Interrelation between environmental conditions, acanthocephalan infection and metal(loid) accumulation in fish intestine: an in-depth study

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Abstract

Metal(loid) bioaccumulation in acanthocephalans (*Dentitruncus truttae*) and intestines of fish (*Salmo trutta*) from the Krka River, influenced by industrial and municipal wastewaters, was investigated in relation to exposure to meta(loid)s from fish gut content (GC), water, and sediment to estimate potentially available metal(loid)s responsible for toxic effects and cellular disturbances in biota. Sampling was performed in two seasons (spring and autumn) at the reference site (river source, KRS), downstream of the wastewater outlets (Town of Knin, KRK), and in the national park (KNP). Metal(loid) concentrations were measured by ICP-MS. The highest accumulation of As, Ba, Ca, Cu, Fe, Pb, Se and Zn was observed mainly in organisms from KRK, of Cd, Cs, Rb and Tl at KRS, and of Hg, Mn, Mo, Sr and V at KNP. Acanthocephalans showed significantly higher bioaccumulation than fish intestine, especially of toxic metals (Pb, Cd and Tl). Metal(loid) bioaccumulation in organisms partially coincided to exposure from water, sediments and food, while in GC almost all elements were elevated at KNP, reflecting the metal(loid) exposure from sediments. Seasonal differences in organisms and GC indicated higher metal(loid) accumulation in spring, which follows enhanced fish feeding rates. Higher number of acanthocephalans in the intestine influenced biodilution process and lower concentrations of metal(loid)s in fish, indicating positive effects of parasites to their host, as supported by high values of bioconcentration factors. Fish intestine and acanthocephalan *D. truttae* were confirmed as sensitive indicators of available metal fraction in conditions of generally low environmental exposure in karst ecosystem. Since metal(loid) accumulation depended on ecological, chemical and biological conditions, but also on the dietary habits, physiology of organisms and parasite infection, continuous monitoring is recommended to distinguish between the effects of these factors and environmental exposure when assessing dietary associated metal(loid) exposure in aquatic organisms.

Keywords: bioaccumulation, biodilution, brown trout, acanthocephalans, trace elements, bioconcentration factors

1. Introduction

Metal(loid)s occur naturally in the environment, but their presence has rapidly increased as a result of anthropogenic activities such as mining, traffic, smelting, agriculture, and the release of industrial or municipal waste. This is of particular concern in freshwater karst ecosystems, characterized by numerous caves, sinkholes, and open fractures in carbonate rocks that, because of high porosity and permeability, allow rapid flow but also significant storage of contaminants, including metal(loid)s, which can be released into the water column over time (Padilla and Vesper, 2018).

The most commonly used bioindicator organisms in freshwater ecosystems are bivalves, crustaceans and fish. In recent decades, parasites have also been recognized as potential bioindicators of water quality, leading to the establishment of the field of Environmental Parasitology (Sures et al., 2017). Among them, acanthocephalans have been proposed as promising bioindicators of metal exposure (Filipović Marijić et al., 2013; Nachev and Sures, 2016; Mehana et al., 2020). Further, accumulated metals in acanthocephalans represent a biologically available fraction, since contaminants have to cross the tegument and membranes and might positively or negatively affect their hosts (Sures et al., 2017; Molbert et al., 2020). Given their widespread distribution and high prevalence, parasites should not be neglected in environmental research as being a crucial link for the correct interpretation of field studies.

Due to the complexity of dietary metal(loid) uptake, correlation to food sources depends on metal(loid) bioavailability in the ingested food and selectivity in metal(loid) accumulation to intestinal tissue in order to maintain homeostasis. Consequently, the uptake and potential toxicity of dietary metal(loid)s have been studied predominantly under controlled laboratory conditions (Ojo and Wood 2007; Creighton and Twining 2010). A drawback of these studies is that they mostly employ higher concentrations of metals and shorter exposure times than in natural conditions (Giguère et al. 2004). There have been limited studies on the metal levels in the intestine of indigenous freshwater fish (Dallinger and Kautzky 1985; Sures et al., 1999; Giguère et al., 2004; Filipović Marijić and Raspor, 2010, 2012; Mijošek et al., 2019, 2022), leaving much of the research on metal uptake from diet under field conditions largely unexplored.

Previous studies involving biota from the Krka River have already confirmed negative impact of urban and industrial wastewaters at the KRK site near the Town of Knin in terms of increased accumulation of metal(loid)s and disruption of biomarker responses in organisms compared to the reference site represented by the Krka River source, which is not under known anthropogenic impact and, according to previous studies, not characterized by stress responses (Dragun et al., 2018; Filipović Marijić et al., 2018; Mijošek et al., 2019, 2022). However, there have been no investigations that concurrently examined the concentrations of metalloids in sediments, organisms, and the intestinal contents of fish within the boundaries of the national park.

As a continuation of the research in this important area, we measured total concentrations of 22 metal(loid)s in the intestine and gut content (GC) of brown trout *Salmo trutta* and in acanthocephalan *Dentitruncus truttae* from three locations with varying levels of contamination (reference site – river source upstream of the wastewater discharge; pollution impacted site near the Town of Knin – downstream of the wastewater discharge; and the most downstream location directly in the Krka National Park). Additionally, due to significance of dietborne metal uptake, metal(loid)s were measured in fish gut contents (GC) to investigate possible bioavailability and metal(loid) transfer from food to fish. Metal(loid) concentrations were also measured in water and sediment samples, and the relation of environmental conditions with accumulation in organisms was evaluated. This comprehensive investigation was conducted with the following objectives: 1) comparison of bioaccumulated metal(loid) concentrations between fish intestine and acanthocephalans; 2) determination of spatial and seasonal variability of metal(loid) concentrations in fish, acanthocephalans and gut content and their connection to environmental metal(loid) exposure (water and sediment); 3) analysis of possible association of bioaccumulated metal(loid) concentrations with the fish size, sex, condition, feeding habits and acanthocephalan infection rate; 4) evaluation of the intestine and acanthocephalans as bioindicators of metal(loid) exposure and their bioavailable levels and 5) assessment of the overall impact of industrial and municipal wastewaters on the Krka National Park.

2. Materials and methods

2.1. Research area

Significant part of the Krka River watercourse is protected as the Krka National Park, which is in last decades influenced by extensive tourism, industrial and municipal wastewaters, agriculture and fertilizers, especially in the upper river basin only 2 km upstream of the border of the Krka National Park (Cukrov et al., 2008; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Based on the results on water quality from previous studies (Filipović Marijić et al., 2018) and current sampling campaigns (Mijošek et al., 2023, Šariri et al., 2024, Table S1), three sites were selected for organisms and sediment sampling – Krka River source (KRS) as the reference site, location downstream of the wastewater outlets and Town of Knin (KRK) as the presumed contaminated site, and the third site in the area of the national park near the Brljan Lake (KNP) (Fig. 1). Detailed description, as well as results on the water analyses were given by Mijošek et al. (2023) and Šariri et al. (2024).

2.2. Sampling procedures

2.2.1. Organisms

Samplings were conducted in April and October 2021 at the three above described sites. Electrofishing, carried out according to Croatian standard HRN EN 14011, was chosen for fish sampling. Fish were kept aerated in the plastic tanks filled with water.

2.2.2. Water

At each location, river water samples were collected in precleaned polyethylene bottles in triplicates. Appropriate aliquots from each bottle were then filtered into pre-cleaned polyethylene bottles using a cellulose acetate filter with a pore diameter of 0.45 μm (Sartorius, Germany) to measure dissolved metal(loid)s, acidified with concentrated HNO_3 (Rotipuran Supra 69%, Carl Roth, Germany) and stored at 4 °C before further analysis.

2.2.3. Sediments

Surface sediments (upper 10 cm) were sampled only once in 2021 from the same sites as fish. We collected approximately 0.5 kg of surface and near surface sediment at each site using plastic spatulas and bags for the storage. Samples were transported to the laboratory at 4 °C and then stored at − 20 °C.

2.3. Procedure of biometric analysis, tissue dissection and storage

Fish were anesthetized immediately after the sampling using tricaine methane sulphonate (MS 222, Sigma Aldrich) according to the Ordinance on the protection of animals used for scientific purposes (NN 55, 2013). Firstly, basic biometric data (total length and body weight) were taken for each fish. Further, organ (liver, gonads, viscera) dissection was performed on ice to collect the data for calculation of biometric indices. Gonads also served for the gender determination. The posterior part of the intestine was isolated, cleaned from the exterior fat and gut content, and examined for the presence of acanthocephalans. The gut content of each fish was separated and stored. After isolating and quantifying acanthocephalans from the intestines of each fish, the prevalence and intensity of infection were calculated. All samples were subsequently stored in liquid nitrogen until transportation to the laboratory, where they were kept at −80 °C until analyses.

2.4. Acid digestion of biological samples and sediments

2.4.1. Biological samples

Prior to acid digestion, acanthocephalans from the same fish were pooled together to enable reliable measurements (3-24 individuals). Fish intestine and acanthocephalans were digested with HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur, Merck, Germany) at 85 °C for 3.5 h, as described by Mijošek et al. (2022). Gut contents of fish were digested under the same conditions using the mixture of HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and HF (Suprapur, Merck, Germany).

2.4.2. Sediments

Sediment samples were air-dried, homogenized in agate mill and sediment subsamples were digested in a two-step digestion procedure performed using microwawe oven (Multiwave ECO, Anton Paar, Austria). Mixture of 4 mL HNO_3 (65%, p.a., Kemika, Croatia), 1 mL HCl (TraceSELECT, Fluka, Germany), and 1 mL HF (TraceSELECT, Fluka, Germany) is used in the first step, followed by the addition of 6 mL H_3BO_3 (Fluka, Switzerland), as described by Fiket et al. (2017).

2.5. ICP-MS measurements

Concentrations of trace and macroelements in water, organisms and GC were measured using an Agilent 8800 instrument (Agilent Technologies, USA) as presented in Table S2. To reduce interference, cell (helium and hydrogen) and reaction (oxygen) gases were used. Ultrapure Mili-Q water (Barnstead Smart2Pure Water Purification System, Thermo Scientific, Langenselbold, Germany) was used for preparation of standards and samples, and washing the dishes and disposable plastics. Acidified water samples underwent analysis without dilution, whereas the biological samples were diluted 10-13 times with a solution containing 1% (v/v) of purified nitric acid [HNO_3, 65%, p.a., Merck; purified by quartz sub-boiling distillation using the Milestone SubPUR system (Milestone S.r.l., Sorisole, Italy)] and hydrochloric acid (HCl, 30%, Suprapur®, for trace analysis, Supelco®). An internal standard solution containing 3 μg L−1 of internal standards (Ge, Rh, Tb, Lu, and Ir) (SCP Science, Canada) was used to correct instrumental drifts and plasma fluctuations. Standard solutions used for external calibration were prepared from individual PlasmaCAL Single-Element Standard solutions which were obtained from SCP Science, Quebec, Canada. Four standard certified reference materials [IAEA-407 (fish tissue), ERM-BB422 (fish muscle), ERM-CE278 (mussel tissue), NIST SRM 1577a (bovine liver)] were analyzed with the samples as part of quality control. The accuracy of the method for the elements in the referent biological samples mostly fell within a range of ±10%, with the recoveries varying from 90.4% for rubidium (Rb) to 110% for cadmium (Cd). Detailed information on the limits of detection (LODs) and the recoveries for each element and certified reference material in water and biological samples are presented in Tables S3 and S4.

Total element concentrations in sediments were measured using triple quadrupole inductively coupled plasma mass spectrometer (ICP-QQQ, Agilent 8900, USA). Analytical quality control was performed by simultaneous analysis of procedural blanks and certified reference materials of stream sediment NCS DC 73309 (GBW 07311) (China National Analysis Center for Iron and Steel, China). Recoveries in the referent sample were in range 91-104%, depending on the element.

2.6. Data processing and statistical methods

2.6.1. Biological and chemical data

Prevalence (number of infected fish) and mean intensity of infections (number of parasites per fish individuals) were calculated to describe acanthocephalan epidemiology. Bioconcentration factors (BCFs) were calculated as ratios between the element concentrations in acanthocephalans and fish intestine. Ratios between metal(loid) concentrations in fish intestine and gut contents, as well as in acanthocephalans and gut contents, were calculated as percentages and considered as the indication of possible meta(loid) bioavailability from the dietborne uptake.

Metal(loid) concentrations are presented as average values ± standard deviation and expressed in μg L^{-1} for water, μg kg^{-1} or mg kg^{-1} of wet weight (w.w.) for organisms and GC, and mg kg^{-1} or g kg^{-1} d.w. for sediments.

2.6.2. Statistical methods

Statistical analyses were done in SigmaPlot 11.0 (Systat Software, USA) and Microsoft Office Excel 2007. Metal(loid) concentrations in fish, acanthocephalans, GC, and water were compared between three sample locations using Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's or Tukey's tests. Mann-Whitney U test was applied to test the data variability between the two seasons. Due to normal distribution of data on sediment samples, spatial differences were tested using one-way ANOVA using Holm-Sidak method. Differences were considered significant when $p<0.05$. Correlations between parameters were calculated using Spearman correlation analysis and correlation coefficients and levels of significance were indicated in the text and all are reported in tables in supplementary material.

3. Results and discussion

3.1. Main biometric characteristics of fish and acanthocephalans

No significant spatial differences were observed in total length (TL), total body mass (TM), and biometric indices of fish. Nevertheless, there were notable seasonal differences in

TL and TM, with higher values observed in autumn, as the spawning period of this species, than in spring at all locations (Table 1). GSI values were also higher in autumn at all locations. However, these seasonal differences were not statistically significant at KNP, likely as a consequence of sporadic sampling of a few small, reproductively immature individuals and generally lower number of fish captured at this site in the spring. There were no significant differences depending on the fish sex.

All fish were infected with acanthocephalan *D. truttae* and there were no significant spatial differences in mean intensity of parasites in any season. The mean intensities and number of parasites were higher in autumn than in spring at all locations (Table 1). Comparable epidemiological characteristics have already been reported for the period 2005-2016 (Vardić Smrzlić et al., 2013; Filipović Marijić et al., 2022; Mijošek et al., 2022), probably all related to the life-cycle of gammarids, intermediate hosts of acanthocephalans. Gammarids reproduce mainly in late summer and autumn, leading to a higher abundance of acanthocephalans due to higher presence of gammarids in fish food.

To describe the relationship of this host-parasite system, we examined the correlations of parasite abundance and fish size and condition (FCI). Given the strong correlation between TL and TW ($r= 0.887–1.000$, $p<0.001$, depending on the site and season), we opted to utilize TM as the variable to describe fish size in order to prevent issues with multicollinearity. For this purpose, all data were tested together. A significant positive correlation ($r=0.478$, $p<0.001$) was established between the abundance of parasites and fish TM, while no correlation was observed between abundance and FCI, confirming that acanthocephalans do not have negative effect on the fish fitness. Similar to findings of Vardić Smrzlić et al. (2013) and Mijošek et al. (2022) for *D. truttae*, we observed higher prevalence of infection in male than in female fish, but not statistically significant.

3.2. Main environmental conditions

3.2.1. Concentrations of dissolved metal(loid)s in water

The recent comprehensive study on the dissolved metal(loid)s in water samples from eight locations along the upper stretch of the Krka River (Mijošek et al., 2023), revealed that industrial wastewaters from the screw factory (IWW) represent the most significant source of metal(loid)s in the Krka River primarily through the Orašnica River, a tributary that is directly influenced by the wastewaters due to potential spillover during heavy rains. Metal(loid) concentrations were also increased near the municipal outlet of the Town of Knin (KRK) in comparison to the river source (KRS) and location in national park (KNP).

At the three locations where fish were sampled, a few distinct patterns of metal(loid)s in the dissolved fraction were observed: As, Fe, Mg, Mn, Na, and Rb were predominantly the highest at KRK, while Ba, Ca, Mo, Sr, and V were the highest at KNP, and Tl was the highest at KRS. (Table S1). Seasonal differences between spring and autumn were not clearly distinguishable between the three sites, which is in line with results of our comprehensive data covering metal(loid) concentrations across eight locations, with no significant differences observed between spring and autumn (Table S1, Mijošek et al., 2023). Furthermore, spatial distribution of metal(loid)s coincided with water physico-chemical parameters, which were below good at KRK due to high concentrations of nutrients and COD in both seasons. Regarding Krka River source (KRS) and location in national park (KNP), mostly very good ecological status was indicated (Mijošek et al., 2023; Šariri et al., 2024). The same pattern was observed for meta(loid) concentrations, wherein the lowest concentrations were found at KRS, followed by the highest concentrations of many elements at KRK and a subsequent gradual decrease at KNP, with the exception of Ba, Ca, Mo, Sr and V, which increased to their maximum concentrations at KNP (Table S1). Cukrov et al. (2008) verified the existence of a self-purification mechanism in the Krka River, starting in the Brljan Lake and progressing through subsequent tufa barriers, resulting in decreased metal(loid) concentrations in water downstream of the pollution sources. Moreover, groundwater input of clean water from the Zrmanja River also contributes to the decrease in water trace metal concentrations in a downstream way of contamination in the Krka River (Cukrov et al., 2008). Hence, elevated concentrations of many elements at KNP in comparison to KRS suggested potential future threats to this protected area, especially since measuring contaminants solely in water is inconclusive due to water discharge fluctuations and short resident times, making it a less reliable method of assessment (Salati and Moore, 2010; Algül and Beyhan, 2020).

3.2.2. Metal(loid) concentrations in sediments

Concentrations of majority of elements in sediment samples showed significant site-specific differences (Table S5). Potassium, Na and all trace elements in sediments exhibited the highest concentrations at the location in close proximity to Brljan Lake in KNP (Table S5). The sole deviations from this pattern were noted for Mg and Ca, with the highest average concentrations at KRS and KRK, respectively. River source is a reference site without the known pollution sources, characterized by the strong flow and low sedimentation rate, which explains the lowest concentrations of most elements at this site, both in sediments and water. However, only few kilometers downstream from the KRS, municipal wastewaters of the Town of Knin cause the increase in metal(loid) concentrations, which is the highest in downstream lake systems which serve as sink for metals due to intensive sedimentation and low flow (Chon et al., 2012). The water flow rate decreases in a downstream direction and lowers the transmission strength and carries smaller sediments particles which sink and deposit over longer time, and during such extensive sedimentation metal(loid)s bind to these particles and precipitate in sediments, consequently reducing metal(loid) concentrations in water in the process of self-purification (Cukrov et al., 2008).

It is obvious that negative influence of anthropogenic activities is observed in the area of the national park according to the sediment analyses (Table S5), which act as a sink, but also the source of many contaminants, including metal(loid)s (Chon et al., 2012). Metal(loid)s are not permanently bound to sediments and can be released into the water at any time depending on pH, temperature or flow which affect metal balance and remobilization between the sediments and water and consequently, cause possible toxic effects in biota. Therefore, both water and sediments, in addition to food can serve as sources of metal(loid)s for organisms, which will be discussed further.

3.3. Metal(loid) concentrations in biota

3.3.1. Metal(loid) accumulation in fish intestine

Metal(loid) accumulation in fish intestine followed the order K>Na>Mg≥Ca for macroelements and Zn>Fe>Rb>Cu≥Se>Mn>Sr>Cd≥Co≥As>Mo>Tl>Ba≥Cr>V≥Hg>Pb≥Cs for trace elements (Figs. 2-5), similar to the brown trout sampled in 2015/2016 (Mijošek et al., 2022). Our findings are consistent with prior research that reported the highest levels of essential elements as Zn, Fe, and Cu in the intestines of various fish species, including perch (Sures, 2002), European chub (Filipović Marijić and Raspor, 2010), or barbel (Nachev and Sures, 2016). When comparing the current state of accumulation of elements in brown trout intestines with that of previous research that focused on two locations in Krka River (KRS and KRK), it is evident that Ca, Cs, K, Mg, Na, Rb and Tl remained relatively constant, whereas bioaccumulation of Cd, Co, Cu, Fe, Mn, Se, Sr and Zn increased with time (Figs. 2-5, Mijošek et al., 2022). Only Pb concentrations were found to be around 3-5 times lower in the present research compared to previous study, owing to possibly lower concentrations of this element in sediment or gut content samples which are related to uptake sources and were not analyzed in the previous study. Given the widespread utilization of Cu, Fe, Mn, and Zn in industrial, municipal, and agricultural processes, our findings suggest the intensifying impact of wastewaters on biota. This is particularly worrisome in unexplored region of the national park, where fish frequently exhibited maximum concentrations of certain elements compared to locations upstream. These patterns are consistent with the analysis of metal(loid)s in water samples, which confirmed that the majority of values in 2021, particularly for Cr, Cu, Fe, and Mn, were higher than those recorded between 2004 to 2015, probably all as a consequence of industrial and agricultural activities in the region (Mijošek et al., 2023).

We further investigated the potential influence of size (represented by TM) and sex on metal(loid) accumulation in the intestine. The influence of FCI was not considered since the differences in FCI between sites and seasons were negligible and not significant (Table 1). Spearman's correlation coefficients for the associations TM and metal(loid) concentrations in fish intestine are presented in Table S6. Only a few notable correlations were observed between the concentrations of elements in the intestine and TM. A statistically significant ($p<0.05$ in all cases) decrease with increasing TM was found for V at KRK (r= -0.886) in spring, Cd and Cs at KRS (r= -0.767, -0.833) in spring, and Cs at KNP in autumn (r= -0.786). Conversely, significant increase was detected for Mg at KRS in spring (r= 0.667) and As at KRK in autumn (r= 0.609). Fish often show variations in metal-size associations, usually as a result of differences in their metabolic rates, detoxification systems or feeding habits (Nikolić et al., 2021). The influence of fish sex on bioaccumulation of metal(loid)s in fish intestine was evaluated using pooled data for each sex. Statistical analysis showed there were no significant differences, although average values of majority of elements were slightly higher in females, possibly due to factors such as food preferences or specificities of the reproductive cycle (Rajkowska and Protasowicki, 2013). Overall, the impact of fish biometric parameters on metal(loid)s accumulation in the intestine of brown trout was mainly negligible.

3.3.2. Metal(loid) accumulation in acanthocephalans

Macroelements followed the order K>Na≥Ca>Mg, and trace elements: Zn>Fe>Cu>Mn>Sr≥Rb>Cd≥Tl≥Se>Pb>Ba>As>Co≥Hg≥Cr≥Mo≥V>Cs (Figs. 2-5), as in previous research from 2015/2016 (Mijošek et al., 2022). Unlike in fish, concentrations of the majority of elements did not vary between the studies, indicating the absence of temporal trends, with the exception of Cd, Cu, Mn, and Zn, which were mostly higher in the current research, implying continuous contamination impact of the screw factory. Our two studies present the only data on metal(loid) accumulation in this endemic acanthocephalan species and both indicated the certain level of environmental deterioration over time. Additionally, concentrations of As, Ba, Cr, Hg, Mo and V in *D. truttae* were now measured for the first time.

Contrary to positive association between parasite abundance and fish size, we observed a negative correlation between metal(loid)s accumulation in acanthocephalans and fish TM for many metal(loid)s, especially in autumn (Table S6). Hence, significant negative correlations were observed for Mn at KRS ($r= -0.800$; $p<0.01$) in the spring, as well as for Tl at KNP ($r= -0.750$; $p<0.05$), Cd ($r= -0.700$; $p<0.05$), Pb ($r= -0.624$; $p<0.05$), Rb ($r= -0.886$; $p<0.05$) and Tl ($r= -0.627$; $p<0.05$) at KRK, and Ba ($r= -0.661$; $p<0.05$), Co ($r= -0.745$; $p<0.05$), Fe ($r= -0.709$; $p<0.05$), Pb ($r= -0.767$; $p<0.05$), Cd ($r= -0.758$; $p<0.01$), Tl ($r= -0.806$; $p<0.01$), Cu ($r= -0.830$; $p<0.001$) and Se ($r= -0.879$; $p<0.001$) at KRS in the autumn. Higher concentrations of metal(loid)s in acanthocephalans from smaller fish may be associated with higher metabolic and feeding rates in smaller specimens, both of which decline with fish aging (Farkas et al., 2003).

Analyses of associations between metal(loid)s accumulation in the intestines of brown trout and in acanthocephalans failed to uncover many consistent correlations when data were stratified by season and location. However, upon analyzing the pooled data, many positive correlations were identified [$p<0.05$ for As ($r=0.369$), and Se ($r=0.360$), $p<0.01$ for Cd ($r=0.441$), Hg ($r=0.400$), Mn ($r=0.468$), Mo ($r=0.372$), Pb ($r=0.331$) and Zn ($r=0.409$), $p<0.001$

for Ba (r=0.615), Ca (r=0.600;), Cs (r=0.794), Cu (r=0.478), Rb (r=0.660), Sr (r=0.819; p), Tl (r=0.511), and V (r=0.519)], suggesting that fish and parasites exhibit similar biological responses. All results on this association are presented in Table S7.

Although various studies (Sures, 2002; Filipović Marijić et al., 2013; Brázová et al., 2015) demonstrated that acanthocephalan-infected fish have lower metal concentrations than the uninfected ones, the 100% prevalence of acanthocephalans in our research prevented us from testing this hypothesis (Table 1). However, we observed notable differences in the accumulation of metal(loid)s in the intestine based on the number of acanthocephalans in fish. In particular, statistically significant inverse relationships between the abundance of acanthocephalans in fish and the concentrations of As (r= -0.545; $p<0.001$), Cr (r= -0.571; $p<0.001$), Mn (r= -0.446; $p<0.001$), Se (r= -0.523; $p<0.001$), V (r= -0.507; $p<0.001$), Mo (r= -0.392; $p<0.01$), Ba (r= -0.326, $p<0.05$), Cu (r= -0.351, $p<0.05$), and Fe (r= -0.339, $p<0.05$) in the intestine of fish was established. Correlation analysis is presented in Table S8. This suggests that fish with greater infection levels absorbed lower concentrations of these elements via the intestinal wall, as consistent with the possible protective and beneficial role for their fish hosts (Sures, 2002; Nachev and Sures, 2016; Mehana et al., 2020).

Continuing to analyze the potential impact of acanthocephalan infrapopulation sizes on metal(loid) accumulation mechanisms within parasites (Table S8), we observed that larger infrapopulations had lower concentrations of Co (r= -0.545; $p<0.001$), Cr (r= -0.500; $p<0.001$), V (r= -0.646; $p<0.001$), Zn (r= -0.475; $p<0.001$), As (r= -0.414; $p<0.01$), Fe (r= -0.453; $p<0.01$), Mg (r= -0.406; $p<0.01$), Mo (r= -0.466, $p<0.01$), Ba (r= -0.333, $p<0.05$) and Se (r= -0.361, $p<0.05$) compared to smaller infrapopulations. Sures et al. (1999) previously reported that concentrations of several elements within the parasite *Acanthocephalus lucii* decreased as the infrapopulation of *A. lucii* in fish increased. Similar findings were reported by Brázová et al. (2015), who recorded that a high intensity of infection was associated with lower heavy metal concentration in *A. lucii* and *Proteocephalus percae*, and by Hassanine et al. (2018) regarding the inverse relationship between metal concentrations in the acanthocephalan *Sclerocollum saudii* and the size of its infrapopulation in fish *Siganus rivulatus.* It is still unclear whether our findings support the traditionally accepted concept of intraspecific competition among acanthocephalans for the absorption of metals from fish intestine (Sures, 2002; Hassanine and Al-Hasawi, 2021) or the more recent hypothesis of biodilution of elements in the fish host proposed by Duarte et al. (2020). The latter concept proposes that in larger parasite populations, elements may be dispersed among all parasite individuals, leading to reduced metal(loid) concentrations in both parasites and fish. In the investigation of the accumulation of 12 trace metals in the muscle, intestine, and liver of the white mullet and its intestinal parasites, Leite et al. (2023) also noted that the size of the parasite infrapopulation had an effect on the concentrations of trace metals in the parasites, with smaller infrapopulations accumulating a greater quantity of metals. Regarding the host, fish with a more severe parasitic infection had reduced concentrations of Cd exclusively in the muscle tissue, while no significant differences were detected with regard to other tissues or metals. They also explained this phenomenon by biodilution, resulting from the increased biomass of the host-parasite system and the fact that after metal is excreted in the intestine, reabsorption by fish does not occur because metal is captured by the parasite at the infection site.

Therefore, as parasites can be found in every free-living organism in all ecosystems there is a need of investigating their impact on environmental health and biological responses of organisms (Timi et al., 2020). Our results confirmed that accumulation in acanthocephalans may have implications for accumulation levels in fish, that they have high accumulation potential and response fast, as well as possible negative and positive effects on intermediate and final hosts (Sures, 2002; Sures et al., 2017). However, it is challenging and almost impossible to distinguish in field conditions acanthocephalan from environmental impact because parameters as season, abundance of intermediate hosts, sex of the hosts, as well as their biometric characteristics may sometimes impact epidemiological characteristics of acanthocephalans and cannot always be excluded. That highlights the complexity of the host-parasite relationship, as well as of the assessment of contamination exposure. Still, good freshwater ecosystem management plans and monitoring programs should always contain physico-chemical factors, water and sediment analyses, fish, but also parasites to interpret the data and results from the field studies as accurate as possible.

3.3.3. Spatial differences in metal(loid) accumulation

Fish intestine and acanthocephalans showed variable spatial differences which were not always statistically significant but could be mostly grouped in four patterns: 1) elements with higher concentrations at the contaminated site, KRK; 2) elements with higher concentrations at the reference site, KRS; 3) elements with higher concentrations at the site in national park, KNP; 4) elements with unclear trends (Figs. 2-5).

Statistically significant differences in metal(loid) accumulation between sites in the fish were observed for Cs, Mn, Rb and Se during both seasons. For As, Co, Fe, Tl, and Zn, these differences were evident only in spring, whereas for Cd and Hg only in autumn (Figs. 2-5). The highest levels of certain elements were recorded at different locations: 1) As, Mn, Se, Zn at KRK; 2) Cd, Cs, Rb, Tl at KRS; and 3) Co, Hg, Mn at KNP. Similar patterns were previously documented for brown trouts from KRS and KRK collected in 2015/2016, with no sampling conducted at KNP during that period (Mijošek et al., 2022). These patterns could not be fully explained by uptake from water or sediments alone, suggesting the importance of long-term dietborne metal(loid) uptake and feeding preferences. Namely, As, Se and Zn were found in higher concentrations in fish from KRK (Fig. 2), but not in the water or sediment samples from that site (Tables S1 and S5). Similarly, Cs and Rb concentrations in fish (Fig. 3b and 3c) did not correspond to their levels in water or sediments; in water, the levels were either similar across sites or the highest at KRK (Table S1) whereas for sediments, they were highest at KNP (Table S5). Bioaccumulation of Co, Fe, Hg and Mn in fish could mostly be explained by their uptake and remobilization from sediments, while concentrations of Tl and Cd in fish from the reference site seemed to be more closely related to their concentrations in water than sediment samples (Tables S1 and S5). Concentrations of Cd and Tl tended to be higher in fish from the KRS (Fig. 3), possibly due the prevalence of Cd-enriched limestones and dolomites in Dinaric karst (Cukrov et al., 2008). In addition to natural and anthropogenic causes, food preferences can significantly affect metal accumulation in the intestine, since dietborne metal uptake may be of equal or greater importance than waterborne uptake for some elements, including Tl (Lapointe and Couture, 2009).

Acanthocephalans showed significant spatial differences for Cs, Fe, and Sr in both seasons, while variations for Cd, Co, Hg, Mo, Na, and V were apparent during only one season (Figs. 2-5), which partially corresponded to patterns in the intestines. Similar to previous research of Mijošek et al. (2022), the highest levels of certain metals in fish parasites were found at the following locations: 1) Co and Fe at KRK; 2) Cd and Cs at KRS; and 3) Mo, Na, Sr, and V at KNP. In contrast to the accumulation of Cd, Co, Cs, and Hg in parasites, which, similar to the intestinal accumulation, was largely unrelated to exposure from water and sediments, the highest accumulation of Mo, Sr, and V at KNP was concurrent with their highest levels in water, sediment and GC samples from that site (Tables S1, S5 and S9). Patterns of other elements might be associated with long-term feeding preferences of their fish hosts or natural geological background. Although there were notable differences in the average values of other elements in acanthocephalans across different sites, these variations were not statistically significant due to the high variability and high standard deviations in metal(loid) concentrations among individual acanthocephalans, as already stated for different acanthocephalan species (Filipović Marijić et al., 2013; Nachev and Sures, 2016; Mijošek et al., 2022).

3.3.4. Temporal differences in metal(loid) accumulation

Seasonal variations of metal(loid) concentrations in the intestine of brown trout were mostly not uniform and could not be extrapolated to all locations (Figs. 2-5). Nevertheless, more elements showed higher levels in spring compared to autumn, with significant differences observed for Ca, Hg, Mg, Mn, Se, and Tl at KRS, As, Co, Hg, Mn, and V at KRK, and As, Co, Cu, Fe, Mn, Mo, and Se in KNP. Conversely, greater accumulation of Cs at KRK, Na at KNP, and Zn at KRS was observed during the autumn (Figs. 2-5). Various biotic and abiotic factors, in addition to exposure conditions, can influence seasonal variability in metal accumulation. For example, differences in growth, metabolism rate or water parameters like temperature, pH, or water hardness may influence bioaccumulation in organisms. Further, food type or feeding rates can also significantly affect bioaccumulation (Farkas et al., 2003) and these differences were clearly observed during fish dissection. Specifically, we observed high gut fullness index in nearly all fish during spring. The feeding intensity was lower in autumn with the higher proportion of half-empty to empty stomachs. Greater diversity of the gut contents was also observed in spring, which contributed to the higher metal(loid) concentrations in fish GC in that season as will be further discussed. This observation is in accordance with research reporting maximum feeding rates of trouts in spring (Debeljak, 1986; Kara and Alp, 2005). Fullness indices of the brown trouts were also already found to be the lowest in autumn, increasing from winter to summer (Kara and Alp, 2005).

Higher accumulation in spring than autumn was also observed in acanthocephalans, although less pronounced and engaged different elements than in fish intestines. Namely, concentrations of Co, Mo, Pb, and V at KRS, Mo and V at KRK, and Co at KNP were significantly higher in spring than in autumn (Figs. 2, 4 and 5). Potassium was significantly higher in autumn compared to spring in acanthocephalans from KRS, while other elements did not display significant seasonal variations (Figs. 2-5). According to Nachev and Sures (2016), seasonality in metal accumulation in parasites can be explained by acanthocephalan transmission, maturation in the intestine of the hosts, or by physiological or behavioral changes of their final hosts, such as mobility or alterations in food preferences.

3.3.5. Bioconcentration factors (BCFs)

To define the relation of metal(loid) concentrations in acanthocephalans and fish intestine, we calculated BCFs (Table 2). Due to the different life spans of acanthocephalans and fish, high BCFs indicate recent metal exposures, while lower ratios represent continuous, longer exposure (Sures et al., 1999).

Our research supported findings of superior efficiency of metal(loid) accumulation in acanthocephalans compared to fish (Filpović Marijić et al., 2013; Nachev and Sures, 2016; Sures et al., 2017; Mijošek et al., 2022). Acanthocephalans showed higher accumulation of As, Ba, Cd, Cr, Cu, Hg, Mn, Pb, Sr, Tl, Ca, and Na in all sites and seasons, and of Co, Fe, and V in majority of cases (Table 2). The only elements with lower accumulation in acanthocephalans than in fish intestine (BCF<1) were Cs, Mo, Rb, Zn and K, with the occasional inclusion of Co, Se, V and Mg in a few instances (Table 2). This is in accordance with a recent research conducted on the Krka River, which found that the same few elements accumulated more in fish intestines than in acanthocephalans (Mijošek et al., 2022). Both investigations confirmed remarkably efficient accumulation of potentially highly toxic elements (Pb, Cd and Tl), which goes in favor of possible protection of the infected fish (Sures, 2002; Filipović Marijić et al., 2013; Mehana et al., 2020; Molbert et al., 2020). Moreover, high BCF values observed for Pb, Cd, Tl, Cu, Ba, Mn, Sr, Ca, and Hg (Table 2) suggest the likelihood of recent high exposure to these elements in the Krka River.

Although we expected to observe some significant spatial or temporal trends regarding BCF values, the number of distinctive patterns was limited (Table 2). Still, the maximum BCFs in both seasons for As, Hg, Mn, and Se were observed at KRS, for Ba and Ca at KRK, and for Sr at KNP, showing that used acanthocephalan species provides a valuable biological response even at the site of the minimal metal(loid) exposure, KRS. Regarding seasonal changes, only the BCFs for Fe, Na, and Sr were higher in spring compared to autumn at all sites, whereas the opposite trend was seen for As, Mn and Pb (Table 2). These trends could not be associated with the metal(loid) exposure from water or sediments, but rather to factors such as age, physiology, reproductive stage or size of the acanthocephalans or fish host (Filipović Marijić et al., 2013; Sures et al., 2017).

The BCF ranges for most elements in the current research were comparable to the 2015/2016 study on the same host-parasite system (Table 2, Mijošek et al., 2022). However, the BCF values for Pb were 2 to 8 times greater in the current study, which can be attributed to the aforementioned decrease in Pb concentrations in fish intestines nowadays. In summary, while there is a lack of additional research pertaining to this particular ecosystem and species, our data suggests that *D. truttae* is a promising indicator, even in ecosystems characterized by low to moderate metal(loid) exposure, such as the karst Krka River.

3.4. Metal(loid) accumulation in gut contents (GC)

Dietborne metal(loid) uptake was considered through measuring metal(loid) concentrations in GC of fish. The sequences of macroelements (Ca>Na>K>Mg) and trace elements (Fe>Zn>Sr≥Mn>Cu>Ba≥V≥Cr≥Co≥Rb≥Pb≥Cd≥As>Se≥Mo>Cs>Tl>Hg) (Table S9) were notably different in comparison to fish intestine (Figs. 2-5), indicating that metal(loid) accumulation depends on the bioavailability of metal(loid)s in fish diet. Further, fish have the ability to regulate metal uptake from food into the tissue depending on the specific requirements of organisms (Clearwater et al., 2000), which is reflected in different efficiency of metal absorption. Similar patterns and differences in metal accumulation (Cd, Cu, Fe, Mn, and Zn) between fish intestine and GC have been reported for the European chub from the Sava River (Filipović Marijić and Raspor, 2012, 2013). As brown trout is an omnivorous species, the gut contents included a variety of species including insect larvae, detritus, plants to gammarids or small mollusks. This led to high variations in metal(loid) concentrations among individual fish (Table S9). For example, the higher average contents of Ca and Sr in GC compared to fish intestine is associated to the prevalence of gammarids as a favored food source for many fish, which exoskeletons are particularly rich in Ca and Sr (Greenway, 2008), but only a small portion of these elements is bioavailable to organisms. To our knowledge, this is the first report on metal(loid)s in GC of brown trouts, so comparison with previous data was not possible.

Spearman correlation analyses revealed sporadic associations between concentrations of metal(loid)s in GC and fish intestine or acanthocephalans. More frequently, we observed negative correlation between metal(loid)s in GC and fish size (TM), which corresponds to higher metabolic needs and feeding rates during the development in early stages (Farkas et al, 2003).

3.4.1. Spatial differences

Statistically significant spatial differences were observed for most elements, except Ba, Ca, Mg, Na, Se, Sr and Tl (Table S9). The levels of most of the remaining elements were highest in GC of fish from KNP, similar to spatial distribution observed in sediment samples (Tables S5 and S9). During dissection, particles of sediments could be observed in GC of many fish, suggesting that sediments play a significant role in the absorption of metals through the diet and may pose a threat to organisms in the area of the national park and sensitive lake systems in case of the prolonged exposure. Exceptionally, Cd concentrations in GC were highest at KRS, while Cu concentrations in GC were highest at KRK, which aligns with the spatial distribution of Cu in fish intestine and acanthocephalans (Figs. 2d and 3a). Overall, metal(loid) concentrations in GC of fish were typically the lowest at KRS, demonstrating the lowest metal(loid) exposure at this location, which corresponded to water and sediment samples.

3.4.2. Temporal differences

The majority of elements in GC had higher concentrations in spring than autumn, with the highest number of statistically significant differences at KRS (Table S9). As already mentioned, higher gut fullness indices were found in spring, a season characterized by maximum feeding rates for this species, as well as greater variability of the prey, which led to the increased metal(loid)s accumulation in spring. A similar pattern was also observed in parasites and intestine, which further suggests that the uptake of metal(loid)s through the diet influences the responses of organisms, prompting us to investigate their relationships further.

3.4.3. Relationships between metal(loid) concentrations in fish intestine and acanthocephalans to gut contents

To compare levels of metal(loid)s in biological samples to those found in GC, as an important uptake route, we calculated average ratios between the concentrations of metal(loid)s in the intestine of fish and GC (INT/GC), as well as in acanthocephalans and GC (AC/GC) (Table S10). With this approach, we were able to evaluate and estimate the potential bioavailability of metal(loid)s from food to organisms. However, it is important to keep in mind that this is only an estimate since the metal(loid)s found in the GC reflect the source of metal(loid)s in the food at the time of sampling, whereas organisms accumulate metal(loid)s over a longer period of time and exhibit a more chronical response.

Nevertheless, it can be presumed that the bioavailability of As, Ba, Ca, Cr, Cu, Fe, Mn, Pb, Sr, and V from food to the fish intestine may be minimal, since the majority of the INT/GC ratios for these elements were <15%, suggesting that their absorption is physiologically regulated and not dependent on their quantity in the food source. Although concentrations of these elements in GC were predominantly highest at KNP, the calculated ratios were either similar across all locations or the lowest at KNP (Table S10). With the exception of Cd and Pb, the lowest values of the AC/GC ratios were found once more for As, Ba, Ca, Cr, Fe, Mn, Sr, and V, confirming low bioavailability of these elements from food. Low values mostly up to 50% were also observed for Co, Cs, and Mo for both fish intestine (INT/GC) and acanthocephalans (AC/GC) (Table S10), suggesting that although concentrations of these elements in the GC are elevated (Table S9), their burden is significantly lower in fish intestine and parasites. This indicates that a large portion of these elements is excreted via the faeces (Dallinger and Kautzky, 1985).

Thallium, Se, Hg, Rb, K and Zn exhibited remarkable luminal absorption efficiency, as evidenced by their substantially higher concentrations in the intestine and acanthocephalans compared to GC (Table S10). In addition, Cd, Cu, and Pb were also accumulated more efficiently in acanthocephalans than GC and fish intestine showing they may selectively accumulate certain elements over others, potentially influencing the overall element distribution within the fish host and providing a protective role for the fish host when exposed to potentially toxic elements (Sures, 2002). Understanding these differences in element accumulation is important since it can provide valuable insights into the dynamics of host-parasite interactions and their impact on element cycling in aquatic ecosystems. To our knowledge, there is no available research that compares metal(loid) accumulation in acanthocephalans and fish gut content. However, Dallinger and Kautzky (1985) published data on Cd, Cr, Cu, Mn, Pb and Zn concentrations in the intestine and GC of species *Salmo gairdneri* from Augraben River and Leiferer Graben River in Italy. The obtained ratios of Cd, Cu, Mn and Zn were similar to those in our research, whereas Cr and Pb appeared to be less bioavailable in our study. Further, Filipović Marijić and Raspor (2012), reported comparable ratios to those found in the present research for European chub (Zn>Cd≥Cu>Fe≥Mn). Prior studies have consistently shown that fish intestines contain very high levels of Zn, serving as an important storage location for Zn in many fish species (Dallinger and Kautzky, 1985; Rajkowska and Protasowicki, 2013). In addition, it is also important to consider the potential influence of thiol-containing proteins, which can be found in even higher quantities in fish intestines compared to liver and gills, as indicated in previous studies on various fish species (Roesijadi et al., 2009; Filipović Marijić and Raspor, 2010), including brown trout from the Krka River (Mijošek et al., 2019). Given the high affinities of Zn and Hg for thiol-containing proteins like metallothioneins (MTs) (Roesijadi et al., 2009), it is possible that MTs may serve as potential binders of Zn and Hg, leading to their substantially higher intestinal concentrations compared to GC. Further, Hg, but also Rb and Se, are among rare elements for which biomagnification in food webs is suggested (Campbell et al., 2005; Córdoba-Tovar et al., 2022). High accumulation and absorption of K in the intestine in comparison with other macrolements has also been previously described by Bucking and Wood (2006). Also, accumulation of K, Se, and Zn over time is probably related to their important biological roles (e.g. as a part of different enzymes, metallothioneins or K^+ channels).

However, it is consistently challenging to make a clear distinction between the uptake through the alimentary tract (dietborne uptake) and absorption through the gills (waterborne uptake) in the field studies. Moreover, the composition and metal(loid) content of fish diet are extremely heterogenous, making it impossible to establish a direct link between a specific food source and the fish.

4. Conclusions

This study provided information on total concentrations of 22 metal(loid)s in the intestine of brown trout *S. trutta*, fish gut content, and acanthocephalan species *D. truttae*. Concentrations of As, Ba, Cr, Hg, Mo, and V in parasites provide the first-ever information on this endemic acanthocephalan species, as well as metal(loid)s in GC of brown trouts, serving as an indication of metal intake through food sources. Although often neglected in fish ecology and ecotoxicology, parasites were integrated in the study to achieve more complete understanding of fish biology and to realize influence of parasitic infections on fish responses. Acanthocephalans and GC were also applied to assess possible bioavailability of metal(loid)s to fish intestinal tissue and intestinal parasites.

Great efficiency of metal(loid) accumulation in acanthocephalans was confirmed for many elements, especially potentially toxic ones such as Pb, Cd, and Tl. With few exceptions, concentrations of various elements were found to be the highest at different sites: As, Ba, Ca, Cu, Fe, Pb, Se, and Zn at KRK; Cd, Cs, Rb, and Tl at KRS; and Hg, Mn, Mo, Sr, and V at KNP. These patterns corresponded only partially to metal(loid) exposure through water and/or sediments, and indicated that differences in metal(loid) bioaccumulation were strongly related to the biology and ecology of host and parasite species, dietary habits, and the presence of acanthocephalans in the fish intestine. Metal(loid) concentrations in GC were generally the highest at KNP, reflecting the spatial distribution of metal(loid)s in river surface sediment. Ratios between metal accumulation in the intestine and GC, and acanthocephalans and GC, indicate potential low bioavailability of As, Ba, Ca, Cr, Cu, Fe, Mn, Pb, Sr, and V from the dietary sources, while high absorption of Tl, Se, Hg, Rb, K and Zn is suggested. However, that should be interpreted with caution since concentrations in GC represent the metal(loid) burden in the moment of sampling, while concentrations in organisms reflect chronic exposure, which might give different response.

We observed a biodilution effect associated with increased acanthocephalan mean intensity. This requires further investigations of other species exposed to different levels to evaluate the potential benefits to hosts and parasites. Comparison with previous research in this area revealed an increase of Cd, Cu, Mn, and Zn concentrations, pointing to the still negative influence of the industrial and municipal wastewater outlets on the Krka National Park.

According to collected data, wastewater discharges near the border of Krka National Park currently have a moderate impact. However, concerning tendencies of frequently highest metal(loid) concentrations in sediments, GC and occasionally even in organisms from the KNP indicate the need for more thorough and frequent monitoring of this area. Besides water and sediment as potential environmental sources of metal(loid)s, future monitoring programs should always consider the dietary effects of metal(loid) exposure and the presence of acanthocephalan parasites as possible important contribution factors to metal(loid) input, which would enable more reliable interpretations of results in all ecosystems. Based on given data, we recommend for future studies to inspect fish for the presence of other parasites like trematodes as well, while for the analysis of dietary habits, it would be relevant to classify the gut content in herbal and animal material to identify the most common groups of insects and gammarids to possibly evaluate the contribution of each group to metal(loid) uptake in fish. Further, estimation of bioavailable, and thus possibly toxic, metal(loid)s which binding to sensitive biomolecules may actually change their molecular function, structure and/or dynamics in the cell is a next crucial step in metal(loid) risk assessment.

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Figure captions:

Figure 1. Study area with marked sampling sites on the Krka River (1 – Krka River source; 2 – Krka River near the Town of Knin; 3 – location in the national park ner the Brljan Lake)

Figure 2. Concentrations (mg kg^{-1} or μg kg^{-1} on wet mass basis) of six elements in the intestine of brown trout *S. trutta* and acanthocephalans *D. truttae* from three sites of the Krka River in two sampling campaigns (spring and autumn) that showed enhanced accumulation at the contaminated site, KRK. Different letters refer to significant differences of metal(loid) concentrations among three locations in spring (capital letters) and autumn (small letters), while statistically significant differences between two seasons at each sampling site are marked with asterisk (*).

Figure 3. Concentrations (μg kg^{-1} on wet mass basis) of four elements in the intestine of brown trout *S. trutta* and acanthocephalans *D. truttae* from three sites of the Krka River in two sampling campaigns (spring and autumn) that showed enhanced accumulation at the reference site, KRS. Different letters refer to significant differences of metal(loid) concentrations among three locations in spring (capital letters) and autumn (small letters), while statistically significant differences between two seasons at each sampling site are marked with asterisk (*).

Figure 4. Concentrations (μg kg^{-1} on wet mass basis) of five elements in the intestine of brown trout *S. trutta* and acanthocephalans *D. truttae* from three sites of the Krka River in two sampling campaigns (spring and autumn) that showed enhanced accumulation at the site in the national park, KNP. Different letters refer to significant differences of metal(loid) concentrations among three locations in spring (capital letters) and autumn (small letters),

while statistically significant differences between two seasons at each sampling site are marked with asterisk (*).

Figure 5. Concentrations (mg kg^{-1} or μg kg^{-1} on wet mass basis) of five elements in the intestine of brown trout *S. trutta* and acanthocephalans *D. truttae* from three sites of the Krka River in two sampling campaigns (spring and autumn) that showed unclear trends of accumulation. Different letters refer to significant differences of metal(loid) concentrations among three locations in spring (capital letters) and autumn (small letters), while statistically significant differences between two seasons at each sampling site are marked with asterisk (*).