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RESEARCH ARTICLE

An integrated study on *Gammarus elvirae* (Crustacea, Amphipoda): perspectives for toxicology of arsenic-contaminated freshwater

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Abstract The Italian region Latium is characterized by extensive quaternary volcanic systems that contribute greatly to arsenic (As) contamination of freshwater, including drinking water supplies. However, knowledge of the possible toxic effects in these aquatic environments is, despite being highly relevant to public health, still limited. In this paper, we approach this issue using *Gammarus elvirae*, an amphipod species that inhabits rivers and streams in central Italy, including Latium. We explored the possibility of using *G. elvirae* in the toxicology of freshwater by addressing the most relevant issues. First, we tested the usefulness of hemocytes from *G. elvirae* in determining non-specific DNA damage by means of the Comet assay after exposure (24 h and 7 days) to different river water samples in Latium; second, we provided an interpretative overview of the usefulness of hepatopancreatic epithelial cells of *G. elvirae* as a means of assessing toxicity after long-term exposure to As and other pollutants; third, the LC (50–240 h) value for *G. elvirae* was estimated for arsenate, which is usually the dominant arsenic species in surface waters. Our study sheds light on *G. elvirae* at different levels, providing a background for future toxicological research of freshwater.

Keywords Arsenate · Comet assay · Elongation factor 1α · Hemocytes · Hepatopancreas · LC50

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Introduction

The growing amount of attention being dedicated to arsenic (As) in the environment in recent years has enhanced the need for detailed toxicological investigations. Indeed, this metalloid may pose a threat to public health, particularly in the long term. Exposure to inorganic forms of As has been associated with considerable adverse health effects, including cancers (Argos et al. 2014; Faita et al. 2013; Xie et al. 2014).

Latium, a region in central Italy, contains As-polluted freshwater largely as a result of geogenic processes, though anthropogenic activities are also likely to represent sources of As water contamination (Davolos and Pietrangeli 2011, 2013, and literature therein). The concentration of arsenic in the water in several areas within this Italian region is high enough (more than 10 As μg L^{-1}; Angelone et al. 2009; Casentini et al. 2010; among others) to raise considerable concern regarding its effects on humans following the introduction of recent European and international regulations for drinking waters. This issue is of great significance because people living in As-contaminated areas within this Italian region may be exposed to arsenic via water and food (Cubadda et al. 2012). However, knowledge of the possible toxic effects in freshwater environments of Latium is still limited.

A growing number of studies have addressed the usefulness of freshwater species belonging to the genus *Gammarus* Fabricius, 1775, of the family Gammaridae Leach, 1813 (Crustacea) for both toxicology and ecological assessment purposes (Kunz et al. 2010). For example, the species complexes *Gammarus fossarum*, *Gammarus pulex*, and *Gammarus roeseli*, which are found in various European regions, are proving to be highly suited to the study of toxic effects in the laboratory setting (Adam et al. 2009; Besse et al. 2013; Böttger et al. 2012, 2013; Bundschuh et al. 2013; Feckler et al. 2012; Lacaze et al. 2011; Peschke et al. 2014; Westram et al. 2013).

Here, we studied *Gammarus elvirae*, an amphipod species distributed in the central Apennines of Italy (Iannilli and Ruffo 2002). *G. elvirae* lives in lotic habitats in which it plays prominent ecological roles, particularly in the breakdown processing of both fresh leaf material and plant debris. Like other gammarid amphipods (see above), *G. elvirae* may be a promising model taxon for research on arsenic in freshwater. However, an adequate investigation on the potential of this crustacean species in the toxicology of the freshwater of Latium is still missing owing to the lack of reliable methods to assay the potential biological effects of arsenic and other pollutants. The present study was therefore designed with the following objectives: first, we tested the usefulness of hemocytes from *G. elvirae* in determining non-specific DNA damage by means of the Comet assay following exposure to a range of inland waters; second, we carried out a histological investigation of the hepatopancreas of *G. elvirae*, since this gland plays a pivotal role in the response/vulnerability to toxic contaminants; and third, we estimated the *G. elvirae* LC (50–240 h) value for arsenate (As(V)), which is the prevalent arsenic species in surface waters.

Materials and methods

Study sites and sampling

Individuals of *G. elvirae* (Iannilli and Ruffo, 2002) were sampled from the following three localities in Latium (Fig. 1): (1) Ciciliano, the type locality of *G. elvirae*, which lies between the Prenestini and Tiburtini Mountains, 619 m above sea level (asl); the animals were collected from a small stream within the drainage basin of the Aniene River; (2) Capo d'Acqua (CDA), a perennial resurgence of the River Amaseno located at the foot of the northern slopes of the Ausoni Mountains, 82 m asl; and (3) Subiaco, Aniene River, located at the foot of the Simbruini Mountains, 408 m asl. The physicochemical values previously obtained by Ronci (2013) for CDA, which can be considered a reference site, and Migliara 55 (M55), which lies in a downstream section of the Amaseno river (Fig. 1; see below), are listed in Table 1.

Individuals of *Gammarus lacustris* G.O. Sars, 1863 were taken from three Italian lakes (Fig. 1): (1) Lake Valparola, 2140 m asl, in Veneto, north-eastern Italy; (2) Lake della Maddalena, 1996 m asl, Maritime Alps, in Piedmont, north-western Italy; and (3) Lake Vivo, Monti della Meta, 1591 m asl, in Abruzzo, central Italy. Samples of *Gammarus italicus* Goedmakers and Pinkster, 1977, were collected in a small stream, River Temo, in northwestern Sardinia, Italy (Fig. 1). *G. lacustris* and *G. italicus* were analyzed only for phylogenetic comparisons (online resource 1 in the electronic supplementary material (ESM)).

The Comet assay

The alkaline Comet assay (single cell gel electrophoresis), performed by applying the procedure as described elsewhere (Frenzilli et al. 2009) with modifications by Ronci (2013), was used to identify and quantify non-specific DNA damage. Briefly, the hemocytes were examined from adult individuals consisting of both females and males of *G. elvirae* collected from CDA, which can be considered a reference site, in the upstream section of the Amaseno river, after 24 h and 7 days of exposure to water from CDA and from M55 in the downstream section of the same river (Fig. 1). A total of 100 randomly captured hemocyte nuclei of *G. elvirae* from each slide (three replicates) were examined after the alkaline Comet assay procedure under ×400 magnification using an epifluorescence microscope (Zeiss Fluorescence Microscope System) connected through a camera to the image analysis system CometScore™ (TriTek Corp). The Comet assay output images were analyzed and then acquired by CometScore™. DNA damage after both 24 h and 7 days of exposure was estimated on the basis of three comet parameters, i.e., tail length, tail intensity (DNA % in the comet tail), as measured by the image analysis software (level of significance set at 5 %), and tail moment. Tail length represents the distance from the center of the head (start of tail) to the end of the tail, while tail % intensity is expressed as a percentage of the comet's total intensity. Tail moment is a product of the tail length and tail intensity. Statistical analysis of Comet output data was conducted in Past (3.01) by applying the non-parametric Kruskal–Wallis test.

Histology

For the histological examination, the specimens of *G. elvirae* (adult individuals, mean body length of 13–15 mm, in intermoult stage, collected from the three localities of Latium examined in the present study (Fig. 1)) were fixed for 24 h in Bouin's solution (see Davolos et al. 2010, among others). The specimens were kept in 70 % ethanol for 1 h and then in 80 % ethanol for 2 days to clear the samples of Bouin's solution. After dehydration in graded concentrations of

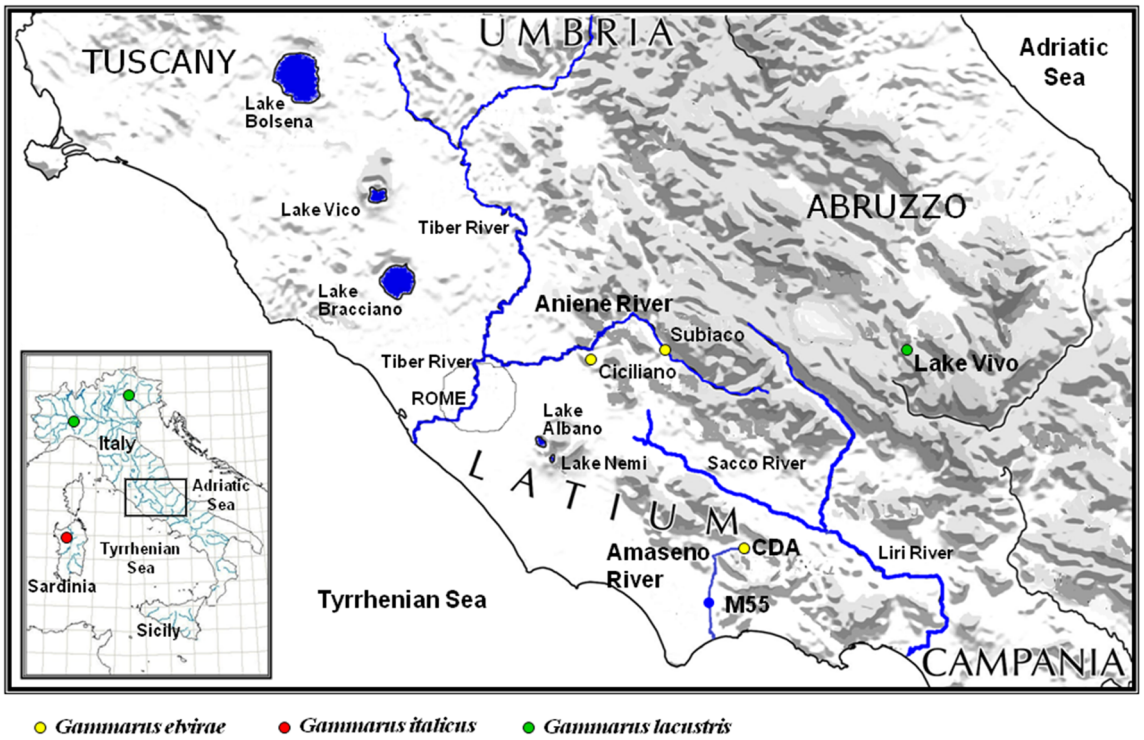

Fig. 1 Site map of the study area and the various sampling sites of *Gammarus elvirae* (*circled in yellow*), *Gammarus italicus* (*circled in red*), and *Gammarus lacustris* (*circled in green*). CDA and M55 in the River Amaseno stand for Capo d'Acqua and Migliara 55, respectively (see text). The part of central Italy corresponding to the Latium region is zoomed in and shown in the inset. For the sake of clarity, the figure shows the sampling location in northern Italy solely in the small scale map of Italy. Note: the River Amaseno downstream of M55 joins the River Ufente to form the River Portatore (features not shown in the map), which flows into the Tyrrhenian Sea

ethanol at 4 °C, the specimens were cleared in histolemon (three times, each 10 min) and finally embedded in paraffin wax at 60 °C (including an overnight step, for better wax penetration). Transverse and longitudinal 6-μm-thick serial sections were made with a microtome using a steel knife. The sections were then rehydrated and stained with hematoxylin-eosin as described by Davolos et al. (2010). Histological sections were examined by bright field light microscopy and digitalized using a digital camera mounted on a Nikon-EFD-3 microscope.

Arsenate toxicity assessment

Amounts (40 mL) of river waters from CDA (Fig. 1; Table 1) were subsequently supplemented with sodium arsenate ($Na_2HAsO_4{\cdot}7H_2O$; Sigma–Aldrich) at different concentrations (approx. from 100 to 1600 arsenate μg L^{-1}). Mortality of *G. elvirae* specimens (adult individuals consisting of both females and males, collected from CDA, which were not fed during the experiment were kept at 10 °C under light and dark conditions at a 16:8-h ratio) was recorded every 24 h up to 240 h of exposure to these water samples (one specimen was maintained for each concentration; experiments performed in three replicates), using the method described by Vellinger et al. (2012). A dose-mortality curve obtained after 240 h of arsenate exposure allowed us to estimate the median lethal concentration LC (50–240 h) value.

Results and discussion

Genotoxicity

Genotoxic effects (DNA damage levels) were evaluated in hemocytes of *G. elvirae* by means of the alkaline Comet assay after exposure to different water samples taken from the River Amaseno (Fig. 1). A higher level of non-specific damage at the DNA level (Table 2) was observed at M55 in the downstream section of the Amaseno river than in the upstream CDA sampling site, which is in keeping with the marked diversification of these two fluvial locations (see below). In particular, a significantly higher level of DNA damage after 24 h and 7 days of exposure was measured using the Comet assay at M55 than at CDA (Table 2). Comparable levels of primary DNA damage, evaluated using the Comet assay, have recently been reported in the hemolymph of *Gammarus balcanicus* (belonging to the Eurasian lineage; see online resource 1 in the ESM) after exposure to heavy metal-rich waters (Ternjej et al. 2014).

Table 1 Summary of the parameters measured in waters from two sites of the River Amaseno in Latium, namely Capo d'Acqua (CDA) in the upstream section of the river and Migliara 55 (M55) in the downstream section (Fig. 1)

Parameter	Unit	April 2011		June 2011		October 2011	
		CDA	M55	CDA	M55	CDA	M55
Arsenic	μg L^{-1}	1	1	1	1	1	1
Barium	μg L^{-1}	21	29	18	43	21	32
Beryllium	μg L^{-1}	0.25	0.25	0.25	0.25	0.25	0.25
Boron	μg L^{-1}	1	30	32	58	1	1
Calcium	mg L^{-1}	61	56	59	56	61	60
Chlorides	mg L^{-1}	9.40	10	8.80	54	9.40	10
Chrome	μg L^{-1}	1.50	1.50	1.50	1.50	1.50	1.50
Copper	μg L^{-1}	2	3.7	6	9	2	2
Fluorides	mg L^{-1}	60	160	50	100	60	70
Iron	μg L^{-1}	1	6	8	16	1	1
Lead	μg L^{-1}	0.50	0.50	0.50	1	0.50	0.50
Lithium	μg L^{-1}	0.50	0.50	0.50	0.50	0.50	0.50
Magnesium	mg L^{-1}	10	11	11	14	10	14
Manganese	μg L^{-1}	0.20	8	1.20	7.90	0.20	1.30
Nitrates	mg L^{-1}	6.70	6.30	5.90	5.20	6.70	3.50
Nitrites	μg L^{-1}	25	25	25	300	25	60
Orthophosphate	μg L^{-1}	30	30	30	30	30	90
Phosphorus	μg L^{-1}	40	30	15	120	40	100
Silicon	μg L^{-1}	200	320	210	320	200	200
Sodium	mg L^{-1}	0.80	8.30	0.93	2.30	0.80	1
Strontium	μg L^{-1}	220	480	260	610	220	730
Sulfur	mg L^{-1}	1.90	1.90	1.90	4.10	1.90	2.10
Sulfate	mg L^{-1}	3.7 0	5.30	3.70	9.30	3.70	4.40
Vanadium	μg L^{-1}	1	3	5	7	1	1
Zinc	μg L^{-1}	0.50	840	5	4	0.50	0.50
pH	CDA	M55					
Mean±SE	7.5±0.2	8.1±0.2					

Each pH value is the mean of the three sampling dates in spring, summer, and autumn (2011), followed by standard error (SE)

Although evidence of nitrate contamination was detected in the Amaseno river (Table 1), probably as a result of widespread contamination sources due to land use practices, concentrations of this substance were lower than the maximum values recommended for rivers by the European community (EU/2000/60/EC-WFD). Moreover, a detailed survey of waters along the Amaseno river (Ronci 2013; data partly extracted from http://www.arpalazio.gov.it) indicates that the chemical and biological quality of the water progressively deteriorates as the river flows downstream, probably owing to untreated agricultural and urban effluents along the river. Indeed, recent studies have shown that the pollution in aquatic environments may result in (geno)toxic effects and alterations in ecosystem functions (Silva et al. 2013; among others). However, the way in which the composition of river water may be related to DNA damage levels is, despite being highly relevant to public health, not yet clearly understood. For instance, some inconsistencies might be ascribed to the presence of mycotoxin contamination related to decaying vegetation in freshwater. Several saprotrophic microfungi distributed in inland water that are known to interact with competing invertebrates, including *Gammarus* species, secrete secondary metabolites such as ochratoxin A (see Davolos and Pietrangeli 2014, and literature therein), which are highly genotoxic (Ali et al. 2014, among others). As the genotoxicology of environmental samples remains a complex discipline, all the aforementioned hypotheses warrant experiment-based verification through the extension of the molecular analysis to other fluvial samples.

Nevertheless, according to our findings, and to those previously obtained in other *Gammarus* species such as *G. fossarum* (the species complex belonging to the European *Gammarus* lineage; see online resource 1 in the ESM), the use of the Comet assay has proved to provide an important means of understanding genotoxicology in these crustaceans (Lacaze et al. 2010, 2011). As regards *G. elvirae*, it is important to

Table 2 DNA damage estimated by the Comet assay in hemocytes of *Gammarus elvirae*. Water samples from two sites of the River Amaseno in Latium, namely Capo d'Acqua (CDA) in the upstream section and Migliara 55 (M55) in the downstream section (see Fig. 1), were examined

Parameter	Mean	SD	SE	Median	25th percentile	75th percentile
CDA						
Tail length (μm) 24 h	0.70*	1.20	0.14	0.46	0.22	1.54
Tail length (μm) 7 days	2.53*	1.40	0.68	1.76	0.25	2.84
Tail intensity (DNA %) 24 h	6.02*	3.20	0.60	5.07	2.60	9.60
Tail intensity (DNA %) 7 days	6.30*	2.80	0.62	5.67	2.83	10.16
Tail moment 24 h	2.90*	1.80	0.43	1.95	0.46	4.33
Tail moment 7 days	4.80*	1.20	0.43	2.00	0.48	5.79
M55						
Tail length (μm) 24 h	10.70*	0.52	0.63	8.71	6.54	14.43
Tail length (μm) 7 days	45.00*	3.44	0.47	37.77	32.56	53.45
Tail intensity (DNA %) 24 h	20.83*	2.40	1.20	11.48	6.92	30.81
Tail intensity (DNA %) 7 days	18.23*	0.50	0.56	10.79	4.93	21.98
Tail moment 24 h	87.64*	12.56	0.36	63.40	35.50	180.77
Tail moment 7 days	113.52*	11.71	37.11	70.01	25.57	136.67

DNA damage was estimated according to tail length (μm), tail intensity (DNA % in the comet tail), and tail moment, after 24 h and 7 days of exposure. Results are based on 100 random measurements (three replicates) of comets (head with DNA migrating into the tail region as a result of primary DNA damage in hemocyte nuclei) of *G. elvirae* per each site. Each value, based on three main parameters (tail length, % tail DNA, and tail moment), is the mean followed by standard deviation (SD) and standard error (SE), and the median followed by quartiles (25 percentile and 75 percentile)

*Significantly different ($P<0.05$) when the non-parametric Kruskal–Wallis test was applied

stress that the percentage and intensity of DNA in the Comet tail detected in the As-unpolluted site (CDA) can, on account of its good chemical and ecological status, be considered in future genotoxic assays of specimens of this species exposed in various ways to As-polluted waters under laboratory conditions.

Histology

As regards the two pairs of hepatopancreatic structures of *G. elvirae*, the pseudostratified epithelium in each tubule was found to consist of different cell types, as previously reported for other amphipods (Correia et al. 2002; Davolos et al. 2010). The distal part of the hepatopancreas was characterized by cuboidal and undifferentiated cells, known as E cells, that were densely arranged around a narrow duct, with a prominent round nucleus containing nucleoli (data not shown). The E cells appeared to be in continuous state of growth, which probably results in their differentiation towards the medial and proximal parts of the hepatopancreas, in which two similar cuboidal cells were detected, namely F cells and R cells, both of which have an apical region in the lumen and a round nucleus towards the central region (Fig. 2; online resource 2 in the ESM). Although no substantial difference at the histological level has been observed between F cells and R cells (which were consequently termed F/R cells) in other amphipods, F cells are likely to be involved in enzyme synthesis, whereas R cells may absorb nutrients from the lumen and play a role in the storage of lipid droplets (see Davolos et al. 2010, and literature therein). Large B cells with a large vacuole and a basally confined nucleus were common in the proximal part of the hepatopancreas, where they were arranged along the apparently irregular lumen (Fig. 2; online resource 2 in the ESM). The contents within the vacuoles of the B cells can be liberated into the lumen of the hepatopancreatic caecum by apocrine, holocrine, and merocrine secretion, while material from the lumen can be taken in by pinocytotic activity at the apical membrane of the B cells (Fig. 2; online resource 2 in the ESM). The low degree of degeneration of the microvilli brush border detected in some B cells appears to be related to the normal cell secretion activity of the large apical vacuole (more pronounced at a stage of cell maturity; online resource 2 in the ESM).

It is important to bear in mind previous studies that have investigated the effects of exposure to various toxicants, including As, on the histological and cellular features of the hepatopancreas in several aquatic crustaceans (Liu et al. 2013; Martìn-Dìaz et al. 2008; Tunca et al. 2013; Williams et al. 2008; Wu et al. 2014). Notably, Mazurová et al. (2010) detected pathological features (including distinct cell lysis) following long-term laboratory exposure to As in the hepatopancreas of *G. fossarum* of the European *Gammarus* lineage (see online resource 1 in the ESM). Moreover, Vellinger et al. (2012, 2013) recently evaluated the toxic effect of arsenate on *G. pulex*, which belongs to the *G. pulex* species complex of the same European lineage (see online resource 1 in the ESM).

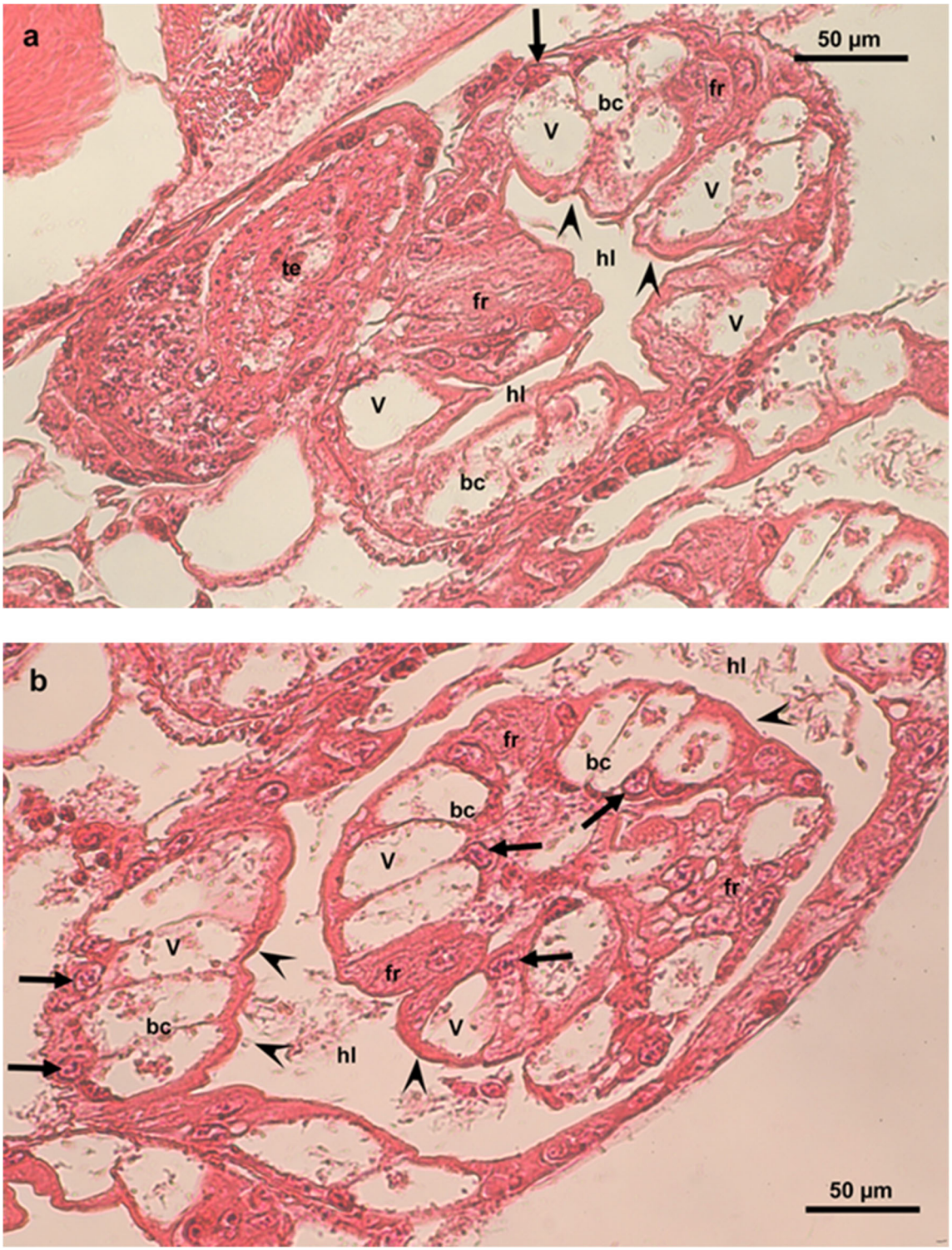

Fig. 2 Transverse sections (**a**, **b**) of the proximal region of two of the four hepatopancreatic tubules (see text) of an adult male of *Gammarus elvirae*. Each hepatopancreatic structure is surrounded by a network of muscle fibers (see Davolos et al. 2010). Note the well-developed epithelium consisting of different cell types; vacuolated B cells are arranged along the lumen, occupying approximately three-quarters of the internal boundaries. *bc*, B cells; *fr*, F/R cells; *te*, testis; *hl*, hepatopancreatic lumen; *v*, vacuole. *Arrows* point to the basally located nuclei (containing nucleoli) of the vacuolated B cells; *arrowheads* point to the apically dense brush border of the B cells

Like other genotoxic agents, arsenic may induce adverse changes from the molecular to the cellular levels, including oxidative stress caused by the overproduction of reactive oxygen species, whose toxic effects may lead to cell membrane

degradation due to lipid peroxidation (Vellinger et al. 2013). These studies suggest that exposure of *G. elvirae* to As is likely to be reflected in changes in the activity and structures of the hepatopancreas, associated with pathological lesions on hepatopancreatic epithelial cells. In particular, it seems reasonable to presume that if hepatopancreatic cells of *G. elvirae* are affected by As, the histological examination will reveal pathological features such as abnormal vacuolization and secretion activity of B cells. Further observations on *G. elvirae* based on the histological methods adopted in this study are needed to identify possible arsenic-related damage of hepatopancreatic cells in exposed individuals. This entails extending fieldwork to a large set of samples and performing dedicated laboratory experiments based on a robust histopathology framework, which goes beyond the scope of the present paper.

As(V) toxicity assessment

Assuming relatively aerobic conditions, As(V) oxy-anions such as $H_2AsO_4^-$ and $HAsO_4^{2-}$ are likely to be the thermodynamically stable arsenic species in surface waters (at pH around 8; see Table 1), whereas arsenite (As(III)) species such as H_3AsO_3 and $H_2AsO_3^-$ are less prevalent (Davolos and Pietrangeli 2013, and literature therein). Consequently, we focused on the assessment of As(V) toxicity. The LC50 value we estimated for *G. elvirae* following 240-h exposure to arsenate was found to be 1000 μg L^{-1}. A 1 % mortality was observed in control reservoirs, while As(V) toxicity appeared to increase when the exposure time was extended (data not shown). It should be noted that the LC50As(V) value we obtained for *G. elvirae* collected from As-unpolluted sites is very similar to the value of 989.75 μg L^{-1} obtained by Vellinger et al. (2012) following 240-h exposure for the phylogenetically distinct *G. pulex* (see online resource 1 in the ESM). This suggests that there may be little interspecies variation within the genus *Gammarus* in response to arsenate toxicity. Overall, the information on the LC50As(V) we obtained in this study for *G. elvirae* and that available in the literature for other freshwater *Gammarus* species point to the usefulness of these crustacean taxa as a means of assessing As(V) toxicity in aquatic systems.

Conclusion

The results of our study enhance knowledge of the ecologically relevant species *G. elvirae* by providing new nuclear gene sequences, genotoxicological results, histological data, and an As(V) toxicity assessment, thereby paving the way for further studies on this species. In particular, the Comet assay warrants consideration in future genotoxic assays for the identification and quantification of damage caused at the DNA level in the hemocytes of *G. elvirae* after exposure to As-contaminated freshwater. Lastly, we hypothesize that As-related damage of hepatopancreatic cells is likely to be observed in experimental organisms belonging to this species when compared with non-exposed individuals. Our integrated study highlights the potential of *G. elvirae* as a means of assessing sensitivity towards (geno)toxic pressure in freshwater. In line with this perspective, we may tentatively conclude that other species of the *G. lacustris* clade, along the phylogenetic diversity of freshwater *Gammarus* species, are candidates for the essays we present here and may constitute valuable models for testing the toxic consequences of polluted freshwater.

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Conflict of interest The authors declare that they have no competing interests.

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