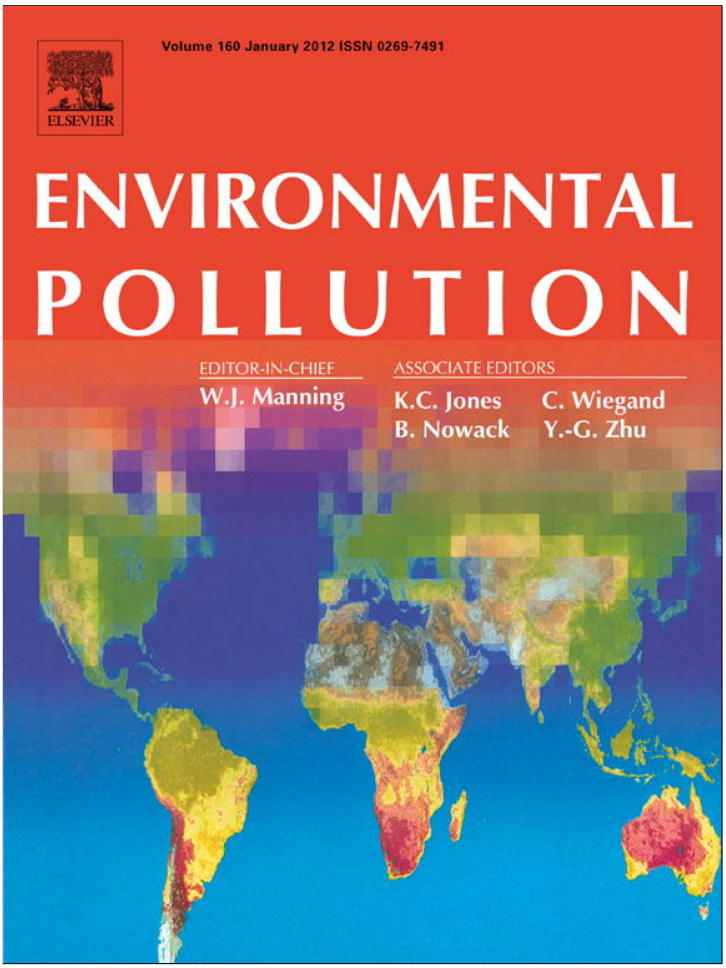

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Micro x-ray absorption spectroscopic analysis of arsenic localization and biotransformation in *Chironomus riparius* Meigen (Diptera: Chironomidae) and *Culex tarsalis* Coquillett (Culicidae)☆

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ABSTRACT

The distribution and speciation of arsenic (As) were analyzed in individuals of various life stages of a midge, *Chironomus riparius*, and the mosquito *Culex tarsalis* exposed to 1000 μg/l arsenate. X-ray absorption spectroscopy (XAS) revealed that *C. riparius* larvae accumulate As in their midgut, with inorganic arsenate [As(V)] being the predominant form, followed by arsenite [As(III)] and an As-thiol. Reduced concentrations of As in pupal and adult stages of *C. riparius* indicate excretion of As between the larval and pupal stages. In adults, As was limited to the thorax, and the predominant form was an As-thiol. In *Cx. tarsalis*, As was not found in high enough concentrations to determine As speciation, but the element was distributed throughout the larva. In adults, As was concentrated in the thorax and eyes of adults. These results have implications for understanding the biotransformation of As and its movement from aquatic to terrestrial environments.

1. Introduction

X-ray spectroscopy is a useful method for toxicology studies with insects because of the non-destructive manner in which samples can be analyzed (Parsons et al., 2002). Micro x-ray fluorescence (μXRF) allows visualization of the spatial distributions of elements within target organs of the insect with micron resolution. X-ray absorption spectroscopy (XAS) may be further utilized to determine speciation and oxidative states of a target element through x-ray absorption near-edge structure (XANES) analysis. This technique is particularly amenable to insect systems given the small size of insects, which allows for the entire animal to be scanned and compartmentalization of a target element to be determined. While XAS has been used in the past to evaluate metal and metalloid accumulation and speciation within insects (e.g. Andrahennadi and Pickering, 2008; Moriarty et al., 2009), it has not been applied to aquatic insects or other invertebrates whose lives are spent in direct contact with arsenic contaminated substrates.

Within biological tissues, the arsenic species most often encountered in XAS analysis are (in order of lowest to highest white line energies): arsenic glutathione [As(Glu)$_3$], monomethylarsenic DMPS (MeAsDMPS), dimethylarsenic 2,3-dimercapto-1-propane sulfonic acid sodium salt (Me_2AsDMPS), monomethylarsonous acid [MMA(III)], arsenite [As(III)], arsenobetaine (AB), arsenobetaine 2 (C2-AB), arsenobetaine 3 (C3-AB), arsenocholine (AC), tetramethylarsonium iodide (Tetra), trimethylarsine oxide (TMAO), (*R*)-2,3-dihydroxypropyl-5-deoxy-5-dimethylarsinyl-β-D-riboside sugar, dimethylarsinic acid [DMA(V)], monomethylarsonic acid [MMA(V)], and arsenate [As(V)] (Smith et al., 2005). Distinguishing between arsenic species is crucial in environmental analyses due to the different modes of toxic action between inorganic forms, such as arsenate [As(V)] and arsenite [As(III)], and organic forms. Inorganic arsenate, the thermodynamically favored form in freshwater systems (Rahman and Hasegawa, 2012), is a chemical analog for phosphate and is taken into cells via phosphate transporters (Zangi and Filella, 2012). Thus, arsenate disrupts glycolysis by replacing phosphate in biochemical reactions, altering the structures of molecular intermediates and disrupting ATP synthesis (Hughes, 2002). Arsenite interacts with the sulfhydryl bonds of proteins, disrupting tertiary structures and enzymatic function as a result (Hughes, 2002). Arsenic may also exist in many organic forms in the environment (Reimer et al., 2010), which may result from microbial activity in aquatic environments (Lloyd and Oremland, 2006).

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Methylated forms are shown to be genotoxic to *Drosophila melanogaster* (Diptera: Drosophilidae), though *D. melanogaster* is not capable of methylating arsenic (Rizki et al., 2006).

Invertebrates posses a variety of methods by which they are generally able to eliminate toxic compounds from their cells, including 1) regulatory mechanisms that balance rates of metal uptake from the environment with excretion rates, 2) intracellular sequestration using metallothioneins and subsequent elimination through the lysosomal endomembrane system, and 3) intracellular sequestration processes involving vacuoles that produce solid metallic phosphorous or sulfur granules that then undergo exocytosis for elimination (Ahearn et al., 2004). Extra cellular sequestration via lipid particles containing iron has also been proposed (Rahman et al., 2009), as well as molting as a means for depuration (Bergey and Weis, 2007). We are not aware of any invertebrates that have been shown to possess all of these mechanisms. There is some evidence for the formation of spherocrystals to regulate excess arsenic ions in *Formica polyctena* (Hymenoptera: Formicidae) (Jeantet et al., 1977). More recently, arsenic susceptibility has been shown to be mediated by the presence of glutathione in insects (Muñiz-Ortiz et al., 2007). Reduction of arsenate and subsequent coordination with sulfur [As(III)-S] has been found in terrestrial insects and other invertebrates (Langdon et al., 2002, 2005; Andrahennadi and Pickering, 2008; Moriarty et al., 2009). There is the possibility that thiols play an important role in mediating this reduction (Thomas, 2010). However, the current body of knowledge regarding potential mechanisms of arsenic reduction and excretion in insects and terrestrial invertebrates is limited to these examples. How invertebrates that spend the majority of their lives immersed in a toxicant enriched environment, particularly aquatics and soil dwelling insects, is of particular interest from a toxicological standpoint given the worldwide nature of arsenic contamination (Ravenscroft et al., 2009) and its status as a priority toxic pollutant (US EPA, 2006). Further, past research has shown that the larvae of aquatic Diptera are able to withstand chronic exposure to relatively high concentrations of arsenic (Mogren et al., 2012; Mogren, unpublished data), though the mechanisms by which they are able to do so are unknown.

In this study, we investigated potential modes of arsenic transformation and excretion in *Chironomus riparius* Meigen (Diptera: Chironomidae) and *Culex tarsalis* Coquillett (Diptera: Culicidae), whose larvae are aquatic and ubiquitous in North America. While *C. riparius* is a benthic detritivore, *Cx. tarsalis* is a filter feeder at the water surface, and both species serve as important food sources for higher trophic levels (Merritt et al., 2008). Because *C. riparius* larvae reside in lake benthos, they often come into contact with contaminants in soil and water, including arsenic (Croisetière et al., 2006). *Culex tarsalis* larvae are found in surface water pools and tolerate a wide range of conditions, including organic enrichment and exposure to industrial effluent (Reisen, 1993). In order to understand the organs responsible for biotransformation and absorption of arsenic in these insects, we used XAS imaging with micro x-ray fluorescence imaging (μXRF) to determine the microscopic distribution of arsenic within intact specimens, in addition to speciation analysis of the same specimens with XANES analysis.

2. Methods

2.1. Insect rearing and sample preparation

2.1.1. Chironomus riparius

Individuals of *C. riparius* were reared following the protocol established in Mogren et al. (2012). Briefly, egg masses were purchased from Environmental Consulting and Testing, Inc. (Superior, WI, USA). A thin layer of pre-rinsed quartz sand (Repti Sand, Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA) was provided as a burrowing substrate for the larvae in 600 ml beakers containing 300 ml of reconstituted water and either 0 or 1000 μg As/l as arsenate in the form of sodium hydrogenarsenate heptahydrate 99.99% (Sigma–Aldrich, St. Louis, MO, USA). The arsenic treatment concentration was chosen because it represents a high yet still ecologically relevant concentration of arsenic that would be found in a contaminated water body (Smedley and Kinniburgh, 2002; Ravenscroft et al., 2009), and to increase the likelihood of detecting reduced or transformed species of arsenic in the samples. According to Ravenscroft et al. (2009) over 45 regions around the world have As levels occurring naturally in surface and ground waters that reach or exceed 1000 μg/l (with many regions exceeding 1500 μg/l).

Ten first instars were transferred to each beaker. Water loss through evaporation was accounted for daily through the addition of Milli-Q HPLC-grade water to maintain the 300 ml volume and one third of the water was replaced daily starting on day five to minimize injury to early instars. Larvae were fed a slurry of TetraMin® Tropical Fish Flakes (United Pet Group, Inc., Cincinnati, OH, USA), made by adding 1 g of flakes to 10 ml of deionized water. Beakers were aerated constantly in an environmental rearing chamber at 23 °C under a 16 L:8 D cycle.

As the larvae grew, individuals were sacrificed at the second instar, fourth instar, pupal, and adult stages (females only) for XAS analysis. Egg masses were also collected from female adults that were allowed to oviposit. Samples were rinsed in deionized water and then frozen at −60 °C prior to being freeze dried.

2.1.2. Culex tarsalis

Egg rafts of *Culex tarsalis* were obtained from colonies maintained at the University of California, Riverside. The use and care of animal hosts was done under Protocol A2010006 approved by the Institutional Animal Care and Use Committee of the University of California, Riverside. Egg rafts were hatched in white enamel pans containing 3 L of tap water (after Van Den Heuvel, 1962) and either 0 or 1000 μg As/l. Pans were maintained in an environmental rearing chamber at 28 °C and 16 L:8 D light cycle. Larvae were fed a ground mouse chow (mouse/rat diet, Harlan/Teklad, Madison, WI, USA) and brewer's yeast (MP Biochemicals, Aurora, OH, USA) 3:1 (wt:wt) mixture as a 10% suspension in deionized water. Three fourth instar larvae and adults were rinsed in deionized water and sacrificed by freezing at −60 °C prior to being freeze dried.

2.2. μXRF mapping and μXANES

Detailed explanations of the mechanisms of XAS may be found in Parsons et al. (2002, 2009) and Smith et al. (2005). Synchrotron-based hard x-ray microprobe measurements of element distributions were conducted at Beamline 2–3 at the Stanford Synchrotron Radiation Lightsource (SSRL), using procedures described by Mayhew et al. (2011).

Experiments were conducted with the SPEAR accelerator ring containing ~350 mA in constant top-off mode. A Si (111) double crystal monochromator fully tuned at 12 keV was used to select the incident energy. Harmonic rejection was accomplished via the micro-focusing mirrors, with an energy cutoff of ~22 keV. The spot size was focused to 2.5 × 2.5 μm using Pt-coated Kirkpatrick–Baez mirrors (Xradia, Inc.). The sample was rastered across the micro-focused x-ray beam at a 45° incident angle, using a pixel step size of 2.5 μm and a dwell time of 100 ms per pixel. A continuous raster scanning mode using single-element Si drift Vortex detector (SII NanoTechnology USA Inc.) was used to generate element maps of As, Ca, Cl, Cu, Fe, K, Mn, P, S, and Zn. Windowed counts for each element extracted from the full x-ray fluorescence spectrum were normalized to the intensity of the incident x-ray beam (I_0). Regions of the sample area of particular interest for arsenic speciation mapping at the arsenic K-edge were identified from an initial 12 keV map. Arsenic K-edge speciation mapping was conducted at 4 discrete energies (11,870, 11,873, 11,875, and 11,880 eV) to determine changes in the arsenic oxidation state as a function of location on the sample. These energies were chosen as they are at energies of unique intensities for arsenic species likely to be encountered in insects ($As(Glu)_3$, As[III], As[V], and a total arsenic energy, respectively) (Andrahennadi and Pickering, 2008).

Multiple energy maps were achieved by raster mapping a single line at each incident monochromator energy and repeating this process at each successive line. The maps were dead time corrected and underwent principal component analysis (PCA) using the MicroAnalysis Toolkit (Webb, 2011) prior to collection of μXANES data. Maps of unique components were used to guide the selection of spots for μXANES investigation, ensuring that the μXANES collected would represent the variety of arsenic phases present in each of the samples. Each μXANES spectrum was collected from approximately 240 eV below the arsenic K-edge to 700 eV above the edge. All μXANES data were dead time corrected, background subtracted, and normalized to unit step edge using standard methods available in the SIXPACK software package (Webb, 2005). The monochromator was calibrated using the first inflection of an arsenate sodium salt as the reference material at 11,880 eV. The short dwell times for μXRF mapping (50–100 μs) and μXANES analyses (8 min) minimized potential radiation damage to the samples.

2.3. Data analysis

Arsenic speciation was determined by non-negative linear least squares fitting of the data. With XANES data, this was performed in the fitting section of SIXPACK,

using model compounds for arsenate, arsenite, and As^{III}-*tris*-glutathione. For XRF image data, the normalized intensities of each of the model compounds was determined at each of the image map energy points (11,870, 11,873, 11,875, and 11,880 eV). A non-negative linear least squares fitting was performed at each image pixel with each of these four energies to the model compounds intensities, giving the overall speciation at each pixel of the image map. Single point XANES spectra were measured to confirm the speciation as determined by the image map analysis. Results typically agreed within the nominal margin of error (±5%).

3. Results

3.1. Chironomus riparius

Arsenic was present in all scans of the different life stages of *C. riparius* in the x-ray fluorescence images (Fig. 1). Although larvae were only exposed to arsenate, which is stable in solution (Al-Sibaai and Fogg, 1973; Liu et al., 2006), x-ray absorption across the arsenic K-edge revealed three species of arsenic in the samples: arsenate, arsenite, and an As-thiol, likely As^{III}-*tris*-glutathione, based on the energy absorbance (Andrahennadi and Pickering, 2008) (Fig. 2). In second and fourth instar larvae, all three forms of arsenic were concentrated in the midgut, with none present in the fore- or hindgut. In the fourth instar larva (Fig. 1a), XANES analysis revealed that 43.4% of whole body arsenic was present as arsenate, 29.2% was present as arsenite, and 27.4% was present as the As-thiol. There is some evidence as well for arsenate and As-thiol assimilation in the anterior region of the body, possibly in the cuticle. In addition to arsenic in the fourth instar larva, Fe, Mn, and Cu were also associated with the alimentary canal of the insect. Sulfur, P, Cl, K, and Zn were found to be constituents of the cuticle. Calcium and Mn were found in the Malpighian tubules (MT). The element pattern distribution was the same for second and fourth instar larvae, but the signal was stronger in fourth instar larvae.

In the pupa, there was less arsenic relative to what was found in the larva, indicating much of the larval body burdens are excreted during the molt to the pupal stage (Fig. 1b). The arsenic appeared in clusters throughout the thorax and abdomen. XANES analysis revealed that 55.1% of whole body arsenic was present as arsenate, 26.2% was present as the As-thiol, and 18.7% was present as arsenite. Arsenic, Fe, S, P, Cl, K, Ca, Mn, and Zn are associated with the cuticle of the pupa. Iron, S, P, Cl, K, along with Cu, are also associated with the head and thoracic region of the pupa, with Fe being especially concentrated in the region developing into the head.

Within the adult female, the majority of arsenic was concentrated in the thorax, likely in the exoskeleton or muscles (Fig. 1c). In contrast to the 4th instar larva, the majority composition of arsenic shifts to As-thiol, constituting 53.3% of the total arsenic, with 23.7% as arsenate and 23.0% as arsenite. Copper was again associated with the alimentary canal. Iron, S, P, Cl, K, Mn, and Zn were also associated with the exoskeleton. Further, there was a strong signal of P, Cl, and K in the head of the adult female, with K, Ca, and Zn exhibiting a strong signal in the abdomen, either in the alimentary canal or reproductive organs.

The egg mass contained very low, yet still detectable, amounts of arsenic present in the gelatinous sheath of the egg mass (not pictured). The signal of arsenic in the sample was not strong enough to differentiate between arsenic species; however, this does provide some evidence for maternal transfer of arsenic. Though arsenic is not present within the eggs themselves, first instar larvae have been observed to feed on the gelatinous sheath upon hatching (C. M., personal observation), and could therefore be exposed to minute quantities of arsenic early on. Phosphorus, Cl, Ca, Mn, and Cu were also found in the gelatinous sheath, while Fe, S, P, K, and Zn were found within the eggs.

3.2. Culex tarsalis

In contrast to *C. riparius*, the larva of *Cx. tarsalis* did not contain a high degree of differentiation with regards to organ structure that was apparent in the XAS images. Arsenic was found to be distributed throughout the body of the fourth instar larva, as opposed to being concentrated in the midgut (Fig. 3a). In the adult female, arsenic was concentrated in the thorax, likely in the exoskeleton, as

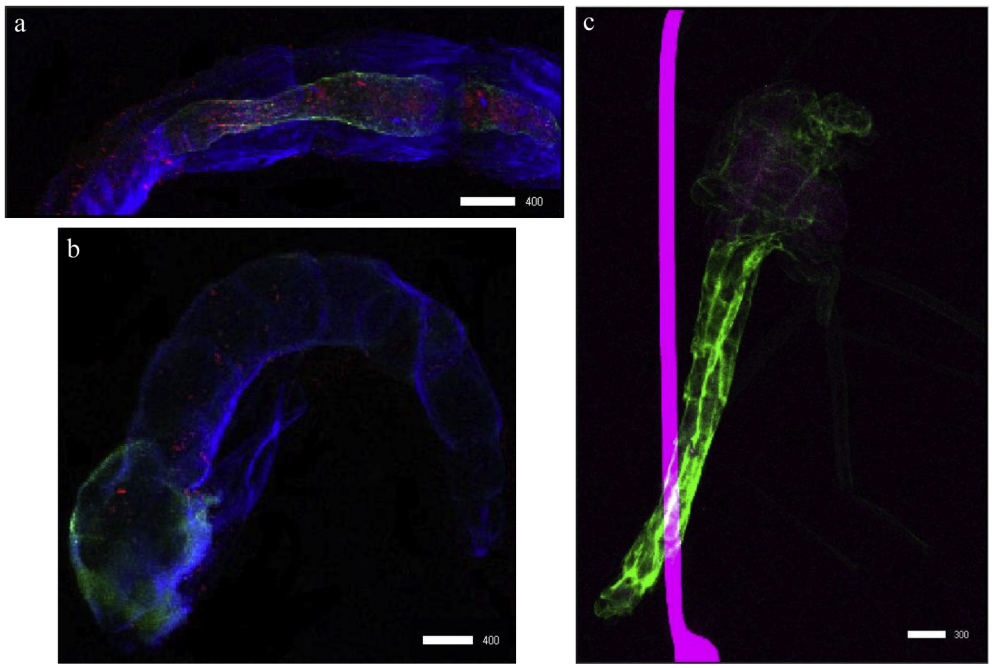

Fig. 1. X-ray fluorescence imaging of *Chironomus riparius*. a) Fourth instar larva. Blue = potassium, Green = copper, Red = arsenic. b) Pupa. Blue = potassium, Green = copper, Red = arsenic. c) Adult female. Green = zinc, Pink = arsenic. The bright purple 'bar' running top to bottom in the picture shows the As in the glass capillary tube upon which the specimen is mounted. Scale bar units are μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

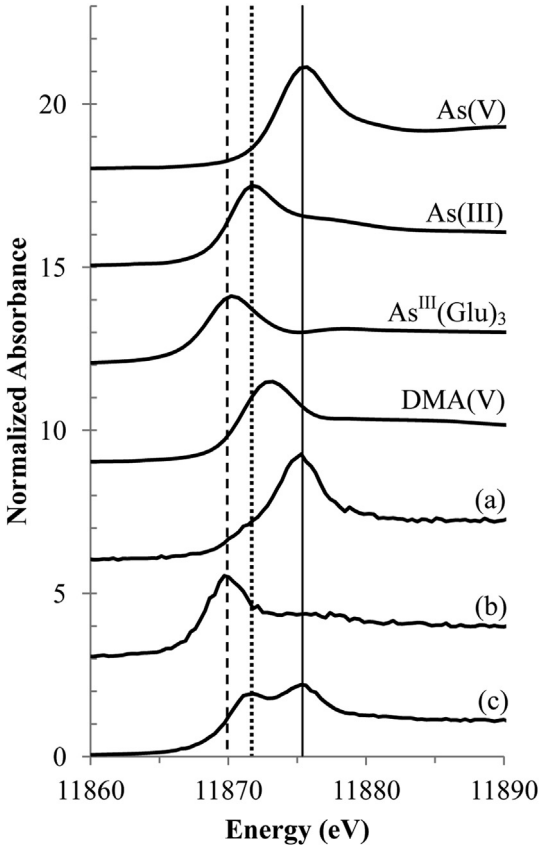

Fig. 2. XANES spectra from arsenic standards, and example spectra from insect tissues. a) Sample spectra of As(V) recovered from adult female chironomid. b) Sample spectra of an As-thiol from chironomid larvae. c) Sample spectra of As(III) and As(V) recovered from chironomid larvae. The solid line indicates the white line for As(V) (11,875.3 eV); the dotted line indicates the white line for As(III) (11,871.7 eV); the dashed line indicates the white line for As^{III}(Glu)$_3$ (11,870.0).

in *C. riparius* (Fig. 3b). Arsenic fluorescence was also seen in the adult eye. For both the larva and the adult, although arsenic was detected in the samples, the fluorescence signal strength was not strong enough to distinguish between different oxidation states.

In immature *Cx. tarsalis*, Fe was found in the alimentary canal; Cu, Ca, K, Cl, S, and P were found in the cuticle, and Cu and Zn were concentrated in the posterior portion of the abdomen, possibly in the MT. In the adult female, Fe, Cu, Ca, K, Cl, S, and P were associated with the cuticle, while Zn, Mn, Ca, and P were associated with the MT.

4. Discussion

Many invertebrates are capable of storing and detoxifying metals and other toxicants they encounter in the environment using a variety of mechanisms (Hopkin, 1989). The ability of insects to detoxify arsenic and the mechanisms by which they do so specifically has not received much attention in the literature. With this study we sought to generate initial information to help elucidate arsenic reduction and excretion pathways in aquatic dipterans. Although XAS analysis cannot provide direct information on molecular detoxification, it can provide corollary information with regards to biotransformation and localization of arsenic within whole insect samples.

In a previous study, *C. riparius* was shown to excrete 72% of total body burdens of arsenic between the fourth instar and adult stages (Mogren et al., 2012). Elimination of other toxicants between larval and adult stages has also been demonstrated (e.g. Cd, Groenendijk et al., 1999), and as much as 75% of total body burdens were excreted during molts of fiddler crabs (Bergey and Weis, 2007). In the present study, we demonstrate that the loss of arsenic in *C. riparius* between the larval and adult stages occurs specifically between the larval and pupal stages, as indicated by the relatively low levels of arsenic recovered in the pupa. However, because very little arsenic was found to accumulate in the exoskeleton of the larvae, we hypothesize that arsenic is not excreted via the exoskeleton, but could be lost in a meconium (defecation between fourth instar and pupal stages). This is evidence of limited movement of arsenic from the aquatic to the terrestrial environment via insects, but additional research is needed to document actual transfer rates.

Specific organs have been proposed as being crucial to detoxification of toxic compounds in insects, such as the Malpighian tubules (MT) and midgut (Hopkin, 1989). In insects, the MT, located at the anterior portion of the hindgut, function in filtering wastes and

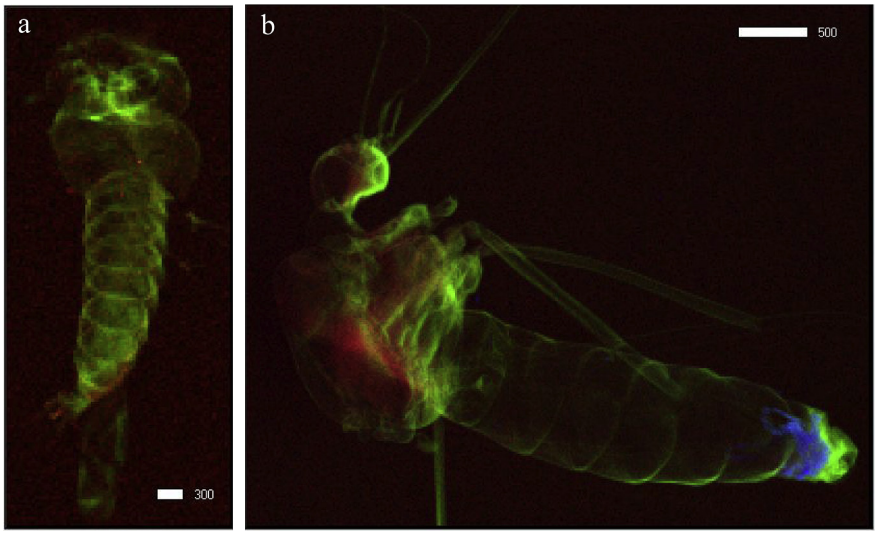

Fig. 3. X-ray fluorescence imaging of *Culex tarsalis*. a) Fourth instar larva. Green = potassium, Red = arsenic. b) Adult female. Blue = manganese, Green = potassium, Red = arsenic. Scale bar units are μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

solutes from the hemolymph; this pre-urine passes into the hindgut and is excreted from the anus (Klowden, 2007). Their ability to filter soluble ions from the hemolymph and form concretions has marked them as detoxification organs. However, in *C. riparius* larvae, Mn is the chief element present in the MT (resulting from Mn exposures in the food), and no arsenic was detected. Therefore, it can be concluded that in this species, the MT do not play a significant role in arsenic detoxification or excretion. The lack of arsenic in the MT of *Cx. tarsalis* further supports the notion that this organ does not play a significant role in arsenic detoxification and excretion in these aquatic Diptera. The absence of As in the MT may be the result of ionic differences between metals (cations) and metalloids (anions).

Although there was no arsenic to be found in the midgut of *Cx. tarsalis*, in *C. riparius* the high concentration of arsenic in the midgut indicates this organ may be important in detoxification. Hare et al. (1991) found that the distribution patterns of trace element contaminants in freshwater insect tissues varied between taxa, but contrary to the present study, reported the majority of arsenic incorporated into the body versus the midgut in *Hexagenia* mayflies. This difference may be due to the longer lifespan of mayflies, which in turn allows for arsenic incorporation into fat bodies. Arsenic being found in the midgut of *C. riparius* may be the result of cellular mineral inclusions, or concretions, sequestering excess arsenic ions in spherocrystals (Ballan-Dufrançais, 2002). Spherocrystals typically trap unusual cations but there is some evidence for limited uptake of arsenic in cells containing mineral inclusions. However, in the case of *Formica polyctena* (Hymenoptera: Formicidae), arsenic was mostly concentrated in organs devoid of spherocrystals (Jeantet et al., 1977). Arsenic in the eye of adult *Cx. tarsalis* may result from incorporation in ommochrome pigment granules, which has been observed in Orthoptera (White and Michaud, 1980).

Because larvae were exposed to inorganic arsenate, which is stable in solution (Al-Sibaai and Fogg, 1973; Liu et al., 2006), it can be concluded that the reduction of arsenate to arsenite in the larval midgut, in addition to the presence of As-thiol species, is the result of biotransformation and/or detoxification pathways in the larvae. Although the reduction of arsenate to arsenite results in a more toxic form of arsenic within the larva, As-thiols are indicative of arsenic binding cysteine residues in either glutathione or metallothionein (Andrahennadi and Pickering, 2008). In their study of *Formica* ants from an old arsenic smelter site, Kuehnelt et al. (1997) reported arsenate and arsenite as the major arsenical compounds recovered from the ants, with dimethylarsinic acid and traces of methylarsonic acid and arsenobetaine as well. More recently, XAS analysis has revealed that *Formica* ants store mainly inorganic arsenate and arsenite, though the concentration of arsenic in the midgut is not apparent (Moriarty et al., 2009). In contrast, midgut differentiation is observed in larvae of the bertha armyworm, *Mamestra configurata* (Andrahennadi and Pickering, 2008). Further, *M. configurata* biotransforms inorganic arsenate to an As-thiol, modeled as As^{III}-*tris*-glutathione (Andrahennadi and Pickering, 2008; Parsons et al., 2009). Sulfur coordination in arsenic metabolism was also documented to occur in the earthworm *Lumbricus rubellus* (Langdon et al., 2002, 2005). In *Lumbricus terrestris*, arsenobetaine, methylarsonate, dimethylarsinate, trimethylarsine oxide, and arsenosugars 1 and 2 were recovered (Button et al., 2011), indicating that even within the same genus, arsenic metabolism may differ. There is also the possibility that the observed thiolation of arsenite in *C. riparius* results from endosymbiont-mediated biotransformations (Basu et al., 2010), though further research is needed to explore this in depth.

XAS analysis did not reveal compartmentalization of arsenic in *Cx. tarsalis* as is observed in *C. riparius*. The greatly reduced concentrations of arsenic in this mosquito species may therefore be due to efficient excretion mechanisms not present in *C. riparius*. Other elements are also accumulated differently (e.g. Cu is not concentrated in the larval midgut), indicating possible physiological differences in the overall handling of metals and arsenic in *Cx. tarsalis*.

With regards to where the other measured elements accumulated within *C. riparius* and *Cx. tarsalis*, a summary is provided in Table 1. There are interesting differences between these two species and other insects that have been evaluated for metals accumulation. In *Formica* ant workers, Zn was largely associated with the MT and cuticle; Cu was associated with the midgut, MT, and cuticle; Fe was associated with the midgut and MT, and Mn was associated with the midgut and MT (Rabitsch, 1997). In *Gammarus pulex*, Mn, Fe, Cu and arsenic were associated with the gut, while Ca and Zn are associated with the cuticle (Schaller et al., 2011; Khan et al., 2012). In the collembolan *Tomocerus minor*, Ca, K, Mn, S, Cl, and P were associated with the midgut (Humbert, 1978). Zinc, Mg, P, Ca, and K were significantly enriched in the MT of the termite *Tumulitermes tumuli* (Stewart et al., 2011). Ballan-Dufrançais (2002) reviews the distributions of metals in the organs of other insects. Whereas Andrahennadi and Pickering (2008) found Mn in the cuticle of *M. configurata*, in this study we found Mn in the MT of *C. riparius* and adult *Cx. tarsalis*. Variations between these different species in metal accumulation and localization may result from differing element makeup in their respective diets, or reflect differences in physiological processing of metals.

With regards to arsenic localization and biotransformation in aquatic Diptera, it appears that in these two species there are marked differences in how the insects are able to mediate toxicity of arsenic. These differences are also reflected in differing distributions of other elements in the midgut and MT. Though the MT do not appear to serve an important role in detoxification of arsenic as it does with other toxicants, the midgut appears to be of particular importance for *C. riparius*. More research is needed to determine if the insect is mediating its own toxicity to environmental toxicants, or if endosymbionts play a role in helping insects to adapt to stressful environments.

Table 1
Element distributions for the a) fourth instar and pupa of *C. riparius* and the fourth instar of *Cx. tarsalis*, and b) the adult females of *C. riparius* and *Cx. tarsalis*.

a)	Alimentary canal		Cuticle		Malpighian tubules	Head	Abdomen	
	C. riparius	*Cx. tarsalis*	*C. riparius*	*Cx. tarsalis*	*C. riparius*	*C. riparius*	*C. riparius*	*Cx. tarsalis*
4th instar larva	As, Cu, Fe, Mn	Fe	Cl, K, P, S, Zn	Ca, Cl, Cu, K, P, S	Ca, Mn			As, Cu, Zn
Pupa			As, Ca, Cl, Fe, K, Mn, P, S, Zn			As, Cl, Cu, Fe, K, P, S	As, Cl, Cu, Fe, K, P, S	

b)	Alimentary canal	Cuticle		Head		Thorax		Abdomen	
	C. riparius	*C. riparius*	*Cx. tarsalis*	*C. riparius*	*Cx. tarsalis*	*C. riparius*	*Cx. tarsalis*	*C. riparius*	*Cx. tarsalis*[a]
Adult female	Cu	Cl, Fe, K, Mn, P, S, Zn	Ca, Cl, Cu, Fe, K, P, S	Cl, K, P	As	As	As	Ca, K, Zn	Ca, Mn, P, Zn

[a] The Ca, Mn, P, and Zn found in the abdomen of *Cx. tarsalis* were associated with the Malpighian tubules specifically.

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