Short communication

**Strategies of *Gammarus pulex* L. to cope with arsenic - results from
speciation analyses by IC-ICP-MS and XAS micro-mapping**

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Abstract

The invertebrate shredder *Gammarus pulex* L. is a key species for aquatic carbon turnover via
litter decomposition and can thrive in high-arsenic (As) environments. To understand their

strategies for coping with increased As concentrations while fulfilling their ecosystem functions, we analyzed the As concentration and speciation in their aquatic habitat and in leaves with heterotrophic biofilms as their natural food source. We also followed the As distribution and speciation on the cuticle and within the body of *G. pulex* by X-ray absorption spectroscopic imaging. Half of the total As on *G. pulex* was found to be associated with the cuticle but was not taken up. Removing this externally bound As yielded only arsenate in the wash solution which reflects the speciation of the surrounding aquatic phase and shows that this As does not undergo any biotransformation. The major pathway into the organism is suggested to be incorporation via food intake, but only very low amounts of As were taken up or translocated from the gut system to other tissues. In one of the main food sources, leaves, 68 % arsenate and 29 % monomethylarsenate were found. After ingestion into the gut system, up to 23 % of the more toxic arsenite was seen, but a substantial share was methylated to dimethylarsenate (46-56 %). Little arsenate and arsenite were found in the adjacent tissues. Besides 76-80 % mono- and dimethylarsenate, 10-21 % of the As was complexed as As(III)-S species. *G. pulex* plays an important role in As cycling and our results indicate that its translocation from the gut to other tissues is minimized, but a transformation to other As-species occurred.

Keywords: amphipods; arsenic speciation; invertebrates; tissues; XANES

Introduction

Elevated As concentrations in surface waters and aquifers are of global concern and can occur as a result of mining activities as well as natural bedrock weathering or atmospheric

deposition. Associated soil and sediment can become contaminated and, as a consequence, they can be a continuous source of pollution through related accumulation and release processes (Cullen and Reimer, 1989). The dominant As species in water are inorganic arsenite and arsenate (Cullen and Reimer, 1989); in organic-rich systems, methylated species like monomethylarsenate (MMA) and dimethylarsenate (DMA) might also be found (Frohne et al., 2011).

Many aquatic organisms seem to be able to cope with increased As concentrations in their habitat and fulfill their ecosystem functions seemingly unaffected (Schaller et al., 2010). Different strategies may help organisms to cope with As toxicity. If As is taken up in aquatic organisms, it can be complexed and metabolized to organoarsenic compounds, such as methylated species, arsenobetaine, and arsenosugars, or bound to glutathione to avoid accumulation (Caumette et al., 2012; Raab et al., 2004). Arsenic can also bind to sulfur or sulfur-containing compounds like glutathione followed by excretion via hepatopancreas (Chavez-Crooker et al., 2003; Raab et al., 2004). If not excreted, As can be translocated from the gut to adjacent tissues and accumulate there, with a possible change in speciation.

Among aquatic organisms, invertebrate shredders are interesting because not only do they take up toxic elements (Rainbow, 2002), but they also decompose aquatic leaf litter with consequences for bioavailability of toxic elements, thereby linking effects on invertebrates to cycling of nutrients and toxic elements in rivers and streams (Zimmer and Bartholme, 2003).

The dominant invertebrate shredder in freshwater systems throughout Europe and central Asia is *Gammarus pulex* L. This invertebrate cuts particulate organic matter (POM) such as leaf litter (~5 cm) into pieces of less than 1 mm. Litter is also ingested and later excreted. It has previously been shown that, because of the remarkable increase of surface area, the capacity of As binding is 670 % higher in these <1 mm pieces than in non-

decomposed leaf litter and litter residuals (Schaller et al., 2011a; Schaller et al., 2010), because of larger surface areas.

G. pulex can bioaccumulate a number of elements, such as cadmium, nickel and lead, from water, binding these metals to high molecular weight compounds and metallothionein-like proteins in their tissues (Geffard et al., 2010). As, on the other hand, is mostly bound to the exterior cuticle of the invertebrates (Schaller et al., 2011b). Physically separating the gut system from the rest of the animal showed high As contents in the gut system (Schaller et al., 2011b).

Little is known to date about the As speciation within invertebrates in freshwater systems. Currently, only a few studies, show the As speciation within aquatic invertebrates, namely, *Daphnia* and copepods. X-ray absorption spectroscopy (XAS), specifically, X-ray absorption near edge structure (XANES) analysis was used for whole-sample speciation analysis and localization of As in the organism, that is, mapping (Caumette et al., 2011; Caumette et al., 2012). However, the modes of life for *D. pulex* and copepods, are highly different from *G. pulex*. *D. pulex* mainly feeds on algae and, being a photoautotroph organism, releases oxygen, and Copepods are carnivores. *G. pulex* mainly feeds on heterotrophic biofilms, consuming oxygen and leading to reduced conditions. Arsenic speciation and potential toxic effects might therefore vary substantially between the different invertebrates.

To understand how invertebrate shredders like *G. pulex* deal with elevated As concentrations, we analyzed the As concentration and speciation in their aquatic habitat and in leaves with heterotrophic biofilms as their natural food source and followed the As distribution and speciation on the cuticle and within the body of *G. pulex* specimens by X-ray absorption spectroscopic imaging. We hypothesize that the As speciation on the cuticle

conforms with that of the surrounding water and that during ingestion and uptake via food the As speciation changes to more reduced species.

Materials and methods

Site properties

The site for invertebrate and leaf sampling is located in Mechelgruen-Neuensalz (Saxony, Germany). The specimens were collected from “Forellenbach”, a small stream with As concentrations of $\sim 29 \mu\text{g L}^{-1}$, high As contents in the organic-rich stream sediment ($\sim 20\text{--}800 \text{ mg kg}^{-1}$ dry matter), and a pH between 7.5 and 7.9, conductivity 800 to $1000 \mu\text{S cm}^{-1}$, Eh of $\sim 252 \text{ mV}$ (all values for the stream water), and a carbon content of 9.5 % in the sediment (Schaller et al., 2010). Leaf samples were collected from stream sediments, too.

Sample collection, preparation and analysis

Adult specimens of *G. pulex* were collected from Forellenbach stream (median size of 1.4 mm and median weight of 5 mg). After collection, five specimens were dried at 40°C . The other specimens were stored separately in a 25 mL polyethylene vessel containing 4 mL of deionized water with an As concentration below $0.2 \mu\text{g L}^{-1}$ (analyzed by ICP-MS). After 10 min of washing, the specimens were removed from solution, and both the specimen of *G. pulex* as well as the desorption water were flash-frozen using dry ice. The desorption water had the same pH as the stream water.

After drying at 40°C , the samples were wet digested in a closed vessel in a MARS5 microwave system (CEM Corp., Matthews, United States) (addition of 3 mL of 65% HNO_3 and 2 mL of H_2O_2). Water samples for total As measurements were acidified with 20 μL 65%

HNO₃ to 2 mL water sample, conforming with DIN-EN-ISO-5667 (2004). Element analysis for all samples was done using inductively coupled plasma-mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher).

Arsenic speciation measurements

Aqueous samples (three from the stream and five from the dissolution experiments using *G. pulex*), two leaves, and two specimens of *G. pulex* were analyzed for As speciation. For As speciation analysis of aqueous samples, flash-frozen samples were thawed and handled exclusively in an oxygen-free atmosphere in a glovebox prior to analysis. For species detection, we used anion-exchange chromatography (Dionex ICS-3000 SP) without anion suppressor coupled to ICP-MS as published previously for methyl(thiol)ated species (Planer-Friedrich et al., 2007).

For As speciation of solid samples, XANES spectra were recorded at the Pacific Northwest Consortium X-ray Science Division (PNC/XSD) facilities, Sector 20 at the Advanced Photon Source (APS), Argonne National Laboratory using methods according to Smith et al. (2005). Leaf samples from the Forellenbach stream were freeze-dried, ground into powder and packed into sample holders for analysis. Analysis was done for a specimen of *G. pulex* previously washed in pure water for 10 minutes as mentioned before. Samples were frozen, and packed into sample holders for analysis at 100 K (-173 °C) on the bending magnet beamline. Five to ten scans of each sample were collected and averaged before data fitting and no beam damage was seen from the first to last scans. For further information please see Caumette et al. (2014). XANES spectra of the As K-edge (11868 eV) were fit within -20 to +30 eV to E0 using Athena software (Ravel and Newville, 2005) using a combination of As standards measured previously (Smith et al., 2005). The fit that produced the lowest reduced

chi-square value was accepted, and included frozen arsenite and arsenate, DMA, MMA, As(III)-S and As(V)-S.

The two-dimensional XRF mapping of *G. pulex* was performed using the insertion device beamline. The beam was focused to $1 \times 2 \mu\text{m}$ spot size. Fluorescence data were collected at $10 \mu\text{m}$ steps with a 3-second integration time. Spectral and statistical analyses were performed as described by Caumette et al. (2012). Photographs of the organism were taken using a Vista Vision stereo-zoom microscope.

Statistics

Student's *t*-test was performed for comparison of the bulk As data of the specimen of *G. pulex* prior to and after desorption using SPSS 21.0.

Results and discussion

Total As distribution in *G. pulex*

The present study uses total concentrations of As in tissues (presented in Figure 1), and As distribution in the organism (presented in Figure 2, a map produced from XAS imaging) to interrogate these pathways. We found significantly higher As values in unwashed ($15.5 \pm 5.2 \text{ mg As kg}^{-1} \text{ DW}^{-1}$) compared to washed ($7.0 \pm 1.7 \text{ mg As kg}^{-1} \text{ DW}^{-1}$) specimens ($p < 0.05$), suggesting that most of the As associated with *G. pulex* was bound to the cuticle (Fig. 1). Furthermore, the As content of the leaves was $40 \pm 17 \text{ mg As kg}^{-1} \text{ DW}^{-1}$ (Fig. 1). These findings verify previous findings of total As in *G. pulex* (Schaller et al., 2011). In the present study, the As species were additionally determined in the cuticle wash solution, and only arsenate was found ($2.6 \pm 0.1 \mu\text{g L}^{-1}$), with arsenite and methylated As species below detection limits ($< 0.1 \mu\text{g L}^{-1}$). The stream water had an As concentration of $28 \pm 0.1 \mu\text{g L}^{-1}$.

and all of this As was in the form of arsenate. Hence, cCuticle-associated As thus reflects the speciation of the stream water, indicating no transformation of this externally bound As. Moreover, this cuticle-associated As probably represents only a minor uptake pathway into the organism, since the thick exoskeleton composed of calcified chitin, fully covering the body of *G. pulex*, is relatively impermeable to toxic chemicals (Rainbow, 1997). Comparing the high amounts of As bound to the cuticle ($7.5 \text{ mg As kg}^{-1} \text{ DW}^{-1}$ as the difference between washed/unwashed specimen, Fig. 1) and the relatively low amounts of As found by XAS-mapping within the tissues adjacent to the cuticle (Fig. 2), confirms that the cuticular pathway is not a major one of As uptake. Our XAS micro-mapping revealed only low As concentrations in tissue adjacent to the gills (Fig. 2, spot 4) (located between the posterior abdominal limbs).

In our study, the XAS in-situ micro-mapping clearly shows a maximum of As concentration along the gut system (Fig. 2), which confirms earlier results showing ~60 % of the total As load in the gut when physically separated from the rest of the animal (Schaller et al., 2011b), and transfer to other tissues was minimal.

Arsenic speciation within *G. pulex*

For information on As speciation within *G. pulex*, bulk XAS analysis was carried out (using XANES) on one specimen and micro-XANES data were collected from different spots of different As intensity on another specimen used for mapping (Table 1). XANES measurements were also obtained for a leaf sample. Bulk XANES measurements of *G. pulex* showed 78 % As(V)-O, 20 % As(III)-O and 2 % MMA (Table 1).

Three micro-XANES spectra were collected within the gut system and another three from the surrounding tissues (Fig. 2) and the corresponding XANES fittings at these locations and for the leaf showed distinctly different As speciation between the food source, the gut, and the surrounding tissues (Fig. S1; Table 1). Leaves coated in heterotrophic biofilms (leaf biofilm is the main food source for *G. pulex*) showed mostly As(V)-O (~70 %) and MMA (~30 %), while in the gut system DMA was the dominant species (46-56 %), followed by As(V)-O (~28-38 %) and As(III)-O (0-23%). In one of the gut spots, As(III) binding to sulfur compounds was observed (6%). In the surrounding tissues, an increased proportion of methylated species was observed (76-80% MMA/DMA). Arsenate (12%) and arsenite (2%) were only detected at one spot in the tissue. Arsenic(III) binding to sulfur was observed at all tissue spots with a share of 10-21% (Table 1). Differences between As speciation in the bulk *G. pulex* sample and the μ -XANES measurements may be related to the heterogeneity of As speciation in both entire *G. pulex* samples.

The differences in As speciation in food, gut and tissue suggest transformations in the organism, as follows. Arsenate as the dominant species in the main food source of *G. pulex* is reduced in the gut, which may initially produce arsenite (Thomas et al., 2001), but it is also further methylated. Translocation to the tissues is quantitatively low and linked to further methylation but also to complexation of arsenite with sulfur. Similar to findings for *Daphnia* we detected As(III)-S binding (Caumette et al., 2012), with significant As(III)-S complexation only observed in the tissues, not in the gut system. Hence, we consider the possibility that the As(III)-S is formed within the invertebrate tissues as part of a possible detoxification strategy, possibly due to binding to glutathione within hepatopancreatic and other cells (Kutlu and Susuz, 2004).

A similar As distribution was found in a comparable study with *D. pulex* with the highest As concentrations within the gut system and low transfer to other tissues (Caumette et al., 2012). However, more As was methylated in *G. pulex* compared with *D. pulex* (Caumette et al., 2012). This increased methylation of As within *G. pulex* can be explained by the differences in food (heterotrophic biofilm on leaves for *G. pulex* compared to autotrophic algae for *D. pulex*). We hypothesize that the heterotrophic biofilms lead to reducing conditions within the gut system, which could result in increased methylation of As.

Conclusions

Studying *G. pulex* in a natural aquatic system with $\sim 30 \mu\text{g L}^{-1}$ As, we found that most of the As associated with the invertebrate shredder was bound as arsenate to the cuticle. *G. pulex*'s ingestion of food (leaf litter) containing As as arsenate was accompanied by arsenate reduction and methylation in the gut system (possibly reducing conditions within the gut system). Arsenic uptake from the leaves via the gut system and translocation to other body tissues was very low and inside these tissues, inorganic As was methylated or complexed by sulfur-containing compounds. Minimal gut passage of As to *G. pulex* tissues, where it would have the potential to cause toxic effects, probably helps this organism to thrive in elevated As environments. The finding of methylated species in *G. pulex* tissues indicates biotransformation, but whether this aids in arsenic tolerance cannot be determined since the toxicity of methylated As species to *G. pulex* is unknown, as is the excretion mechanism of As. Because of *G. pulex* reducing As accumulation, this species contributes to arsenic cycling in two ways: first, by increasing arsenic sorption to the sediment when shredding organic material (leaf litter) and, second, by transferring cuticle-bound arsenic to its predators at higher trophic levels in the food web.

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301 Table 1: Arsenic speciation within different tissues of *G. pulex* and for leaf bulk samples

302 obtained from XANES measurements.

Micro XANES spots/bulk		Arsenic speciation (%)						Reduced Xi-square
		As(V)- O	As(III)-O	MMA	DMA	As(III)-S	As(V)-S	
Leaf	Bulk	68		29			3	0.010
<i>G. pulex</i>	Bulk	78	20	2				0.017
Gut area	Spot1	38			56	6		0.009
	Spot2	32	22		46			0.007
	Spot3	28	23		49			0.010
Tissues	Spot4			80		20		0.018
	Spot5				79	21		0.003
	Spot6	12	2		76	10		0.013

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307 Fig. 1: Arsenic concentration in leaves as the main food source and washed/unwashed

308 *G. pulex*.

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Fig. 2: Mapping of As in *G. pulex* specimen (top). Signal is shown as a gradient of concentration (right), with the corresponding photograph of the *G. pulex* (left). Spots 1-3 are located within the gut systems, spot 4 is located near the gills and spots 5-6 are possibly located within storage tissues or muscles.

Arsenic concentration and speciation within *Gammarus pulex*