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Accumulation of metal(loid)s in the digestive gland of the mussel *Unio crassus* Philipsson, 1788: a reliable detection of historical freshwater contamination

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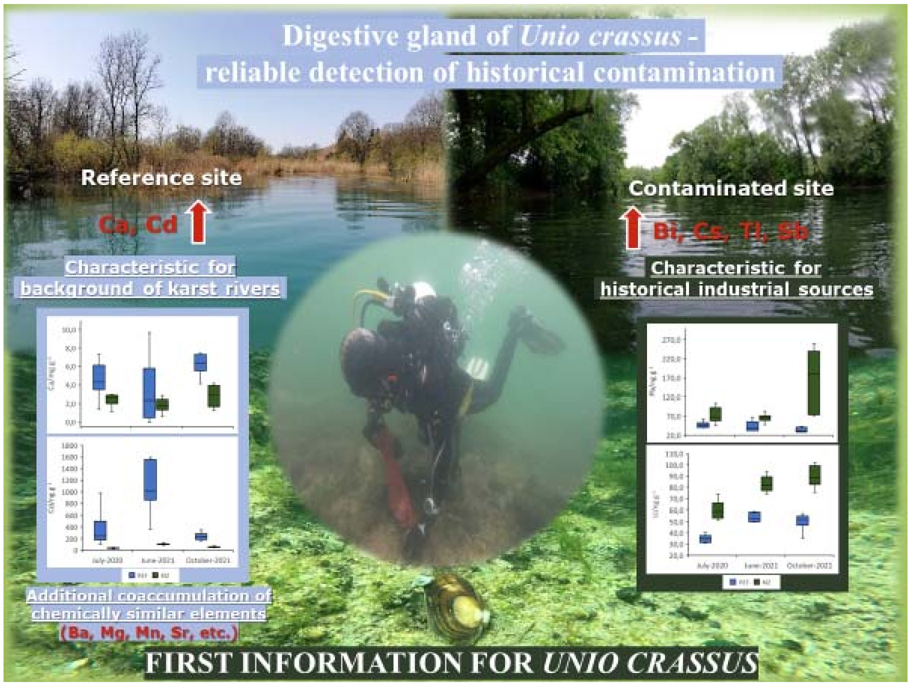
Graphical abstract



Highlights

- First report on total/cytosolic metal(loid) accumulation for *Unio crassus* mussels
- Mussel digestive gland – reliable detection of historical freshwater contamination
- Cytosolic levels reveal metal(loid) bioavailability better than total tissue loads
- Importance of considering metal coaccumulation due to element chemical similarity
- Seasonal changes of metal loads in digestive gland caused by reproductive cycle

Abstract

The possible influence of historical contamination of water/sediments on the metal(loid) bioaccumulation in the digestive gland of mussel *Unio crassus* Philipsson, 1788, from two differently contaminated sites at the Mrežnica River was studied in three seasons. The first data for this species on total/cytosolic concentrations of 27 (non)essential elements were obtained by HR ICP-MS. Higher bioaccumulation was observed at the historically contaminated site, with several nonessential elements (Bi, Cs, Pb, Sb, Tl, U) found in 5-6 times higher concentrations compared to the reference site. Although both total and cytosolic levels revealed the influence of water/sediment contamination, the latter showed association between bioaccumulation and exposure for larger number of studied elements. At the reference site, several elements (Ba, Ca, Cd, Cr, Mn, Sr) were also found in 2-10 times higher concentrations compared to contaminated one, but it was attributed to background levels characteristic for karst rivers (for Ca and Cd), and to coaccumulation due to chemical similarity (for Ba, Cr, Mn, Sr). The seasonal variability was also observed, with generally highest metal(loid) concentrations in mussel digestive glands found in autumn which was associated to mussels reproductive period. Our results confirmed that sediment-dwelling mussels, specifically *U. crassus*, represent a good bioindicators for detection of historical pollution due to their direct contact/exposure to contaminants stored in sediments, with concurrent consideration of physiological/chemical factors. Historical contamination potentially can have serious impact on freshwater environment even long time after its cessation, and, therefore, a careful continuous monitoring is recommended.

Key words: bioaccumulation, bivalve, freshwater, long-term pollution, subcellular distribution, trace elements

1. Introduction

Freshwater ecosystems are under constant pressure from multiple sources of metal contamination - urbanization, industrialization, and anthropogenic activities, all of which contribute to water/sediment contamination and to decline in biodiversity (Dudgeon et al., 2006). Metal(loid)s bound to the sediment can be remobilized and become repeatedly available to benthic organisms, such as mussels. Freshwater mussels, as sedentary, long-living sediment-dwelling filter feeders with a great capacity for metal accumulation, could be useful indicators of water and sediment pollution with metals in a given area over an extended period of time (Naimo, 1995). Therefore, freshwater mussels are used in many biomonitoring programs (Falfushynska et al., 2009; Khoma, 2019; Yoloğlu et al., 2018).

This study was conducted on *Unio crassus* Philipsson, 1788, thick-shelled river mussels from the Croatian river Mrežnica. The freshwater mussel *U. crassus* is widely distributed in central, southeastern, and northern Europe, including Croatia, and is a representative species in the Mrežnica River (Lopes-Lima et al., 2017). The Mrežnica River is a karst/lowland river of great national and international importance and is a part of the Natura 2000 network, which aims to protect natural habitats and species. As previously minutely described (Dragun et al., 2022), this river has been exposed to various sources of pollution in the past, especially in lower part of its watercourse. From 1884 to early 2000s, the river was exposed to the effluents of the textile industry in the Duga Resa town. Recent studies have shown that some contamination with pollutants from the textile industry, such as Pb and Zn, whose salts were used for softening and dyeing textiles, and Cu and Cr as components of dyeing and bleaching effluents (Hurley et al., 2017, and the references therein), can still be detected, especially in the river sediments (Frančišković-Bilinski et al., 2017; Dragun et al. 2022). In addition to the common contamination from the textile industry, this factory burned coal for 110 years, until 1994, and discharged coal slag and ash directly into the Mrežnica River (Frančišković-Bilinski, 2008), which could likely be the source of elevated concentrations of U and Fe not only at sites near the former textile factory, but also downstream of the factory (Frančišković-Bilinski et al., 2017). In addition, numerous industrial facilities were built on both watersides of the Mrežnica River in the industrial area of the city of Karlovac, some of which date back to the 1930s.

About ten years ago, many of these facilities were connected to the Karlovac city wastewater treatment plant, and the treated wastewater is now discharged in the Kupa River downstream from Karlovac (Dragun et al. 2022). However, a recent study of Dragun et al. (2022) revealed that some elements such as Al, Cr, Cs, Li, Mo, and Ni were still present in the sediment in 5-17 times higher concentration at the location near the industrial area of the city of Karlovac compared to the reference location. So, all studies conducted upstream or nearby the contaminated sampling site of this study have shown that remnants of past contamination can still be detected, even though a number of plants have closed or stopped discharging their effluents directly into the river more than a decade ago (Dragun et al., 2022), and thus could potentially have negative impacts on the aquatic biota of the Mrežnica River, including mussels.

When bivalves are used as bioindicators, a commonly used tissue to assess environmental quality is their digestive gland, the major storage and detoxification organ that plays an important role in detoxification and long-term storage of metals (Faggio et al., 2018 and references therein; Gupta and Singh, 2011). Thus, it is often a more sensitive target organ for monitoring water pollution with metals than the whole organism (Ivanković et al., 2005; Regoli and Orlando, 1994), especially in the cases of long-term exposure in the past. Regardless, the studies conducted so far on metal bioaccumulation in the digestive gland of bivalves of the family Unionidae have encompassed only total concentrations of a limited number of elements (Falfushynska et al., 2014, 2013; Khoma, 2019).

Such data, to the best of our knowledge, are not available for the digestive gland of *U. crassus*. Furthermore, the soluble cytosolic fraction of digestive gland tissue, containing various metalloproteins and compartmental vesicles, is thought to represent the metabolically available and thus potentially toxic fraction of tissue metal(loid)s (Amiard et al., 2006; Naimo, 1995; Viarengo and Nott, 1993). Accordingly, analysis of the concentrations of metal(loid)s in cytosolic tissue fraction may provide significant information on potential metal toxicity, and serve as an important supplement to the analysis of total tissue metals. Cytosolic metal concentrations are also often a better indicators of actual water contamination than total metal levels (Langston et al., 1998), although metals in the soluble fraction sometimes present only a small fraction of the total metal load. Our main goal was, thus, to provide comprehensive data sets on metal(loid) concentrations in the digestive gland of *U. crassus*, both total and cytosolic, that could serve as a basis for comparison in future monitoring studies. The data presented here are the first data of such kind on the total/cytosolic concentrations of twenty-seven metal(loid)s in the digestive gland of *U. crassus*. In addition, for each metal(loid) analyzed, we calculated the proportion of the cytosolic metal(loid) fraction in the total tissue load, to determine whether the proportion of the cytosolic metal(loid) fraction changed with increasing exposure/bioaccumulation.

Our further objective was to compare bioaccumulated metal(loid) concentrations (both total and cytosolic) at two sampling sites on the Mrežnica River characterized by different anthropogenic influences previously described in detail in the second paragraph of this section, in order to determine whether the long-term historical pollution of the Mrežnica River resulted in an increased bioaccumulation of metal(loid)s in the digestive gland of the mussel *U. crassus*, as a representative bioindicator organism. Also, we wanted to determine whether the cytosolic concentrations of elements in the digestive gland of *U. crassus* might be a better indicator of water pollution than their total concentrations.

Since there are many environmental and biological factors that can influence fluctuations in element concentrations both in the environment and in the organisms (Erk et al., 2018; Ravera et al., 2007; Regoli and Orlando, 1994), seasonal variability in metal(loid) concentrations in the digestive gland of *U. crassus* has also been studied.

2. Materials and methods

2.1. Study area and periods of mussel samplings

The sampling of thick-shelled river mussels (*U. crassus*) was performed at two sites in the Mrežnica River in Croatia (Fig. SI. 1). The first sampling site, located about 2 km upstream from the town of Duga Resa, represented the site of expectedly low anthropogenic influence (i.e. the REF site, N 45° 26' 28,40'' E 15° 30' 15,39''), and the second, a contaminated site, was located in the industrial zone

Mala Švarča of the town of Karlovac (i.e. the KIZ site, N 45° 27' 49,05" E 15° 32' 30,09") (Fig. SI. 1). The detailed information on the geographical characteristics of the studied section of the Mrežnica River, as well as hydrological and physico-chemical data referring to the time of mussels' sampling are presented in our previous paper (Dragun et al., 2022). Samples of *U. crassus* were collected by scuba-diving in three sampling campaigns (early summer - mid-July 2020; late spring - early June 2021; and early autumn - early October 2021). Mussels were transported to the laboratory in dark and cool containers where they were depurated 24 hours prior to dissection.

2.2. Dissection and preparation of the mussel digestive gland samples

The digestive glands were removed, frozen into liquid nitrogen, and later kept at -80 °C until further treatment. To obtain sufficient tissue for analysis, composite samples were prepared, each containing digestive gland from four to five mussels. After the samples were cut into small pieces, cooled homogenization buffer (20 mM Tris-HCl buffer, pH 8.6, 22 °C) containing 0.01% β-mercaptoethanol and protease inhibitors (0.006mM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride) was added (w/v 1:4). Following step was homogenization with a Potter-Elvehjem homogenizer (Glas-Col, USA). An aliquot of each homogenate was set aside for subsequent acid digestion. The rest of each homogenate was centrifuged at 50,000 x g for 2 hours at 4 °C (Avanti J-E centrifuge, Beckman Coulter). Thus, the supernatant, the soluble cytosolic fraction of the digestive gland, was obtained.

2.3. Digestion of homogenates and cytosolic fractions prior to metal(loid) measurement

Digestive gland homogenates were digested by the addition of an oxidation mixture (v/v 1:3), which contained concentrated HNO_3 (Normatom® 67–69% for trace element analysis, VWR Chemicals, UK) and 30% H_2O_2 (Suprapur®, Merck, Germany) (v/v 3:1). Cytosolic fractions were digested with the same oxidation mixture but in a ratio to the cytosol of 1:1 (v/v). Digestions were performed in a laboratory dry oven at 85 °C for 3.5 h. After digestion, samples were diluted with Mili-Q water, 1:4 prior to Ca and trace element analysis, and 1:19 for Na, K, and Mg analysis.

2.4. Metal(loid) analysis

Twenty-seven (non)essential elements were analyzed using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany) equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister. Measurements of ^{82}Se, ^{85}Rb, ^{98}Mo, ^{109}Ag, ^{111}Cd, ^{121}Sb, ^{133}Cs, ^{205}Tl, ^{208}Pb, ^{209}Bi and ^{238}U, were operated in low resolution mode; of ^{23}Na, ^{24}Mg, ^{27}Al, ^{42}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{86}Sr and ^{138}Ba in medium resolution mode; and of ^{39}K and ^{75}As in high resolution mode. External calibration mode was applied using adequate dilutions of multielement stock standard solution (Analitika, Czech Republic) in 2% (vol.) HNO_3 (Normatom® 67–69%, VWR Chemicals, UK) supplemented with standard solutions of Cs (Fluka, Germany), Rb and U (Aldrich, USA), and Sb (Analytika, Czech Republic). Separate external calibrations were made using multielement standard for macro elements (Fluka, Germany) and standard solution of Ag (Fluka, Germany). Before measurement, indium (1 µg/L; Fluka, Germany) was added in all samples and calibration standards as an internal standard. All measurements were performed in duplicate. For checking the accuracy of HR ICP-MS measurements, quality control samples obtained from UNEP/GEMS (Burlington, Canada) were used. A generally good agreement was observed between our data and certified values (Table SI.1). Limits of detection (LOD) for all elements in homogenates and cytosolic fractions, were calculated as three standard deviations of ten metal(loid) measurements in the blank sample (supplemented homogenization buffer, as described above), digested in the same manner as homogenates/cytosols of the digestive gland tissue (Table SI.1). The results obtained by measurement in digested homogenates are referred to as total metal(loid) concentrations, whereas the results obtained by measurement in cytosolic fractions (supernatants) are referred to as cytosolic metal(loid) concentrations.

2.5. Data processing and statistical analysis

SigmaPlot 11.0 and Statistica 12.5 for Windows were used for statistical analyses. Basic calculations were performed in Microsoft Office Excel 2011, and graphs were created in Microsoft Office Excel 2011 and Statistica 12.5 for Windows. Comparisons of total and cytosolic metal(loid) concentrations in the digestive gland of *U. crassus* at two sampling sites within each season were performed using the Mann-Whitney U test. To compare total and cytosolic metal(loid) concentrations between seasons at each sampling site, a Kruskal-Wallis ANOVA test was performed in combination with a Dunn post-hoc test. A Spearman correlation analysis was used to determine the degree of association between element concentrations in the digestive gland of *U. crassus* and their concentrations in river water/sediment. The level of significance was set at 95% ($p < 0.05$) for all tests applied.

3. Results and discussion

<u>*3.1. Total metal(loid) concentrations in the digestive gland of U. crassus*</u>

3.1.1. Spatial variability and relationship to metal(loid) concentrations in the environment

Based on metal(loid)s' role in mussel organisms, they are classified as essential or non-essential elements (Ali et al., 2019). We used the same classification when evaluating our data. The total concentrations of the analyzed twenty-seven elements in the digestive gland of *U. crassus* sampled from two sites of the Mrežnica River are shown in Figs. 1 and 2 and can be classified for essential elements as follows: 5 mg/g > Ca > 1 mg/g > Fe, K, Mg, Mn, Na > 100 µg/g > Zn, Cu > 1 µg/g > Co, Mo, Se, V > 0.030 µg/g; and for non-essential elements, as follows: 15 µg/g > Al, Ba, Cr, Sr > 1.5 µg/g > As, Cd_{REF}, Ni, Pb_{KIZ}, Rb > 0.100 µg/g > Ag, Cd_{KIZ}, Pb_{REF}, U > 0.010 µg/g > Bi, Cs, Sb, Tl > 0.001 µg/g.

Comparison of total (non)essential element concentrations in the digestive gland of *U. crassus* from two sampling sites revealed three spatial patterns: 1) some elements had comparable concentrations at both sites; 2) some elements had higher concentrations at the KIZ site; and 3) some elements had higher concentrations at the REF site (Figs. 1 and 2).

In most cases, comparable concentrations between two sites were found for five essential elements (Fe, K, Se, V, and Zn, Fig. 1) and four non-essential elements (Ag, Al, Ni, and Rb, Fig. 2). This could be due to the fact that metal loadings in the water of the Mrežnica River did not differ sufficiently between two sampling sites. However, our previous study (Dragun et al., 2022) revealed that river water, and even more the sediment, of the Mrežnica River had higher concentrations of some of these elements at the KIZ than at the REF site; several were increased even in the dissolved form in the water, and almost all in the sediment. Thus, another explanation is plausible, that the physiological regulation of the above essential and non-essential elements was quite effective in the *U. crassus* mussel species. It is in accordance with the known fact that mussels possess the ability to regulate certain essential and non-essential elements, up to a certain threshold, through various biological mechanisms (enzymes, active membrane ion pumps, etc.) (Thorsen et al., 2006).

The total concentrations of two essential elements (Cu and Mo, Fig. 1) and six non-essential elements (Bi, Cs, Pb, Sb, Tl, and U, Fig. 2) in the digestive gland of *U. crassus* from the KIZ site were significantly higher compared to the REF site. The values of essential elements Cu and Mo were up to two times higher; while of non-essential Bi, Cs, Pb, Sb, Tl, and U were up to five to six times higher at the KIZ site compared to the REF site. The higher bioaccumulation of these elements in the digestive gland generally corresponded to their higher concentrations in the sediment and in some cases also in the water of the Mrežnica River at the KIZ site (Dragun et al., 2022). Although many industrial facilities (e.g. former textile industry in Duga Resa) upstream of the KIZ site have not discharged their effluents into the Mrežnica River for more than a decade, elevated concentrations of various elements, including those mentioned above, have been continuously observed in the water and/or sediment at this site (Dragun et al., 2022; Frančišković, 2008; Frančišković-Bilinski et al., 2017). Among the elements increased at the KIZ site, the most prominent differences in total concentrations in the digestive gland of *U. crassus* between the two sites were observed for Bi, whose use has greatly increased in various spheres of life, especially in medicine (Das et al., 2006). Hospitals and the medical industry have been discharging their effluents into the Mrežnica River for a long time at the KIZ site (Dragun et al., 2022), which could explain almost six times higher total concentrations of Bi in the digestive gland of *U. crassus* sampled at the KIZ site compared to the REF site in October 2021. Furthermore, the information about Bi biological role in mussels is very poor and should be further investigated. Thus, our results confirmed that even long after the cessation/reduction of the activities of the river pollutants, the accumulation of non-essential elements, such as Bi, Cs, Pb, Sb, Tl, and U, in the digestive gland of *U. crassus* can be increased, which, considering the fact that they are potentially toxic, could have harmful consequences for this mussel species, and even indicate a potential threat to the other aquatic organisms. In addition, the multiple increases in the concentrations of aforementioned elements in the digestive gland of *U. crassus* could indicate tendency of this species to accumulate metal(loid)s in their main digestive organ, as well as confirm the absence of effective regulatory mechanisms for studied non-essential elements, and even some essential, in *U. crassus*. Accordingly, the almost twofold increases in Cu and Mo concentrations, although essential elements, suggested that their concentrations are only partially regulated in the digestive gland of *U. crassus*. And, despite their essential roles as cofactors for a number of enzymes (e.g., Cu in cytochrome oxidase, superoxide dismutase, etc.), in higher concentrations even essential elements can exerts a toxic effect (e.g. Cu - by producing reactive oxygen species) (Gomes et al., 2011). Hence, Cu bioaccumulation tendency and toxicity potential were demonstrated in a laboratory study on the digestive gland cells in the swollen river mussel, *Unio tumidus* Philipsson, 1788, which showed that at a certain Cu concentration level, the antioxidant capacity decreases, and apoptosis and necrosis of the cells are induced (Labieniec and Gabryelak, 2007). Toxic effects of Cu were further observed in the duck mussel *Anodonta anatina* (Linnaeus, 1758) and swan mussel *Anodonta cygnea* (Linnaeus, 1758) exposed to increased environmental/laboratory concentrations of Cu (Falfushynska et al., 2013; Huebner and Pynnonen, 1992). Constant exposure to metal contaminants certainly burdens mussels' defensive systems which could have a harmful effect on these organisms even during sensitive, larval stages of life and thus have a negative impact on the entire mussel population (Naimo, 1995).

On the other hand, six essential elements (Ca, Co, Mg, Mn, Na, and Se) (Fig. 1) and five non-essential elements (As, Ba, Cd, Cr, and Sr) (Fig. 2) generally had higher total concentrations in the digestive gland of *U. crassus* at the REF site than at the KIZ site. The differences were statistically significant for Ba, Ca, Cr, Mn, and Sr in all sampling campaigns and amounted to two to four times, depending on the campaign and element. In its upper section, the Mrežnica River is a carbonate-rich river, which can explain relatively high Ca concentrations in sediment at relatively clean sites (Dragun et al., 2022), as well as a higher level of bioaccumulation in *U. crassus* digestive gland at the same site. However, the increased bioaccumulation of the majority of remaining elements (Ba, Cr, Mn, and Sr) at the REF site could not be connected to sediment contamination (Dragun et al., 2022), but rather to calcium mimic hypothesis, according to which mussels coaccumulate some trace elements, such as Sr, Ba, Mg, and Mn, due to their chemical similarity to Ca, regardless of the physiological needs of the organism and/or environmental exposure (Beone et al., 2003; Markich and Jeffree, 1994). However, the most significant differences between sites were found for Cd. Cadmium concentrations in the digestive gland of *U. crassus* were about 10 times higher at the REF site in the spring and summer campaigns, and four times in the autumn. It was consistant with the occasionally higher Cd concentrations in sediment of the Mrežnica River at the REF site compared to the KIZ site (Dragun et al., 2022). Higher Cd concentrations were also found in several karst areas in central and eastern France (Rambeau et al., 2010) and in southwestern China (Zhan et al., 2021), regardless of pollution sources, indicating higher geological background levels of Cd in karst water systems, which may explain higher Cd concentrations in bedrock and consequently mussels at REF site. Karst landscapes are characterized by soluble carbonate rocks such as limestone or dolomite, which often have high Cd concentrations (Wu et al., 2020). These rocks are susceptible to dissolution and weathering, and carbonate weathering is considered a major cause of Cd enrichment in karst watersheds (Wu et al., 2020). In addition, in carbonates and hydrous oxides of karst waters Cd is bound in the less stable and exchangeable form, and thus can be easily mobilized (Kubier et al., 2003).

As filter-feeding organisms, bivalves are exposed to both dissolved pollutants and pollutants bound to dispersed particles or deposited in the sediment (Naimo, 1995). Since bivalves are exposed to different species of the elements present in different riverine compartments (dissolved and particulate phases of water, as well as the sediment), we performed a Spearman correlation analysis to determine if there was a relationship between the bioaccumulated amount of elements in the digestive gland of

U. crassus and the element concentrations in each of the above river-water compartments (Table SI. 2). We wanted to determine which river compartment might contribute more to the bioaccumulation of certain elements in the digestive gland of *U. crassus* mussels. Data on the metal(loid) concentrations in these compartments of the Mrežnica River in the time of mussel samplings were previously reported by Dragun et al. (2022). The analysis showed a relatively high degree of association between the bioaccumulation of elements in the digestive gland of *U. crassus* and their concentrations in the ambient environment. Thus, 17 of the 27 elements analyzed in the digestive gland showed a positive correlation with metal(loid) concentrations in one or more of the river-water compartments studied. Six bioaccumulated elements, Cs, Sr, Al, Rb, Ca, and Mg, showed moderate ($r = 0.5$-0.7) to strong ($r > 0.8$) positive correlations with their concentrations in the dissolved water fraction. Three bioaccumulated elements (Mo, Pb, and U) showed a strong correlation ($r = 0.8$-1) with their concentrations in the particulate fraction of surface water, and another five bioaccumulated elements (Cd, Co, Cu, Mn, and V) showed low to moderate correlation. ($r = 0.4$-0.5) with this water fraction, and a larger number of bioaccumulated elements (nine) showed a strong positive association with their concentrations in the sediments: Cu, Sb, and U ($r > 0.8$); Bi, Cs, Pb, and Tl ($r = 0.7$-0.8); and Ca and Mo ($r = 0.5$-0.6), indicating that exposure from sediment/resuspended sediment has probably mostly influenced the metal(loid) bioaccumulation in the mussels. These findings can be associated with the fact that the surface water contamination with metals in the Mrežnica River was less notable in the dissolved phase than in the sediment/particulate matter, which is characteristic for the historically contaminated sites. Thus, the mussels have reflected the source of metals they were exposed to. As mussels live/thrive by filter feeding, i.e. by passing water through the gills and collecting food particles from the water (Smith et al., 2012), it could have been expected that they will be more susceptible to contaminants bound to the particles and stored within the sediment compared to the other aquatic organisms. Thus, they present an excellent bioindicator species specifically for the monitoring of the contamination of the sediment, which is typical for the historical freshwater pollution.

To put our results in a broader context, we compared total metal(loid) concentrations in the digestive gland of *U. crassus* with the available information on metal(loid) concentrations in the digestive gland of freshwater mussels from differently contaminated ecosystems worldwide. As there is no data available on metal(loid) bioaccumulation for *U. crassus*, our results being the first of this kind for this species, the comparison was made with other species from the same family, i.e. Unionidae (Table 1). The information were generally gathered on the accumulation of Cd, Cu, and Zn in the digestive gland of freshwater mussels, as most of these studies were focused on metallothioneins (MTs) – low molecular weight proteins responsible for homeostasis and detoxification of the aforementioned elements (Amiard et al., 2006). Therefore, it should be emphasized that our study provided, for the first time, the information for a much larger set of elements than currently available in the literature for any member of the family Unionidae. The only study that included somewhat more extensive set of eight elements, namely As, Cd, Cr, Cu, Fe, Ni, Pb, and Zn, was found for the freshwater mussel, *Unio mancus* Lamarck, 1819, sampled from the Atatürk Dam Lake, at the sites polluted with either agricultural, or municipal and industrial effluents (Yoloğlu et al., 2018). Several of these elements, namely As, Ni, Pb and Zn, were either comparable or even lower in *U. crassus* in our study compared to *U. mancus*, regardless of the type of contamination (Table 1). Contrary, the remaining four elements were generally higher in *U. crassus* in our study compared to *U. mancus*; specifically, Cr and Fe at both REF and KIZ sites, indicating higher exposure to these elements in the Mrežnica River independently on the studied area; on the other hand, higher concentrations of Cd and Cu in *U. crassus* compared to *U. mancus* were confined to REF and KIZ site, respectively. The comparisons were further made for Cd, Cu, and Zn from three other studies on mussel species from Unionidae family (Table 1). The concentrations of Cd recorded at the KIZ site in the digestive gland of *U. crassus* were lower compared to all freshwater mussels presented in Table 1. On the other hand, Cd concentrations in the digestive gland of *U. crassus* at the REF site were generally higher compared to those found in freshwater mussels sampled at sites with agricultural activities, or, more specifically, they were higher than Cd in *U. tumidus* from Seret and Zhvanchyk rivers (Khoma, 2019), and *A. cygnea* from Dniester river basin (Falfushynska et al., 2009) (Table 1). It was, however, somewhat lower compared to *A. anatina* from Dniester river basin, for which Falfushynska et al. (2014) reported twice as high Cd concentrations compared to our study even for specimens from the forestry site, which was reported as the reference site. When compared to other freshwater mussels sampled at sites of various types of contamination, both Zn and Cu concentrations were lower in *U. crassus*, up to ten and six times, respectively (Table 1). The only exceptions were almost twice higher values of Cu in *U. crassus* at the KIZ site compared to *U. tumidus* (Khoma, 2019; Table 1). It should be noted, however, that differences in the bioaccumulation of certain elements may vary among closely related bivalve species, i.e., species of the same bivalve family, as it depends on the growth rate of the bivalves, nutritional habits, and other inherent factors specific for each mussel species (Pourang et al., 2010). Thus, discussed differences in bioaccumulated levels of metal(loid)s between *U. crassus* and other mussels from Unionidae family could be regarded as a combined effect of the differences in the metal(loid) exposure and biological diversity, only further emphasizing the importance of establishing the baseline values for each bioindicator species. Still, based on the data obtained and the above presented comparisons with other published reports of metal(loid)s in the digestive gland of freshwater mussel species, it can be concluded that chronic exposure to some metal contaminants have resulted in increased bioaccumulation which consequently may have a negative impact on the freshwater ecosystem.

3.1.2. Seasonal differences in total metal(loid) concentrations

Various biotic and abiotic factors (reproductive cycle, metabolism, ambient temperature, dissolved oxygen in the water, etc.) can affect the physiological requirements and environmental bioavailability of metals, which may lead to seasonal differences in the metal concentrations of mussel organisms independent of the pollution of the aquatic system (Erk et al., 2018; Ravera et al., 2007; Regoli and Orlando, 1994). In this study, total metal concentration data collected from three sampling campaigns at different times of the year (early summer - mid-July 2020; late spring - early June 2021; and early autumn - early October 2021) indicated significant seasonality of total metal(loid) concentrations in the digestive gland of *U. crassus* (Figs. 1 and 2). In general, higher element concentrations were measured in the autumn sampling campaign in comparison to the spring and summer. Significantly higher values in the autumn compared to the spring campaign were observed at the REF site for two essential (Co, and Fe, Fig. 1) and three non-essential elements (Ba, Cr, and Rb, Fig. 2); and compared to the summer sampling campaign for six essential (Cu, Fe, Mg, Mn, Na, and Zn, Fig. 1) and two non-essential elements (As, and U, Fig. 2). In addition, four essential (Fe, Mg, Na, and Zn, Fig. 1) and three non-essential elements (Ag, Ni, and Tl, Fig. 2) were significantly higher in the autumn compared to the summer and spring sampling campaigns at the KIZ site. Contrary, significantly higher total concentrations in the spring compared to summer and autumn sampling campaigns were observed for four elements, two essential (Mo, and V, Fig. 1) and two non-essential (Bi, and Cd, Fig. 2) at the REF site, while only Bi had significantly higher concentrations in the spring compared to the other two sampling periods at the KIZ site (Fig. 2). Our results are consistent with the study of Ravera et al. (2007), who also observed a similar seasonal pattern of metal accumulation in shells and whole tissues of two *Unio pictorum mancus* Lamarck, 1819, populations. An explanation for the generally highest element bioaccumulation during autumn seasons could be found in the reproductive cycle and/or feeding behavior (Almeshal et al., 2022). The reproductive season of *Unio* spp. is quite short, lasting from April to mid-August, when glochidia are released immediately after maturation (Hochwald, 2001). A studies on marine bivalves, the blue mussel *Mytilus edulis* Linnaeus, 1758 (Regoli and Orlando, 1994), and the Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819 (Raspor et al., 2004), showed a decrease in metal concentrations in mussel digestive gland during gametogenesis. The probable cause of this could be the invasion of gonadal tissue into the digestive gland during the breeding season. It is well known that mussels' digestive gland mass varies greatly during the year due to development of gonadal tissues which penetrate into the digestive gland and make difficult to completely physically separate the two tissues by dissection (Regoli and Orlando, 1994; Raspor et al., 2004). Therefore, gonadal development biologically dilutes the total concentrations of (non)essential elements during the warmer seasons, even in contaminated areas (Ivanković et al., 2005). These seasonal variations in total metal concentrations should be taken into account when assessing the contamination status of the sampled area. Unfortunately, fluctuations in total (non)essential element concentrations during the spawning season cannot be readily predicted because spawning in *U. crassus* species occurs asynchronously and several times during the reproductive season (Hochwald, 2001). In addition, degeneration of the structure of the digestive tubules and an increase in autophagic processes occur during the reproductive season (Regoli and Orlando, 1994, and references therein), and all of this may have a significant effect on total metal(loid) concentrations in the digestive gland of *U. crassus*. The lower nutrient levels measured in the autumn compared to other sampling campaigns in the Mrežnica River (Dragun et al., 2022) could also affect the composition of the digestive gland. When the food supply is lower, the energy reserves stored in the digestive gland are used for the metabolic needs of the organisms (Saout et al., 1999). Therefore, even if the content of (non)essential elements remains the same, a higher energy release from the digestive gland (Paulet et al., 2006; Ravera et al., 2007) could cause an apparent increase in the total element concentration. Based on the data obtained and the information on seasonal variations in other mussel species, it can be concluded that for a correct interpretation of the analytical values, the combined effect of the physical environment and the biological state of the organism should be taken into account. However, although a seasonal trend in accumulation was observed for most of the metal(loid)s studied, it should be noted that the seasonal variability in total metal(loid) concentrations did not mask the observed differences in metal(loid) accumulation between the two sampling sites (Figs. 1 and 2).

3.2. Cytosolic metal(loid) concentrations in the digestive gland of U. crassus

3.2.1. Spatial variability and relationship to total metal(loid) concentrations

In addition to total element levels, the concentrations of 27 (non)essential elements were also measured in the soluble, cytosolic fractions of the digestive gland of *U. crassus* which represent metabolically active, potentially toxic tissue metal(loid) compartment (Amiard et al., 2006; Naimo, 1995; Viarengo and Nott, 1993). Presented cytosolic concentrations (Tables 2 and 3) are the first data of such kind for any organ of *U. crassus* mussel species, and specifically for its digestive gland. Furthermore, data for cytosolic concentrations in the digestive gland of other members of the family Unionidae are also rather scarce, and available for only few elements (Cd, Cu, and Zn) (Bonneris et al., 2005; Campbell et al., 2005).

The cytosolic concentrations of analyzed essential elements were found in the following ranges:

K (286-334 µg/g) > Na (140-174 µg/g) > Ca (82.9-139 µg/g) > Mg (58.1-72.9 µg/g) > Fe (8.64-17.3 µg/g) > Zn (6.73-8.75 µg/g) > Mn (1.52-4.13 µg/g) > Cu (1.41-3.67 µg/g) > Se (0.530-0.672 µg/g) > Co (0.105-0.177 µg/g) > V (0.102-0.166 µg/g) > Mo (0.099-0.144 µg/g).

The cytosolic concentrations of analyzed non-essential elements were found in the following ranges: Cd (0.037-1.14 µg/g) > Al (0.196-0.874 µg/g) > Ba (0.138-0.492 µg/g) > As (0.277-0.463 µg/g) > Rb (0.252-0.454 µg/g) > Cr (0.038-0.109 µg/g) > Sr (0.083-0.108 µg/g) > Ni (0.028-0.093 µg/g) > Pb (0.019-0.066 µg/g) > U (0.018-0.044 µg/g) > Ag (0.004-0.012 µg/g) > Bi (0.002-0.004 µg/g) > Cs (0.001-0.003 µg/g) > Sb, Tl (0.001-0.002 µg/g).

Since cytosolic concentrations for mussels belonging to the family Unionidae so far were not reported for such a large set of elements, it was not possible to made adequate comparisons, but rather to set an initial data base which will serve for comparisons in the future monitorings.

Cytosolic concentrations of five essential (Cu, Fe, Co, Mo, and V, Table 2) and 11 non-essential elements (Ag, Al, As, Bi, Cr, Cs, Ni, Pb, Sb, Tl and U, Table 3) were significantly higher in *U. crassus* digestive gland at the KIZ site compared to the REF site. On the other hand, cytosolic concentrations of Mn and two non-essential elements (Ba and Cd) were significantly higher in *U. crassus* digestive gland at the REF site compared to the KIZ site. Once again, concentrations of Cd were up to 12 times higher at the REF location. For most of the elements, the spatial patterns of cytosolic concentrations in the digestive gland of *U. crassus* were generally similar to those of their total concentrations. The correlations of cytosolic concentrations with element concentrations in the three riverine compartments were also similar to those observed for the total concentrations in mussel digestive gland for most elements. The exceptions were cytosolic Al, As, Ba, Co, Cr, Fe, K, and Mg, which, unlike their total levels, showed a positive association with concentrations of corresponding elements in the particulate water fraction and/or sediment (Table SI. 2). The obtained data indicated that a larger number of elements reflected the environmental exposure when measurements were performed in the cytosolic tissue fraction compared to total tissue load. Cytosol of the mussel digestive gland thus could be recommended as a target fraction for monitoring of metal(loid) bioavailability, i.e. the contamination status of the aquatic environment.

3.2.2. Partitioning of metal(loid)s in the soluble, cytosolic fraction

Campbell et al. (2005) observed a gradual increase in metal concentrations in the soluble fraction of freshwater mussels' tissues with increasing environmental metal levels. Therefore, as a next step, the proportions of each element in the soluble tissue fraction were evaluated, based on the ratios between cytosolic and total metal(loid) concentrations in *U. crassus* digestive gland (Tables 2 and 3), separately for the REF and KIZ sites. The percentages of analyzed elements present in the soluble, cytosolic digestive gland fraction of *U. crassus* from the Mrežnica River reference area (the site REF) decreased in the following order: Bi, Na, Cd (~100%) > K, Rb, Se (80-99%) > Pb, Mo, Cs, Cu, Zn, V (50-79%) > U, Sb, Tl, As (40-49%) > Co, Mg, Ag, Ni, Al, Fe, Cr, Ba, Ca, Sr, Mn > 0.7% (Tables 2 and 3). The percentages of analyzed elements present in the soluble, cytosolic digestive gland fraction of *U. crassus* from the Mrežnica River contaminated site (the site KIZ) decreased in the following order: Na (~100%) > Sb, K, Cd, Se, Rb (80-99%) > Cs, Cu, Mo, Pb, Zn, Bi, Co, As, V, U (50-79%) > Ni, Tl, Ag, Mg (40-49%) > Cr, Al, Ba, Fe, Ca, Sr, Mn > 1% (Tables 2 and 3).

Similar to reports by Campbell et al. (2005), in our study eight non-essential elements (Ag, Al, As, Cr, Cs, Ni, Sb, and U, Table 3) were more present in the soluble fraction of the digestive gland of the mussels with the higher cytosolic metal(loid) load, i.e. at the KIZ site of the Mrežnica River compared to the REF site. High presence of metal(loid)s in the soluble tissue fraction could represent the potential for toxic effects since those elements are more available for participation in the various metabolic reactions. In our study, Cd was almost completely present in the soluble cytosolic digestive gland fraction (≥93%) (Table 3) of *U. crassus* at both REF and KIZ sites of the Mrežnica River. Cadmium is a well-known inducer of MTs, proteins important for the regulation and detoxification of several (non)essential elements (Amiard et al., 2006), which lessens to a certain degree the posibility of Cd toxicity associated to its high presence in the tissue cytosol. A number of studies (Falfushynska et al., 2009, 2013, 2014; Khoma, 2019) on freshwater mussels have shown a positive correlation between Cd and MT concentrations in the digestive gland. However, although some tolerance to metal toxicity has been associated with MT induction (Amiard et al., 2006, and references therein), Campbell et al. (2005) reported that there is no threshold concentration below which Cd is completely detoxified in bivalves of the Unionidae family, i.e. some portion of Cd is always expected to bind to other cytosolic components than MTs. Since Cd competes for binding sites on important cytosolic biomolecules with several essential elements (e.g. Ca, Cu, Zn), excessive accumulation of this non-essential element can cause physiological impairments and reduce the filtration rate in freshwater mussel species (Das and Jana, 1999). In addition to Cd, four essential elements (K, Na, Rb, and Se) were also almost completely present in the soluble, cytosolic digestive gland fraction (≥86%) (Table 2). This finding, on the other hand, can be associated with their functions within the cells. Sodium and K, for example, partake in neurotransmission and ionic regulation, as well as in the activity of Na^+/K^+ ion pumps, which are also important for exchange reactions regarding the accumulation or rejection of certain contaminants (da Silva and Williams, 2001). Disruption of Na^+ and K^+ levels, moreover, can be caused by elevated Pb concentrations (Mosher et al., 2012), which is the reason why rather high presence of Pb in the cytosolic fractions of *U. crassus* digestive gland (up to 85%) following the increase in bioaccumulation can be regarded as a potential risk for mussels health status.

Contrary, several elements are present in the cytosol of digestive gland of *U. crassus* in rather small percentages. Less than 5% of Ca, as an essential element, is present in the cytosol, which is consistent with its function in bivalves shell formation (Mosher et al., 2012, and references therein). In addition to Ca, two other essential (Fe, and Mn, Table 2) and four non-essential elements (Al, Ba, Cr, and Sr, Table 3) were almost completely present in the insoluble fraction (less than 15% in the cytosol) of the digestive gland of *U. crassus*. A positive correlation between Ca tissue concentrations and levels of alkaline-earth metals (e.g. Ba, Sr) was previously reported for two freshwater mussels, indicating that these metals are metabolically regulated in a manner analogous to Ca (Jeffree et al., 1993). Furthermore, various types of inclusions and lysosomal granules are present in the digestive gland of bivalves. One of the inclusions is the Ca/Mg carbonate-based inclusion, which is thought to be responsible for Ca metabolism and binding of a number of transition metals, thus protecting against metal toxicity (Langston et al., 1998). Although mussels have protection mechanisms against metal toxicity, excess metal concentrations may lead to the binding of metals to sensitive molecules (enzymes, DNA, and RNA) and potentially disrupt their functions. Our results show that a number of non-essential elements tend to be more present in the soluble, cytosolic fraction when metal(loid) contamination in the ambient environment is increased and possibly troublesome. Therefore, the high presence of non-essential elements, such as Ag, Cd, Pb, Sb, and U, in soluble fractions could have adverse effects on mussel physiology and potentially disrupt cellular defensive mechanisms and homeostasis.

4. Conclusions

This study provided the first ever information on 27 total and cytosolic metal(loid) concentrations in the digestive gland of *U. crassus* mussels, providing thus fundamental data required for future assessment of the quality status of freshwater ecosystems and of the influence of anthropogenic pollution sources on the availability and accumulation of metals in these aquatic organisms. At the historically contaminated section of the Mrežnica River in Croatia, increased accumulation of several non-essential and potentially very toxic elements (Bi, Cs, Pb, Sb, Tl, and U) in the *U. crassus* digestive gland was observed. Despite the cessation of some and the reduction of discharges of the other sources of pollution more than a decade ago, the effect of the past contamination, which is to this date present in the Mrežnica River sediments, is still recognizable in the metal(loid) bioaccumulation patterns observed for the studied mussel species. Accordingly, sediment-dwelling mussels, specifically *U. crassus*, due to their high exposure to contaminants through sediments, were confirmed as excellent bioindicators of the past pollution. In addition, cytosolic concentrations were demonstrated as a better indicator of metal(loid) bioavailability in the aquatic environment than the total metal(loid) load in the digestive gland of mussels. Further studies of the effects these elements might have on numerous proteins in the cytosolic fraction of the cell are strongly recommended. Moreover, finding of several elements, namely Ca, Ba, Cr, Mg, Mn, and Sr, in higher concentrations at the site upstream of the known pollution sources, was connected to high Ca background at that site, since Mrežnica is a typical karst river in its upper part. Bioaccumulation of Ba, Sr and other elements in higher concentrations than could be explained by the exposure levels was attributed to their coaccumulation with Ca, influenced by their physico-chemical forms and their chemical similarity. Our results, thus, further emphasized the importance of considering the coaccumulation process, inherent chemical properties and interactions among elements, in the assessment of the freshwater pollution. Total concentrations of studied elements additionally showed marked seasonal variability, i.e. they were generally higher in the autumn compared to the summer and spring sampling campaigns, most probably as a result of the invasion of gonadal tissue into the digestive gland during the breeding season in the warmer periods of the year, and consequent dilution of the total concentrations of (non)essential elements. Although seasonal variations in metal(loid) bioaccumulation were noted, the observed spatial differences in metal(loid) concentrations in the digestive gland of *U. crassus* were more prominent. Therefore, the environmental status of the Mrežnica River, as well as of the other freshwaters worldwide which are contaminated by historical sources, should be more rigorously and regularly monitored.

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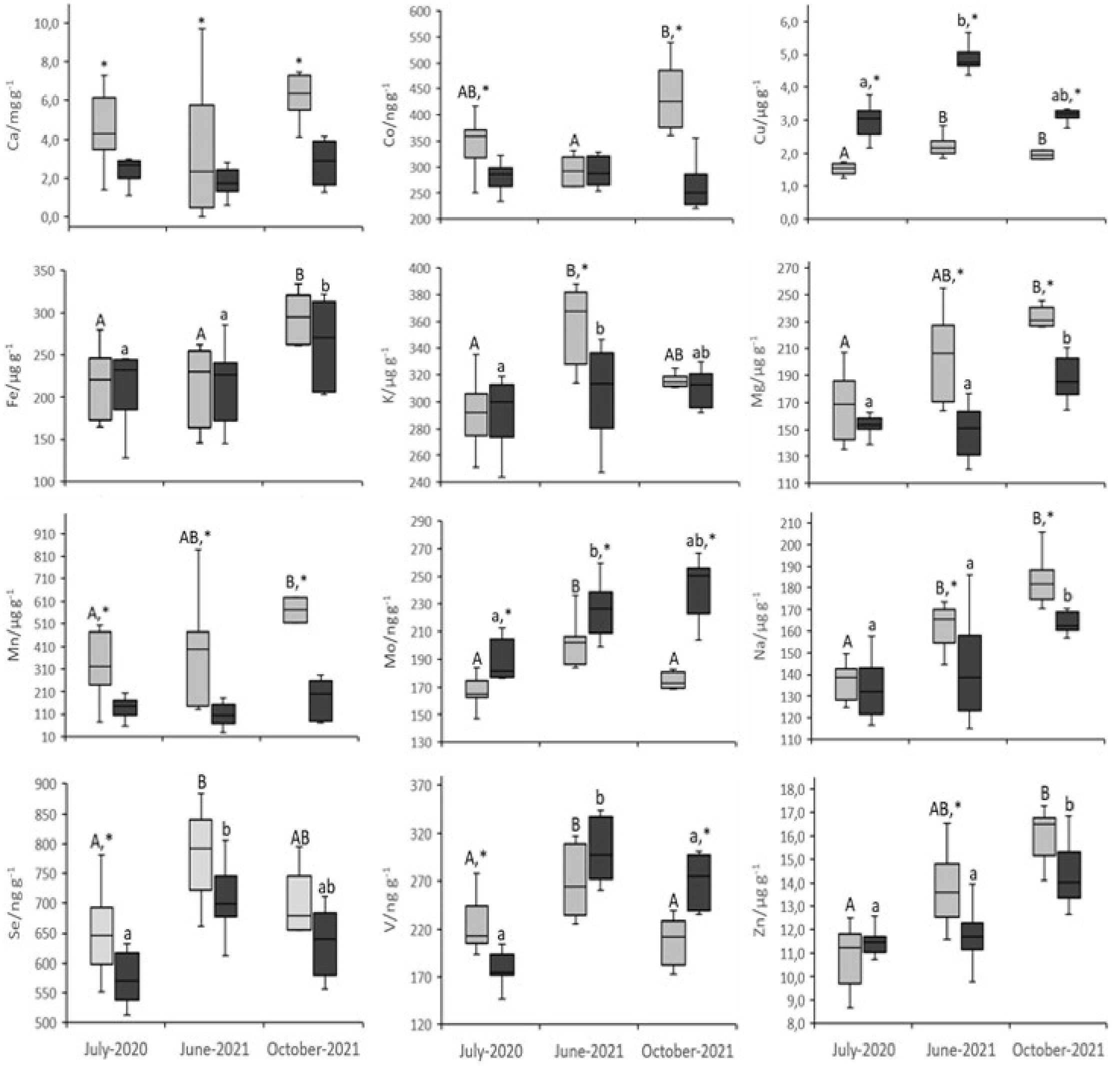
Figure captions

Figure 1. Total concentrations (ng/g or µg/g on wet mass basis) of essential elements in the digestive gland of the mussel species *Unio crassus* Philipsson, 1788, sampled at the two sites in the Mrežnica River (reference site (REF): upstream of the town of Duga Resa; contaminated site (KIZ): Karlovac city industrial zone) in three sampling campaigns in different seasons (July-2020, June-2021, and October-2021). In each sampling campaign, the light box indicates the REF site, and the dark box indicates the KIZ site. Significant differences between sites within each sampling campaign are indicated with an asterisk (*) (Mann-Whitney U test, $p < 0.05$). Significant differences between three sampling campaigns within a site (Kruskal-Wallis ANOVA and Dunn's post-hoc test; $p < 0.05$) are indicated by different letters (A, B for site REF; a, b for site KIZ).

Figure 2. Total concentrations (ng/g or µg/g on wet mass basis) of non-essential elements in the digestive gland of the mussel species *Unio crassus* Philipsson, 1788, sampled at two sites in the Mrežnica River (reference site (REF): upstream of the town of Duga Resa; contaminated site (KIZ): Karlovac city industrial zone) in three sampling campaigns in different seasons (July-2020, June-2021, and October-2021). In each sampling campaign, the light box indicates the REF site, and the dark box indicates the KIZ site. The results of the statistical analyses are presented same as in the Fig. 1.

Figure 1.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4,0
2,0
0,0
*
*
*
Co/ng g⁻¹
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550
500
450
400
350
300
250
200
AB,*
A
B,*
Cu/µg g⁻¹
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5,0
4,0
3,0
2,0
1,0
0,0
A
a,*
B
b,*
B
ab,*
Fe/µg g⁻¹
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300
250
200
150
100
A
a
A
a
B
b
K/µg g⁻¹
400
380
360
340
320
300
280
260
240
A
a
B,*
b
AB
ab
Mg/µg g⁻¹
270
250
230
210
190
170
150
130
110
A
a
AB,*
a
B,*
b
Mn/µg g⁻¹
910
810
710
610
510
410
310
210
110
10
A,*
AB,*
B,*
Mo/ng g⁻¹
290
270
250
230
210
190
170
150
130
A
a,*
B
b,*
A
ab,*
Na/µg g⁻¹
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140
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110
A
a
B,*
a
B,*
b
Se/ng g⁻¹
900
850
800
750
700
650
600
550
500
A,*
a
B
b
AB
ab
V/ng g⁻¹
370
320
270
220
170
120
A,*
a
B
b
A
a,*
Zn/µg g⁻¹
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17,0
16,0
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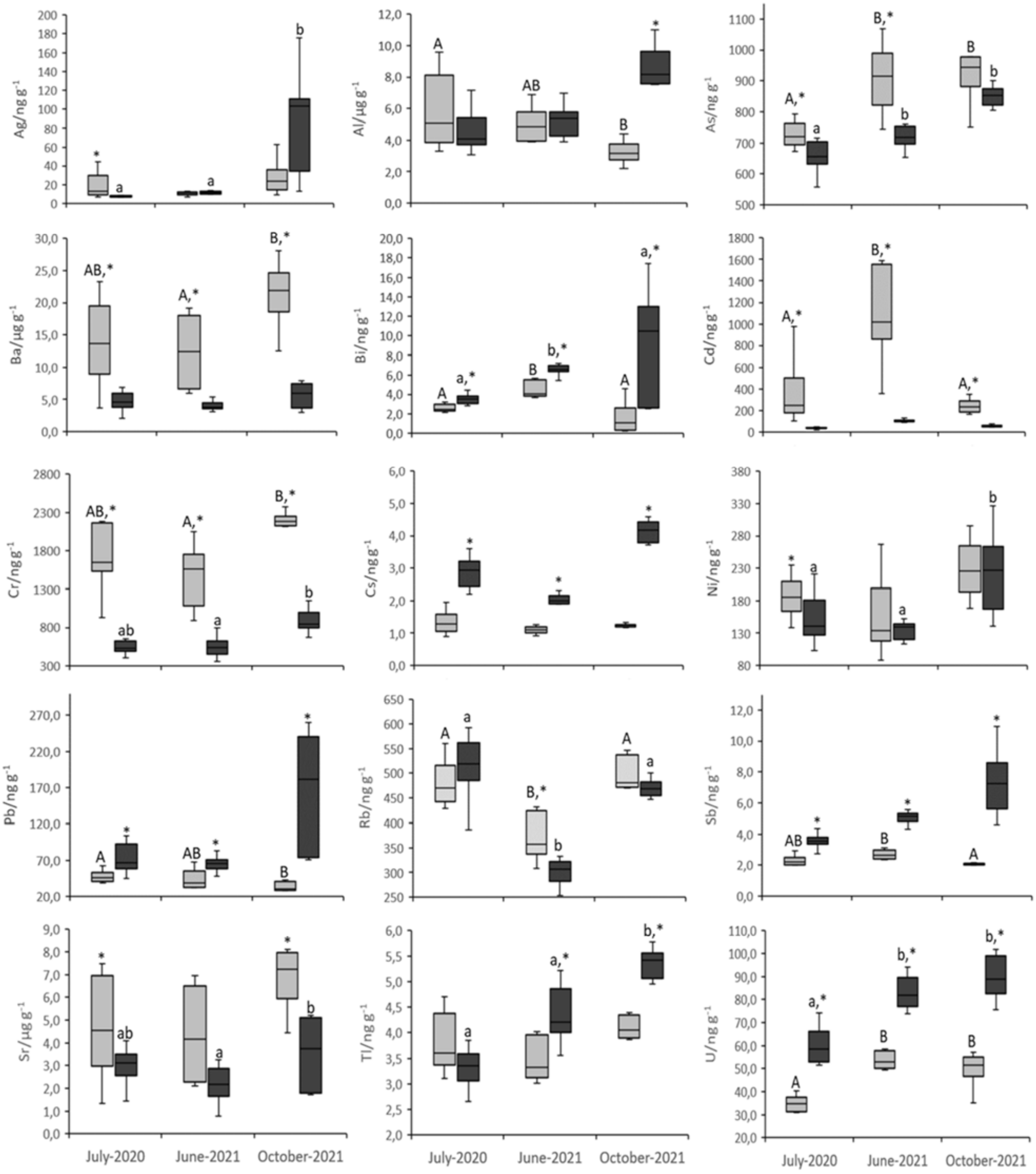
14,0
13,0
12,0
11,0
10,0
9,0
8,0
A
a
AB,*
a
B
b
July-2020
June-2021
October-2021
July-2020
June-2021
October-2021
July-2020
June-2021
October-2021

Figure 2.


Ag/ng g-1
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b
Al/µg g-1
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AB
B
*
As/ng g-1
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a
B,*
b
B
b
Ba/µg g-1
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A,*
B,*
Bi/ng g-1
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A
a,*
B
b,*
A
a,*
Cd/ng g-1
0 200 400 600 800 1000 1200 1400 1600 1800
A,*
B,*
A,*
Cr/ng g-1
300 800 1300 1800 2300 2800
AB,*
ab
A,*
a
B,*
b
Cs/ng g-1
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*
*
*
Ni/ng g-1
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*
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a
b
Pb/ng g-1
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*
AB
*
B
*
Rb/ng g-1
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A
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B,*
b
A
a
Sb/ng g-1
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AB
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B
*
A
*
Sr/µg g-1
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a
*
b
Tl/ng g-1
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a
a,*
b,*
U/ng g-1
20,0 30,0 40,0 50,0 60,0 70,0 80,0 90,0 100,0 110,0
A
a,*
B
b,*
B
b,*
July-2020
June-2021
October-2021

Table 1. Comparison of metal(loid) concentration ranges (µg/g on wet mass basis) in the digestive gland of different freshwater mussel species from differently contaminated ecosystems worldwide.

Mussel species	**Sites**	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Ni**	**Pb**	**Zn**	**References**
Unio crassus Philipsson, 1788	REF	0.497-1.23	0.106-1.59	0.895-2.37	1.24-2.82	145-334	0.088-0.295	0.028-0.067	8.68-17.3	Present study
	KIZ	0.588-0.966	0.021-0.132	0.354-1.15	2.15-5.66	127-335	0.103-0.326	0.045-0.259	9.76-16.8	
Unio tumidus Philipsson, 1788	A	-	0.290-1.250	-	1.37-3.39	-	-	-	62.9-98.2	Khoma, 2019
Unio mancus Lamarck, 1819	A	1.56-2.09	0.129-0.131	0.230-0.240	2.07-2.53	178-225	0.370-0.490	0.050-0.170	11.7-26.5	Yoloğlu et. al., 2018
	B	0.930-1.90	0.050-0.070	0.140-0.320	1.23-2.44	84.4-165	0.230-0.340	0.030-0.170	7.14-11.8	
Anodonta anatina Linnaeus, 1758	B	-	1.10-2.40	-	5.50-31.5	-	-	-	95.0-175	Falfushynska et. al.,2014
	C	-	0.70- 3.50	-	1.50-4.50	-	-	-	25.0-80.0	
Anodonta cygnea Linnaeus, 1758	A	-	0.220-0.700	-	1.31-20.7	-	-	-	19.3-26.8	Falfushynska et. al.,2009
	B	-	0.220-0.620		0.900-6.73	-	-	-	14.4-166	

REF – reference site; KIZ – contaminated site; A – rural/agricultural site; B – urban/industrial site; C – forestry site

Table 2. Cytosolic concentrations (expressed on wet mass basis; average ± SD) of essential elements in the digestive gland of the mussel species *Unio crassus* Philipsson, 1788, sampled at two sites in the Mrežnica River (reference site (REF): upstream of the town of Duga Resa; contaminated site (KIZ): Karlovac city industrial zone) in three sampling campaigns (July 2020, June and October 2021). The proportions of the total tissue metal (expressed as percents, %) present in the cytosolic fraction of the digestive gland are also given.

		Reference site (REF)		**Contaminated site (KIZ)**	
		Cytosolic metal(loid) concentration	**% of the total metal(loid) load**	**Cytosolic metal(loid) concentration**	**% of the total metal(loid) load**
Ca (mg/g)	July 2020*	0.14±0.02^{a}	3.56±1.42	0.11±0.02	4.50±1.47
	June 2021*	0.08±0.013^{b}	3.26±1.43	0.11±0.02	5.45±2.07
	October 2021*	0.13±0.01^{a}	2.25±0.32	0.10±0.01	4.39±1.85
Co (ng/g)	July 2020*	118±9.63^{a}	34.6±5.31	169±15.2^{a}	59.2±7.70
	June 2021*	123±13.6^{a}	39.7±8.12	178±12.4^{a}	61.3±5.54
	October 2021*	105±4.66^{b}	24.7±3.68	140±16.2^{b}	53.8±8.39
Cu (µg/g)	July 2020*	0.84±0.08^{a}	56.1±7.11	2.24±0.48^{a}	75.3±4.88
	June 2021*	1.41±0.21^{b}	65.4±9.46	3.67±0.57^{b}	76.0±6.05
	October 2021*	0.99±0.13^{ab}	49.1±7.58	2.02±0.25^{a}	64.1±4.99
Fe (µg/g)	July 2020*	8.64±1.80	4.24±1.61	12.4±2.03^{a}	5.98±2.09
	June 2021*	8.93±1.82	4.04±1.52	16.9±2.93^{b}	7.96±1.48
	October 2021*	9.26±0.87	3.17±0.43	17.3±0.97^{b}	6.79±1.37
K (µg/g)	July 2020	290±19.1	100±7.44	286±19.1^{a}	98.1±6.72
	June 2021	293±28.0	92.0±9.51	334±36.6^{b}	98.5±5.01
	October 2021	287±12.3	90.9±3.18	298±15.5^{ab}	96.3±2.98
Mg (µg/g)	July 2020	58.1±4.16	35.9±7.10	60.5±2.99^{a}	38.9±2.73
	June 2021	61.8±5.45	30.1±9.40	61.4±7.02^{a}	42.9±6.96
	October 2021*	63.4±2.27	28.1±2.45	72.9±4.08^{b}	39.2±5.97
Mn (µg/g)	July 2020*	3.09±0.45^{ab}	1.23±0.71	1.67±0.23^{ab}	1.31±0.39
	June 2021*	2.76±0.42^{a}	2.14±1.22	1.52±0.31^{a}	3.19±1.80
	October 2021*	4.13±0.85^{b}	0.77±0.14	1.99±0.39^{b}	1.35±0.63
Mo (ng/g)	July 2020*	120±15.9^{ab}	72.1±7.27	137±13.3	73.2±3.70
	June 2021	131±16.6^{a}	67.4±6.92	140±11.1	62.4±3.71
	October 2021	99.4±14.1^{b}	58.2±5.09	143±11.4	59.3±1.92
Na (µg/g)	July 2020*	155±7.82^{a}	113±7.90	140±9.82^{a}	105±5.92
	June 2021*	165±19.9^{ab}	101±11.6	161±16.7^{b}	113±5.53
	October 2021	174±11.7^{b}	95.2±2.91	164±4.77^{b}	100±2.93
Se (µg/g)	July 2020*	605±58.2^{ab}	93.6±9.12	533±44.6^{a}	93.3±3.63
	June 2021	672±83.7^{a}	88.3±7.62	623±61.6^{b}	88.2±4.38
	October 2021	530±37.2^{b}	76.1±5.05	562±10.3^{ab}	89.2±7.89
V (ng/g)	July 2020	120±22.2^{ab}	53.9±5.79	113±15.5^{a}	62.2±4.14
	June 2021*	129±8.60^{a}	53.4±6.82	166±18.3^{b}	55.0±3.63
	October 2021*	102±20.4^{b}	49.0±5.51	140±16.0^{ab}	52.1±4.25
Zn (µg/g)	July 2020	6.73±0.32^{a}	62.6±8.17	7.11±0.49^{a}	62.1±6.18
	June 2021	7.66±0.45^{b}	56.5±9.02	7.30±0.40^{a}	62.9±5.34
	October 2021	7.71±0.71^{b}	48.2±2.21	8.75±0.59^{b}	61.4±9.34

[*] statistically significant differences in cytosolic concentrations between sites within each season based on Mann-Whitney U test ($p < 0.05$)

[A,B] statistically significant differences between seasons at site REF according to Kruskal-Wallis ANOVA and Dunn's post hoc test ($p < 0.05$)

[a,b] statistically significant differences between seasons at site KIZ according to Kruskal-Wallis ANOVA and Dunn's post hoc test ($p < 0.05$)

Table 3. Cytosolic concentrations (expressed on wet mass basis; average ± SD) of non-essential elements in the digestive gland of the mussel species *Unio crassus* Philipsson, 1788, sampled at two sites in the Mrežnica River (reference site (REF): upstream of the town of Duga Resa; contaminated site (KIZ): Karlovac city industrial zone) in three sampling campaigns (July 2020, June and October 2021). The proportions of the total tissue metal(loid) load (expressed as percents, %) present in the cytosolic fraction of the digestive gland are also given.

		Reference site (REF)		**Contaminated site (KIZ)**	
		Cytosolic metal(loid) concentration	**% of the total metal(loid) load**	**Cytosolic metal(loid) concentration**	**% of the total metal(loid) load**
Ag (ng/g)	July 2020	4.40±0.84^{ab}	32.6±17.8	4.12±0.53^{a}	52.7±15.7
	June 2021*	3.81±0.55^{a}	35.8±7.25	5.47±0.79^{b}	47.9±90.5
	October 2021*	5.22±1.13^{b}	25.7±15.6	11.8±5.47^{b}	22.4±15.8
Al (µg/g)	July 2020*	0.49±0.08^{a}	9.40±4.56	0.67±0.06^{a}	15.8±3.61
	June 2021*	0.39±0.07^{ab}	8.28±2.24	0.87±0.09^{b}	17.1±4.15
	October 2021*	0.20±0.04^{b}	6.16±1.18	0.62±0.04^{a}	7.27±1.08
As (ng/g)	July 2020*	277±32.6^{a}	38.5±2.40	364±41.8^{a}	55.5±2.43
	June 2021	442±54.9^{b}	49.5±6.93	457±30.0^{b}	62.1±5.34
	October 2021*	316±15.5^{ab}	33.8±5.03	463±33.1^{b}	54.5±4.50
Ba (µg/g)	July 2020*	0.46±0.04^{a}	4.36±3.21	0.34±0.07^{a}	8.64±6.46
	June 2021*	0.49±0.05^{a}	4.51±2.69	0.37±0.03^{a}	12.3±6.30
	October 2021*	0.20±0.03^{b}	0.98±0.38	0.14±0.01^{b}	2.83±1.21
Bi (ng/g)	July 2020*	2.04±0.31^{a}	80.0±9.83	2.46±0.38^{a}	70.4±4.59
	June 2021*	2.55±0.34^{b}	59.5±13.2	3.89±0.34^{b}	55.2±13.1
	October 2021*	2.37±0.57^{ab}	412±338	3.72±0.78^{b}	60.9±41.2
Cd (ng/g)	July 2020*	432±319^{a}	117±9.58	36.9±9.69^{a}	102±5.61
	June 2021*	1149±284^{b}	94.7±3.42	96.0±12.3^{b}	90.5±4.72
	October 2021*	222±47.8^{a}	93.0±7.83	49.9±7.86^{ab}	88.3±7.33
Cr (ng/g)	July 2020	55.3±6.18^{a}	3.47±1.28	64.8±6.21^{a}	11.9±3.44
	June 2021*	83.2±9.55^{b}	5.88±3.03	97.3±6.80^{b}	18.8±4.23
	October 2021*	37.9±2.54^{a}	1.81±0.32	109±51.3^{ab}	12.2±5.86
Cs (ng/g)	July 2020*	0.84±0.15^{ab}	64.0±12.6	2.39±0.32^{a}	83.7±6.12
	June 2021*	0.67±0.11^{a}	60.4±15.9	1.54±0.21^{b}	76.8±6.20
	October 2021*	0.86±0.06^{b}	70.0±6.93	2.95±0.30^{a}	71.3±6.28

[*] statistically significant differences in cytosolic concentrations between sites within each season based on Mann-Whitney U test ($p < 0.05$)

[A,B] statistically significant differences between seasons at site REF according to Kruskal-Wallis ANOVA and Dunn's post hoc test ($p < 0.05$)

[a,b] statistically significant differences between seasons at site KIZ according to Kruskal-Wallis ANOVA and Dunn's post hoc test ($p < 0.05$)

Table 3.-continued Cytosolic concentrations (expressed on wet mass basis; average ± SD) of non-essential elements in the digestive gland of the mussel species *Unio crassus* Philipsson, 1788, sampled at two sites in the Mrežnica River (reference site (REF): upstream of the town of Duga Resa; contaminated site (KIZ): Karlovac city industrial zone) in three sampling campaigns (July 2020, June and October 2021). The proportions of the total tissue metal (expressed as percents, %) present in the cytosolic fraction of the digestive gland are also given.

		Reference site (REF)		**Contaminated site (KIZ)**	
		Cytosolic metal(loid) concentration	**% of the total metal(loid) load**	**Cytosolic metal(loid) concentration**	**% of the total metal(loid) load**
Ni (ng/g)	July 2020*	72.9±19.0^{a}	39.5±11.0	92.2±16.2^{a}	62.6±9.15
	June 2021*	47.5±7.61^{b}	32.8±10.0	72.9±5.33^{b}	54.2±4.40
	October 2021*	27.6±5.89^{b}	12.4±3.03	60.4±5.49^{b}	28.5±6.35
Pb (ng/g)	July 2020*	39.6±6.23^{a}	84.8±8.28	53.6±15.1	74.2±10.3
	June 2021*	31.7±6.58^{ab}	73.6±10.8	48.2±8.14	73.8±5.21
	October 2021*	18.6±1.31^{b}	56.3±8.11	66.5±18.8	45.4±14.3
Rb (µg/g)	July 2020	0.44±0.02^{a}	93.4±6.80	0.45±0.05^{a}	88.6±3.21
	June 2021*	0.31±0.03^{b}	88.9±9.95	0.25±0.02^{b}	83.5±5.50
	October 2021	0.41±0.02^{ab}	83.6±5.39	0.41±0.02^{a}	87.4±7.14
Sb (ng/g)	July 2020*	1.35±0.36^{a}	58.5±10.0	2.47±0.41	69.1±5.57
	June 2021*	1.06±0.10^{ab}	34.5±14.1	2.41±0.14	47.7±2.33
	October 2021*	0.77±0.08^{b}	36.9±4.67	2.27±0.17	32.7±7.17
Sr (µg/g)	July 2020	0.11±0.01	2.73±1.45	0.10±0.02	3.57±1.52
	June 2021	0.10±0.02	2.38±1.14	0.08±0.01	4.40±2.03
	October 2021	0.11±0.01	1.62±0.32	0.11±0.01	3.53±1.50
Tl (ng/g)	July 2020	1.65±0.31	43.7±5.62	1.63±0.26^{a}	49.0±4.32
	June 2021*	1.42±0.28	41.2±5.44	1.96±0.24^{ab}	45.2±2.46
	October 2021*	1.52±0.12	36.9±2.02	2.33±0.19^{b}	43.6±4.80
U (ng/g)	July 2020*	18.6±2.22^{a}	53.2±5.24	38.9±4.13	64.8±4.59
	June 2021*	24.4±3.07^{b}	46.9±5.65	43.6±3.84	52.7±2.13
	October 2021*	17.5±2.93^{a}	35.0±2.76	41.1±2.87	46.2±5.62

* statistically significant differences in cytosolic concentrations between sites within each season based on Mann-Whitney U test ($p < 0.05$)

^{A,B} statistically significant differences between seasons at site REF according to Kruskal-Wallis ANOVA and Dunn's post hoc test ($p < 0.05$)

^{a,b} statistically significant differences between seasons at site KIZ according to Kruskal-Wallis ANOVA and Dunn's post hoc test ($p < 0.05$)

Supplementary materials

Figure SI.1. Study area with marked sampling sites on the Mrežnica River in Croatia (1 – reference site upstream from Town of Duga Resa (REF); 2 – Karlovac industrial zone Mala Švarča (KIZ)).

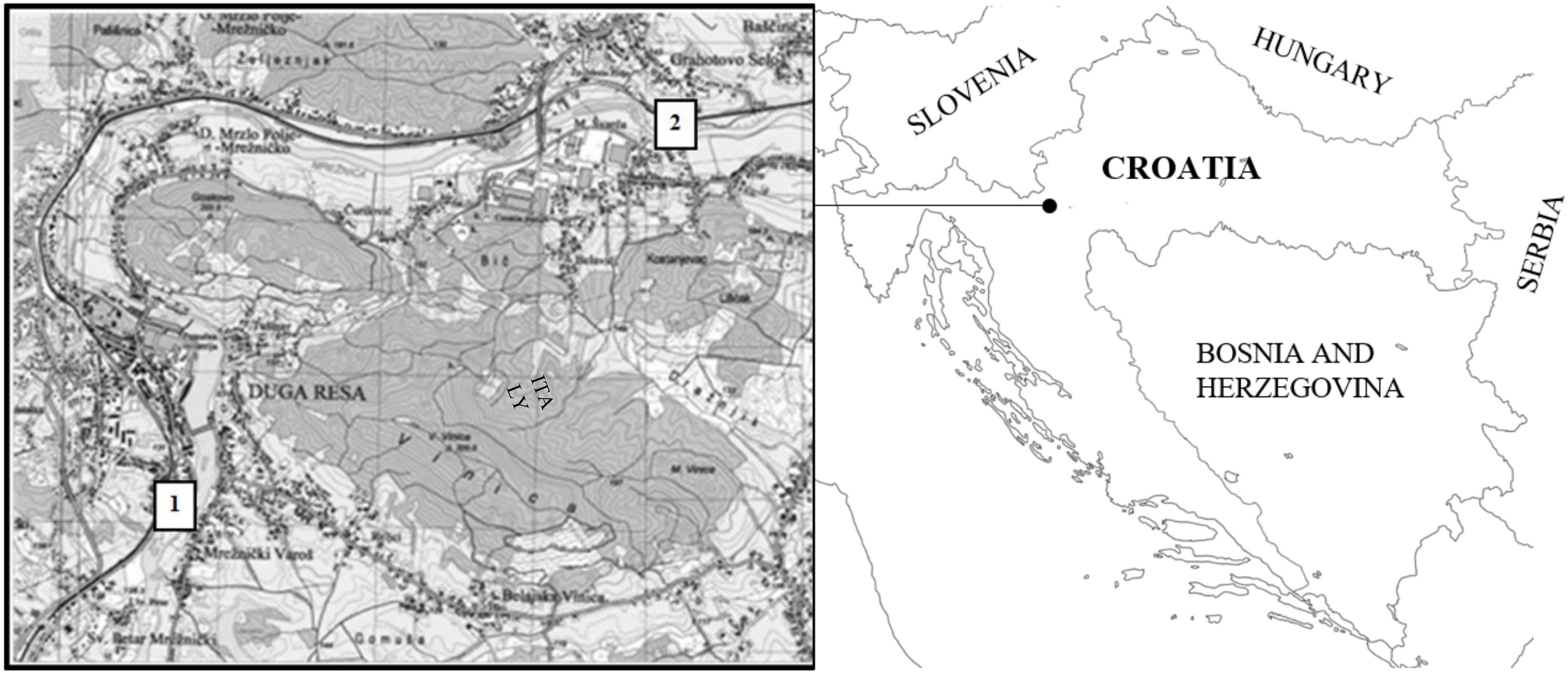

Table SI.1. Limits of detection (LOD) for total and cytosolic concentrations (measurement in homogenates and supernatants, respectively) in digestive gland of *Unio crassus*, and the results of the accuracy control based on three to five measurements in control sample (UNEP/GEMS, Canada) for macro and trace elements.

	LOD for total concentrations	**LOD for cytosolic concentrations**		**Recovery / %**
Ca / μg g^{-1}	2.39	1.53	**Ca**	97.6±0.8
K / μg g^{-1}	14.34	0.536	**K**	98.2±3.2
Mg / μg g^{-1}	3.74	0.564	**Mg**	100.2±1.5
Na / μg g^{-1}	13.04	0.320	**Na**	102.2±0.6
Ag / ng g^{-1}	0.132	0.106	**Ag**	-
Al / ng g^{-1}	158	122	**Al**	98.2±2.6
As / ng g^{-1}	1.33	0.988	**As**	104.9±5.1
Ba / ng g^{-1}	4.00	0.014	**Ba**	101.1±2.3
Bi / ng g^{-1}	0.292	0.394	**Bi**	-
Cd / ng g^{-1}	0.212	0.106	**Cd**	101.3±1.8
Co / ng g^{-1}	0.276	0.310	**Co**	103.6±4.4
Cr / ng g^{-1}	1.10	2.36	**Cr**	103.0±1.9
Cs / ng g^{-1}	0.001	0.001	**Cs**	-
Cu / ng g^{-1}	45.0	6.00	**Cu**	102.6±2.5
Fe / ng g^{-1}	80.0	101	**Fe**	98.4±3.8
Mn / ng g^{-1}	4.00	12.0	**Mn**	101.8±2.3
Mo / ng g^{-1}	0.583	0.348	**Mo**	100.9±2.0
Ni / ng g^{-1}	4.20	2.50	**Ni**	103.2±3.4
Pb / ng g^{-1}	5.04	5.54	**Pb**	100.4±0.4
Rb / ng g^{-1}	0.405	0.362	**Rb**	-
Sb / ng g^{-1}	0.802	0.221	**Sb**	95.4±1.5
Se / ng g^{-1}	2.26	2.41	**Se**	-
Sr / ng g^{-1}	4.00	3.00	**Sr**	105.9±1.9
Tl / ng g^{-1}	0.001	0.001	**Tl**	-
U / ng g^{-1}	0.210	0.063	**U**	-
V / ng g^{-1}	0.581	0.168	**V**	-
Zn / ng g^{-1}	195	389	**Zn**	105.2±3.0

Table SI.2. Correlation coefficients (r; Spearman analysis) between total/cytosolic element concentrations in the digestive gland of *U. crassus* and element concentrations in the dissolved fractions of surface water (DW) / particulate fractions of surface water (PW) / sediments (n = 6 for elements in the DW and in sediments, n = 4 for elements in the PW). Bold numbers indicate statistically significant positive correlations ($p < 0.05$).

	Correlation coefficient (r)	
	Total element concentration vs.	**Cytosolic element concentration vs.**
	DW/ PW / sediment	
Al	0.62 / -0.39 / -0.12	0.67 / 0.43 / 0.58
Ag	- / -0.45 / -	- / -0.23 / -
As	-0.21 / -0.79 / -0.35	-0.44 / 0.76 / 0.71
Ba	-0.41 / -0.31 / -0.81	-0.36 / 0.86 / -0.34
Bi	-0.54 / 0.07 / 0.70	-0.30 / 0.14 / **0.81**
Cd	0.49 / 0.59 / -0.05	0.48 / 0.59 / 0.00
Co	0.07 / 0.85 /-0.70	-0.24 / -0.16 / 0.80
Cr	-0.36 / -0.45 / -0.80	0.48 / 0.24 / 0.79
Cs	0.70 / -0.52 / **0.83**	0.81 / -0.49 / **0.89**
Cu	0.61 / 0.29 / 0.76	0.60 / 0.37 / 0.70
Fe	-0.15 / -0.74 / -0.15	-0.03 / 0.33 / **0.84**
Mn	-0.42 / 0.35 /-0.93	-0.44 / 0.46 / -0.88
Mo	-0.11 / 0.78 / 0.44	-0.46 / 0.85 / 0.63
Ni	-0.75 / -0.99 / -0.22	0.52 / 0.63 / 0.41
Pb	- / 0.67 / -	- / 0.90 / -
Rb	0.77 / -0.92 / -0.37	0.70 / -0.95 / -0.46
Sb	0.41 / - / **0.89**	0.36 / - / 0.41
Se	-0.70 / - / -	-0.35 / - / -
Sr	0.66 / 0.21 / -0.67	0.68 / 0.61 / -0.43
Tl	0.04 / -0.66 / **0.82**	-0.14 / -0.40 / 0.71
U	0.31 / **0.95 /** 0.42	-0.03 / 1**.00 /** 0.73
V	0.34 / 0.67 / 0.03	0.51 / 0.65 / 0.30
Zn	-0.33 / - / -0.24	-0.17 / - / 0.22
Ca	0.36 / -0.65 / 0.49	-0.16 / 0.03 / 0.47
K	0.01 / -0.26 / 0.24	-0.21 / **0.96** / 0.51
Na	0.09 / -0.52 / -0.13	0.05 / -0.67 / -0.16
Mg	0.67 / 0.06 / -0.26	0.55 / 0.73 / 0.42

Missing r-values: element concentrations in the environment were below the detection limit (Ag and Pb in DW) or were not determined (Sb, Se and Zn in PW; Ag and Se in sediments