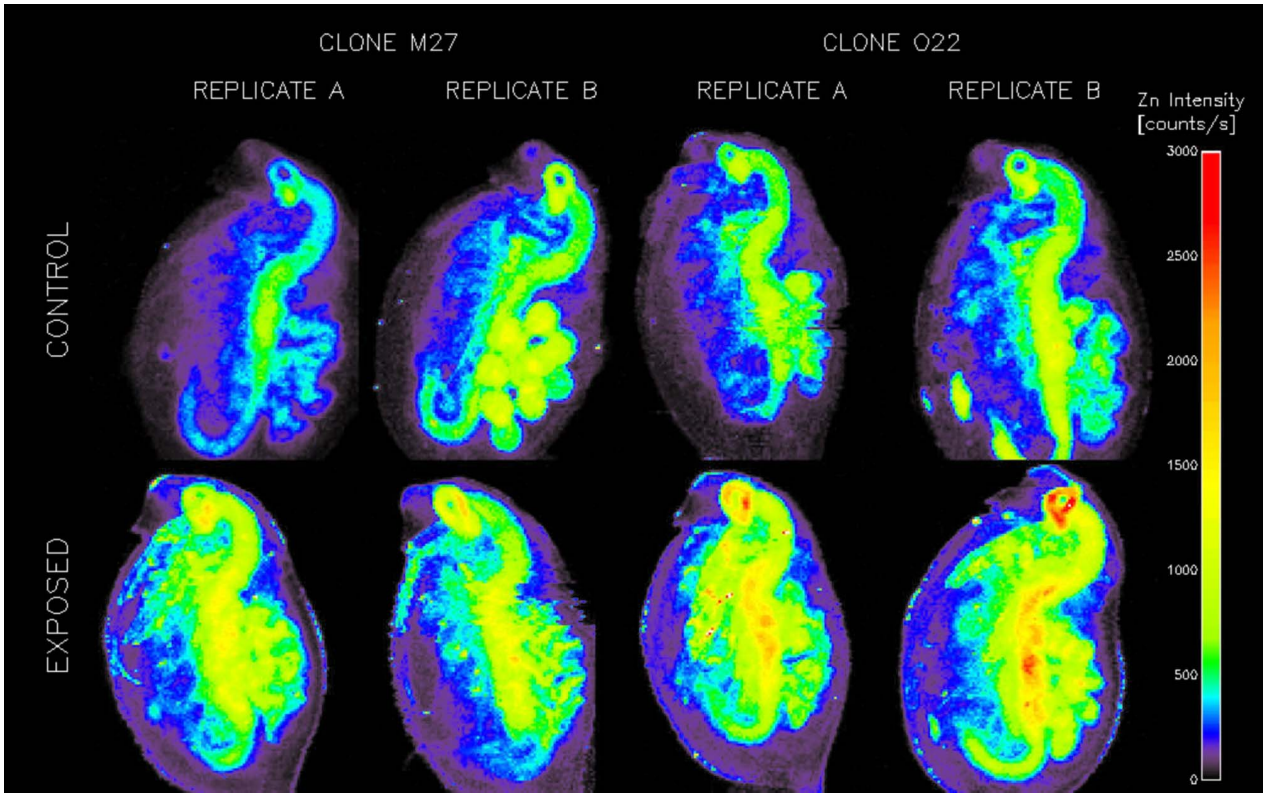

Figure 4. (Color online) Elemental distribution of different metal sensitive *Daphnia magna* genotypes (clones M27 and O22) under different exposure conditions (control medium and 200 μg/L Zn) obtained under cryogenic conditions.

emental sinograms, the elemental distributions within a virtual cross section through *Daphnia magna* could be reconstructed as shown in Figure 6. When the sinograms originating from the single detectors are reconstructed, only half of the calcium containing exoskeleton can be reconstructed. However, the summed sinograms provide a full reconstruction for the calcium distribution within the sample. Also here, some absorption effects can be observed for the virtual cross section of Cu and Zn which are disappearing when using the information originating from both detectors.

An interesting development is the combination of elemental information obtained by micro-XRF tomography with the morphology of the sample, which can be obtained by means of laboratory absorption micro-CT. Figure 7 shows the result of such a "cross-fertilization" of both techniques. Here, it can very clearly be illustrated that the exoskeleton contains Ca, the gut region contains Zn, and Fe hot spots coincide with the gill tissue. The laboratory microabsorption CT thus provides an accurate frame of reference, which allows to virtually dissect the tissue-specific metal distributions within the biological sample.

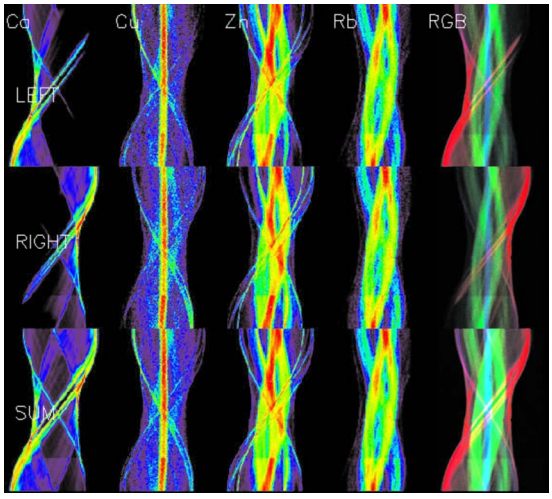

Figure 5. (Color online) Separate and summed elemental sinograms originating from the individual and both detectors of Ca, Cu, Zn, and Rb from a cryogenic frozen *Daphnia magna*. An RGB representation reveals the combined presence of Ca, Cu, and Rb, respectively.

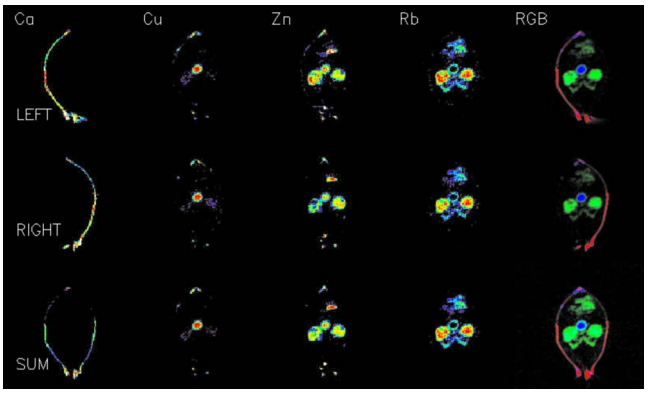

Figure 6. (Color online) Reconstructed cross section of the separate and summed elemental sinograms from both detectors. An RGB representation shows the complementary presence of Ca, Cu, and Rb, respectively.

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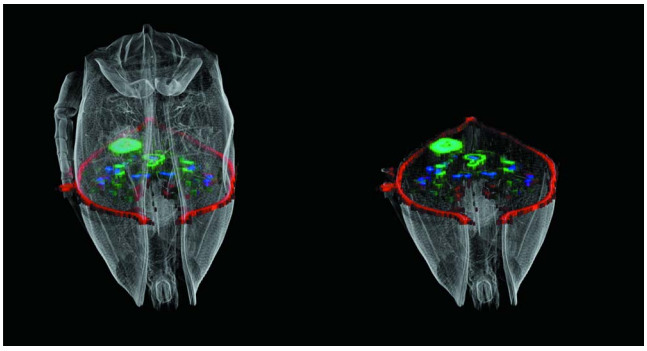

Figure 7. (Color online) RGB representation of the metal distribution (Ca, Zn, and Fe) obtained by micro-SR-XRF combined with laboratory based absorption micro-CT.

VI. CONCLUSION

The analysis of metals within biological model organisms using synchrotron radiation micro-X-ray fluorescence imaging is a quickly developing field of research. Biological samples pose challenges in this context due to their high water content requiring dehydration procedures which may alter their chemical composition. A cryogenic sample environment allowing the analysis of the sample under frozen conditions is put forward as an alternative but rarely implemented on a routine basis. In this article we report the use of a cryostream instrument at a microfocus beamline enabling micro-XRF cryomapping and cryotomography in a dual detector arrangement on biological specimens. No vibration of the sample on the microscale was observed and due to the absence of ice deposition no heating of the sample carrier was required. A comparison between a chemically fixed sample and a sample analyzed under cryogenic conditions can provide valuable information regarding the possible metal relocalisation. The dual detector arrangement can be used to correct for self-absorption effects and for providing fast scanning results. The technique is thus ideally suited to analyze the metal distributions of biological samples in their frozen, native state. The combination with laboratory absorption micro-CT, moreover, allows extending the interpretation of metal distribution towards the tissue-specific level.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Fund for Scientific Research-Flanders (FWO-Vlaanderen), Ghent University "Special Research fund" (BOF), Ghent University "Concerted Research Action" (GOA) fund, and the Institute for Science and Technology (IWT) for financial support.

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