Thallium accumulation in different organisms from karst and lowland rivers of Croatia under wastewater impact

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Abstract

The aim of the present research was to investigate the bioaccumulation of Tl, technology-critical element, in fish intestine and muscle, gammarids and fish intestinal parasites, acanthocephalans, and to evaluate their potential as indicators of metal exposure in the aquatic environments. Moreover, total and cytosolic (metabolically available and potentially toxic fraction) Tl concentrations were measured and compared between intestine of brown trout (*Salmo trutta* Linnaeus, 1758) from the karst Krka River and Prussian carp (*Carassius gibelio* Bloch, 1782) from the lowland Ilova River. Since there is a scarcity of information on subcellular metal partitioning in the fish intestine, results on Tl concentrations in digested intestinal tissue, homogenate and cytosol represent preliminary data on Tl diet borne uptake in salmonid and cyprinid fish. In both rivers, samplings were performed upstream (reference site) and downstream (contaminated site) of the wastewater impact in autumn and spring. Total Tl concentrations were much higher in brown trouts than Prussian carps, as well as proportions of cytosolic Tl concentrations in intestinal tissue of brown trout (45-71%) than Prussian carp (32-47%), both showing species- and site-specific differences. Considering different bioindicator organisms, the most effective Tl accumulation was evident in acanthocephalans compared to the fish tissues and gammarids, confirming the potential of fish parasites as bioindicators of metal exposure. Trends of spatial and temporal Tl variability were mostly comparable in all indicator organisms and of total and cytosolic Tl concentrations in intestine of salmonid and cyprinid fish species, confirming their application as useful biological tools in metal exposure assessment.

Introduction

Thallium (Tl) is a rare trace metal, but its high toxicity, water solubility and tendency of bioaccumulation made it a US EPA priority pollutant. Although naturally present in environment in low concentrations, human activities have greatly increased its presence in nature. The most common anthropogenic sources of Tl are coal burning, metal mining and smelting, but also a variety of other applications yielding thallium as a byproduct (Karbowska 2016; Peter and Viraraghavan 2005). Despite the fact that Tl toxicity is comparable or even higher than toxicity of mercury, lead or cadmium (Peter and Viraraghavan 2005; Zitko 1975), the studies considering its ecotoxicological relevance are rare and most of the existing studies were conducted only as laboratory exposure experiments (Couture et al. 2011; Lan and Lin 2005; Lapointe and Couture 2009; Pickard et al. 2001; Ralph and Twiss 2002; Xiao et al. 2004; Zitko 1975). In nature, Tl occurs in two oxidation states: monovalent Tl(I) and trivalent Tl(III) (Cheam et al. 1995; Lan and Lin 2005; Ospina-Alvarez et al. 2015). Compared with trivalent Tl, monovalent Tl is thermodynamically more stable and less reactive, so in aquatic environments, dissolved thallium mostly appears as Tl(I) (Couture et al. 2011; Ospina-Alvarez et al. 2015). Toxicity studies using unicellular algae *Chlorella* (Ralph and Twiss 2002) and cladoceran *Daphnia magna* (Lan and Lin 2005) showed that Tl(III) is more toxic than Tl(I). As one of the technology-critical elements, Tl has a potential of being used in the development of emerging key technologies such as energy efficiency, electronics or acoustic-optical measuring devices (Cobelo-García et al. 2015), but potential biological and human health threats need to be further explored.

A common approach in biomonitoring studies of metal exposure in the aquatic environment involves measurements of total metal concentrations in different target organs of bioindicator organisms, usually liver/hepatopancreas and/or gills of bivalves, crustaceans and fish (Dragun et al. 2018; Geffard et al. 2010; Langston and Bebiano 1998). However, as total tissue concentrations do not represent metabolically available metal levels, since portion of the metals is bound to metal-rich granules or detoxified by metal binding proteins (metallothioneins), the information on metal partitioning and potentially toxic metal fractions can be obtained by determining metal concentrations at the subcellular level (Barst et al. 2016). After entering the organisms, some portions of metals are sequestered to few detoxified forms like metal-binding proteins or granular structures and are therefore indicated as biologically detoxified metals (Wallace et al. 2003). In contrast, some other metal portions are incorporated into non-detoxifying components, such as sensitive biomolecules, which could lead to possible toxic effects (Mijošek et al. 2019b; Urien et al. 2018). Untill now, subcellular metal partitioning has been investigated mainly in bivalves (Bonneris et al. 2005; Wallace et al. 2003), crustaceans (Geffard et al. 2010) and fish liver, gills and gonads (Barst et al. 2016; Giguère et al. 2006; Krasnići et al. 2018; Urien et al. 2018). Despite its importance in diet borne metal uptake (Clearwater et al. 2000), intestine of freshwater fish was rarely applied as a bioindicator tissue and existing studies presented only total metal concentrations (Andres et al. 2000; Dallinger and Kautzky 1985; Filipović Marijić and Raspor 2010, 2012, 2014; Giguère et al. 2004; Jarić et al.

2011; Nachev and Sures 2016; Staniskiene et al. 2006; Yeltekin and Sağlamer 2019), while subcellular metal partitioning in fish intestine has not been conducted yet. Thus, due to the lack of information on Tl accumulation in freshwater organisms from natural habitats, especially considering dietary Tl uptake in fish, the aim of the present study was to compare Tl concentrations in different bioindicator organisms and in intestinal tissue of salmonid and cyprinid fish from rivers impacted by a wastewater discharge.

In Croatia, the lower part of the karst Krka River was proclaimed national park in 1985, but its upper part is still directly influenced by industrial (factory of metal based products) and municipal wastewaters, which are released without proper treatment in the river water. In the Krka River, salmonid fish are highly infected with intestinal parasites, acanthocephalans, which enabled comparison of Tl content in fish intestine and its intestinal parasites, acanthocephalans, as well as in gammarids, therefore comprising organisms involved in parasite life cycle (crustaceans as intermediate and fish as definitive host). Moreover, acanthocephalans were reported to effectively accumulate metals, orders of magnitude higher than other indicator organisms (Filipović Marijić et al. 2013; Sures 2001), but Tl concentrations were rarely evidenced (Sures et al. 1999; Thielen et al. 2004). Therefore, in the Krka River total Tl concentrations were compared among acanthocephalan *Dentitruncus truttae* Sinzar, 1955, its intermediate host *Gammarus balcanicus* Schäferna, 1922 and intestine and muscle of its definitive hosts *Salmo trutta* Linnaeus, 1758. Lowland Ilova River is a part of the Lonjsko polje Nature Park, and is threatened by the wastewater influence, mostly produced by a fertilizer factory. In the Ilova River, the dominant fish species was *Carassius gibelio* Bloch, 1782, a mostly herbivorous species not involved in the acanthocephalan life cycle, but was used to compare diet borne Tl concentrations in intestine of cyprinid fish from the lowland river with salmonid fish from the karst river. In addition, in both fish species, total Tl concentrations in digested intestinal tissue (total Tl in tissue) were compared with its concentrations in homogenates (total Tl in homogenate) and cytosolic fraction, which contains heat-denatured proteins (HDP, such as enzymes), lysosomes and microsomes (biologically available and partially toxic metal fraction) and heat-stable proteins (HSP), such as metallothioneins (detoxified metal fraction) (Bonneris et al. 2005; Urien et al. 2018; Wallace et al. 2003).

Therefore, additional goals of the presented study were to estimate Tl distribution in the intestinal tissue (digested tissue, homogenates and cytosols) of fishes from the karst and lowland rivers, involving spatial (reference and contaminated site) and temporal (autumn and spring) variability. Moreover, the potential of Tl accumulation in acanthocephalans, gammarids and fish intestine and muscle was compared and used as a tool in metal exposure assessment of the Krka and Ilova rivers.

Experimental

Study areas and sampling

The two study areas were selected as freshwater ecosystems of specific ecological characteristics, karst Krka River and lowland Ilova River. At each area, the sampling was carried out at two sampling sites (reference and contaminated) and in two seasons (autumn and spring).

Krka River is a typical karst river in Croatia, known by travertine waterfalls and exceptional natural beauty, which is threatened by the technological and municipal wastewaters discharges, only 2 km upstream of the park border. Based on the physico-chemical water parameters and dissolved metal levels in the river water (Filipović Marijić et al. 2018; Sertić Perić et al. 2018), Krka River source was chosen as a reference site, while the contaminated site was located downstream of the wastewater outlets near the town of Knin. Bioindicator organisms from the Krka River were salmonid fish brown trout (*Salmo trutta* Linnaeus, 1758), gammarid species *Gammarus balcanicus* Schäferna, 1922 and acanthocephalan *Dentitruncus truttae* Sinzar, 1955. Sampling campaigns were performed in autumn 2015 and spring 2016.

Ilova River is a lowland river in a central continental part of Croatia and its lower course is influenced by municipal (the town of Kutina) and industrial (petrochemical processing of natural gas in production of fertilizers) wastewaters. The site near the Ilova village was taken as a reference site and it is located upstream of the known pollution sources, while the contaminated site is situated near the Trebež village and 8 km downstream of the confluence of the Kutinica River that discharges industrial wastewater originating from a fertilizer factory (Radić et al. 2013). Contaminated site is located in wetland area protected as a Lonjsko Polje Nature Park. Bioindicator organism was cyprinid fish Prussian carp (*Carassius gibelio* Bloch, 1782). Sampling campaigns at the Ilova River were conducted in autumn 2017 and spring 2018.

Fish sampling was performed by electro-fishing, following the Croatian standard HRN EN 14011 (2005). Captured specimens of fish were kept alive in aerated water tank till further processing in the laboratory. Gammarids were collected at the same locations as fish, using benthos hand net (625 cm^2 and mesh size 250 μm).

The river water samples were collected in triplicates in acid pre-cleaned polyethylene plastic bottles at each site. Immediately after sampling water was filtered through a 0.45 μm pore diameter cellulose acetate filter (Sartorius, Germany) mounted on syringes. Aliquots of filtered samples for metal analyses were transferred into acid pre-cleaned 20 mL polyethylene plastic bottles and acidified with 400 μL of concentrated nitric acid (HNO_3) (Rotipuran® Supra 69%, Carl Roth, Germany) and stored at 4 °C until metal measurement.

Dissection and sample preparation

Before tissue dissection, basic fish biometric parameters, total length and body mass, were recorded and condition indices were calculated. Individuals of both fish species were euthanized with freshly prepared anesthetic tricainemethane sulphonate (MS 222, Sigma Aldrich) in accordance to the Ordinance on the protection of animals used for scientific purposes (European Union 2010). Muscle and intestinal tissue were dissected and weighed for further Tl determination. Gonads and liver were also weighed in order to calculate gonadosomatic and hepatosomatic indices. In the Krka River, fish intestinal parasites were isolated from the intestine and counted in each specimen. Gammarids were cleaned and pooled for further analyses due to their small sizes and masses. In autumn, 14 and 16 pooled samples of gammarids from the reference and contaminated site were obtained, respectively and in spring 10 and 17 pooled samples from the reference and contaminated site, respectively. All samples were stored at -80 °C until further analyses.

Acid digestion of muscle and intestinal tissue, gammarids and acanthocephalans

All samples were digested in a drying oven at 85 ºC for 3.5 h using HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and H_2O_2 (Suprapur®, Merck, Germany) in a volume ratio of 2:1 for fish muscle and intestine and 3:1 for gammarids and acanthocephalans. Before Tl measurements, all samples were 5 times diluted with Milli-Q water.

Homogenization, digestion and preparation of cytosolic fractions of fish intestinal tissue for Tl measurement

Samples of intestinal tissue of both fish species were homogenized in 5 volumes of buffer which contained 100 mM Tris-HCl/base (Merck,Germany, pH 8.1 at 4 °C) with 1 mM dithiothreitol (DTT, Sigma, USA) as a reducing agent and 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, USA) and 0.006 mM leupeptin (Sigma, USA) as protease inhibitors. Tissues were homogenized by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice-cooled tube at 6000 rpm. One part of the homogenate was digested in order to determine the total metal content (insoluble and soluble tissue fraction). Rest of the homogenate volumes were centrifuged in the Avanti J-E centrifuge (Beckman Coulter, USA) at 50,000×g for 2 h at 4 °C to obtain the soluble tissue cytosolic fractions (Urien et al. 2018; Wallace et al. 2003).

Intestinal homogenates and cytosolic fractions were digested by addition of oxidation mixture, which contained concentrated HNO_3 and 30% H_2O_2 (v/v 3:1) in a laboratory dry oven at 85 °C for 3.5 h. Before Tl measurement, cooled samples were five times diluted with Milli-Q water. Acid digestion efficiency was checked by digestion of fish muscle certified reference materials for trace metals (DORM-2, National Research Council of Canada, NRC, Canada). Certified value for Tl is 0.004 mg kg^{-1}, while the average value of five performed measurements was 0.004±0.0005 mg kg^{-1}, giving the recovery of 100%.

HR ICP-MS measurement of Tl concentrations

Thallium concentrations were measured in prepared samples 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). Measurement was operated in a low- resolution mode. Calibration solution was prepared by dilution of 100 mg L^{-1} multielement stock standard solution (Analitika, Czech Republic) and indium (1 µg L^{-1}, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added as an internal standard to all samples and solutions. The accuracy of HR ICP-MS measurements was tested using quality control sample QC trace metals (catalogue no. 8072, UNEP GEMS, Burlington, Canada). Limit of detection (LOD) was calculated as three times the standard deviation of the mean of ten blank determinations (100 mM Tris-HCl/Base, 1 mM DTT) and amounted 0.001 ng g^{-1}.

Data processing and statistics

Results on Tl concentrations in digested homogenates (total Tl content) and cytosols (metabolically available Tl content) were compared between salmonid and cyprinid fish species. In order to enable appropriate comparison of Tl accumulation in organisms involved in acanthocephalan life cycle, Tl contents were presented as total concentrations in digested fish muscle and intestinal tissue and in the whole gammarids and acanthocephalans from the Krka River. All concentrations obtained in this study are presented as µg kg^{-1} of wet tissue weight (w.w.).

Basic calculations were made in Microsoft Excel 2007, while the significance of differences between seasons or locations was tested in SigmaPlot 11.0 (Systat Software, USA) by application of Mann-Whitney U-test, since assumptions of normality were not always met. The level of significance was set to 95% ($p<0.05$) and is indicated in the tables or text. Data are presented as mean ± standard deviation.

Fish indices were calculated according to the following equations: Fulton condition index ($FCI = (W/L^3) \times 100$; Ricker 1975), hepatosomatic index ($HSI = (LW/W) \times 100$; Heidinger and Crawford 1977) and gonadosomatic index ($GSI = (GW/W) \times 100$; Wootton 1990), where W is the fish mass (g), L is the total length (cm), LW is the liver mass (g) and GW is the gonad mass (g).

The levels of parasite infection were quantified by prevalence, which describes the number and percentage of infected fish, and by mean intensity of infection, which represents an average number of parasites per fish host (Bush et al. 1997). Bioconcentration factors (BCF) were calculated according to Sures et al. (1999) as the ratio of the element concentration in the parasites and the concentration in the host tissue.

Results and discussion

Thallium concentrations in the karst and lowland river water

Thallium concentrations in water were rather low in both, Krka and Ilova rivers, with the average concentrations 5-13 ng L^{-1}. At the Ilova village site, comparable Tl concentrations were recorded with those at both sites in the Krka River (5-8 ng L^{-1}), while concentrations were slightly higher at the contaminated site near the Trebež village (9-13 ng L^{-1}) (Fig. 1). Therefore, spatial Tl differences were considered significant only in the Ilova River, as higher Tl concentrations in water from the contaminated site in both seasons compared to the reference site. Seasonal differences were significant at the Trebež village, as higher Tl concentration obtained in autumn than spring. In the Krka River, only slightly higher average Tl values were evident at the contaminated site near the town of Knin compared to the reference site in both seasons, but without significant differences (Fig. 1). The contaminated site near the Trebež village receives contaminating inputs of a fertilizer factory (nitrogenous fertilizers, mineral NPK fertilizers, carbon black, bentonites and additives for foundries, cattle feed additives) and runoff from soil contaminated by agriculture (Radić et al. 2013), which all possibly serve as significant Tl source in water.

Although Tl is a United States Environmental Protection Agency priority pollutant (EPA 2012), it is not considered as a part of the European Union Water Framework Directive and as such, there are no official European environmental quality standards for Tl in aquatic systems (Commission of the European Communities 2006). The Canadian Water Quality Guideline, however, proposes the total dissolved Tl levels in freshwaters of 0.8 µg L^{-1} (CCME 2003). Observed Tl concentrations in Croatian rivers are comparable to the average Tl values usually reported for freshwater ecosystems around the world, which range 5-10 ng L^{-1} in clean waters (Belzile and Chen 2017; Karbowska 2016; Peter and Viraraghavan 2005). Nielsen et al. (2005) proposed the global average Tl range of 6±4 ng L^{-1}, based on analyses in 12 large rivers. Other studies reported various Tl concentration ranges in freshwater ecosystems, for example 5-17 ng L^{-1} in river waters in Poland (Karbowska 2016), 2-443 ng L^{-1} in United Kingdom (Law and Turner 2011) , about 13 ng L^{-1} in a river whose catchment contained no metal mines to 2640 ng L^{-1} in water taken directly from an abandoned mine shaft in Cornwall in England (Tatsi and Turner 2014), 120-570 ng L^{-1} in the Tunuyán River in Argentina (Escudero et al. 2015). Therefore, in both of our investigated freshwater ecosystems, Tl concentrations in water are lower or in accordance with the range reported for world rivers.

Thallium bioaccumulation and distribution in the intestine of brown trout (*Salmo trutta*) and Prussian carp (*Carassius gibelio*)

Fish biometry

Considering brown trouts in the Krka River, average total length and body mass did not show spatial differences but pointed to seasonal differences in both sites, with significantly higher fish biometric parameters in autumn. A similar trend was observed in carps from the Ilova River, with higher average total length and body mass in autumn, although significantly only for body mass of fish from the Ilova village (Table 1). Higher values of GSI were observed in spawning periods of both species, late autumn for brown trout (Mrakovčić et al. 2006) and April-July for Prussian carps (Sasi 2008). On the other hand, FCI and HSI values declined in spawning periods of both species (Table 1), probably as a result of the mobilization of energy reserves needed for reproductive development (Moddock and Burton 1999). Although lower levels of FCI were often observed in the polluted sites (Couture and Rajotte 2003; Jenkins 2004; Zhelev et al. 2018), in our case, values were significantly higher at the contaminated sites in comparison to the reference sites in both rivers (Table 1), which could suggest that the contamination impact in both rivers was not so high to induce additional defence mechanisms which would consequently result in the decline of FCI values. Therefore, higher values at the contaminated sites might be associated with a better availability of nutrients (Lambert and Dutil 1997).

Comparison of total and cytosolic Tl concentrations in the intestine of salmonid and cyprinid fish

Thallium concentrations in digested tissues and homogenates of brown trout intestine from the Krka River ranged from 18.7-48.7 µg kg^{-1}, depending on the location and season (Fig. 2a, b). Cytosolic Tl concentrations were slightly lower (8.7-30.8 µg kg^{-1}), since cytosolic fraction involves only part of the total metal levels accumulated in the cell cytosol, which comprises HSP (metals bound to MT) and HDP (metals bound to enzymes) (Fig. 2c). A pattern of significantly higher Tl concentrations in intestine of brown trout from the Krka River source compared to the contaminated site was observed in all fractions in both seasons (Fig. 2a-c), while there were no significant seasonal differences. On the other hand, 20-30 times lower average Tl concentrations were observed in all intestinal fractions of the Prussian carp from the lowland Ilova River compared to brown trout. Obtained range in homogenates and digested tissue of Prussian carp was 1.2-2.1 µg kg^{-1}, and in cytosols 0.5-0.9 µg kg^{-1} (Fig. 3a-c). Thallium concentrations were significantly higher at the contaminated site near the Trebež village compared to the reference site in autumn in all fractions, but at the reference site only in cytosols in spring. Considering temporal variability, significantly higher Tl concentrations were observed in spring in all intestinal fractions of Prussian carp from the reference site, while in cytosols in autumn at the contaminated site (Fig. 3a-c). As already mentioned, Tl concentrations in water samples were low and mostly comparable in both rivers, so dissolved metal levels in the river water cannot explain such high variability in Tl accumulation between the two species (Fig. 1). Thus, emphasis should be put on the sediments and dietary metal uptake as possible reasons of these differences. Furthermore, while brown trout is omnivorous, actually mostly a carnivorous species (Mrakovčić et al. 2006), Prussian carp is mostly herbivorous fish species (Kottelat and Freyhof 2007), which might influence different Tl accumulation and detoxifying mechanisms in fish intestine.

Despite species-specific differences in Tl concentrations, the ratio of Tl levels between digested homogenates and intestinal tissues was comparable in both species, ranging from 85-103% (Table 2). Both Tl values, in digested tissue and homogenate, represent total metal levels, only obtained by different procedures, which were confirmed as reliable methods for tissue preparation prior to total metal analyses (Table 2). The portion of metabolically available Tl content in fish intestine was evaluated based on the ratio between cytosolic and total metal concentration in the intestinal tissue of both fish species. These ratios revealed that Tl is present in the metabolically available fraction (cytosol), which contains the potentially toxic metal fraction, from 45 to 71% in brown trout and from 32 to 47% in Prussian carp, depending on the location and season (Table 2). However, high percentage in cytosolic fractions does not completely reflect potentially toxic Tl levels in the cells, because besides microsomes, lysosomes and heat-denaturable proteins, cytosols also involve heat-stable proteins, like metallothioneins, which can contain a metal detoxified fraction (Bonneris et al. 2005), so at least some part of Tl present in the cytosol will probably still be detoxified.

In the same specimens of brown trouts, similar proportions of cytosolic and total Tl concentrations were reported for the liver tissue, in which proportions were around 67% and 63% at the reference and contaminated site, respectively (Dragun et al. 2018). To our knowledge, the data on cytosolic Tl concentrations in the intestinal tissue of freshwater fish species have not been published yet, so we cannot discuss on the regular distribution of Tl in the fish intestine. There is general scarcity of information on Tl distribution at the subcellular level in aquatic organisms. In few existing studies, Lapointe et al. (2009) and Lapointe and Couture (2009) reported that Tl mostly binds to the heat-stable proteins and granules in the liver of *Pimephales promelas*, while Rosabal et al. (2015) and Barst et al. (2016) confirmed dominant binding of Tl to the heat-stable proteins (HSP) in the liver of *Anguilla anguilla*, *Anguilla rostrata* and *Salvelinus alpines*, respectively. Moreover, Barst et al. (2016) observed the differences in Tl detoxifying mechanisms of *S. alpines* inhabiting four lakes with different environmental conditions, possibly the same as for the observed differences between the two fishes from the Krka and Ilova River. Regarding invertebrate species, in *Chironomus riparius* and *Daphnia magna* exposed to Tl-contaminated food, >55% and >40% of the total Tl levels were found to be bound to HSP, respectively (Dumas and Hare 2008). A major association of Tl with HSP corresponds to the relatively high presence of this metal in the fish intestinal cytosols in our study, while differences between the two fish species might be associated to the different biology and ecology of salmonid and cyprinid fish, as well as to the natural and anthropogenic site differences.

Comparison of Tl bioaccumulation among fish, gammarids and acanthocephalans from the karst Krka River

Biological characteristics of acanthocephalans

Almost all brown trout individuals from the Krka River were infected with parasites, except one fish from the reference site in spring (Table 3). Total number and mean intensities of infection were higher in autumn than spring in both locations, and this difference was much more pronounced at the contaminated site near the town of Knin (Table 3), with 6 times higher total parasite number at that site in autumn than in spring. Vardić Smrzlić et al. (2013) have already reported high acanthocephalan prevalence and similar trends in brown trout from the Krka River during 11 sampling campaigns (2005-2008). In their research, the highest mean intensity of *D. truttae* infection was also in the autumn period, similarly to the study of Paggi et al. (1978), who reported high prevalence (90.9–100%) of *D. truttae* infection in *S. trutta* from the Tirino River in

Italy, with the highest prevalence and intensity of infection between August and October. Increase in parasite abundance during autumn could be due to the life-cycle of their intermediate hosts, mostly gammarids, which reproduce in late summer and autumn, possibly influencing the highest acanthocephalan prevalence and abundance in that period (Kennedy 1985). Regarding ecotoxicological studies, high prevalence and number of acanthocephalans is actually in accordance with relatively low dissolved Tl concentrations in the river water, because abundance and species richness of endoparasites with indirect life cycles, such as acanthocephalans, tend to decrease under very stressful conditions and significantly increased level of pollution (MacKenzie 1999; Marcogliese 2004).

Prevalence and total parasite numbers in our study were higher than in other studies in Croatia, i.e. compared to chubs infected with *Pomohorhynchus laevis* and *Acanthocephalus anguillae* from the Sava River, which prevalence and total parasite numbers were 53% and 167 for *P. laevis*, respectively, and 47% and 120 for *A. anguillae*, both obtained during fish spawning period (Filipović Marijić et al. 2014). As the spawning period for the brown trout occurs in late autumn (Mrakovčić et al. 2006), higher infection rates during the spawning period were confirmed in our research as well. Obtained infection rates were also higher in comparison to *S. trutta* from other rivers, for examples from three streams in northern Italy (Brenta River) with reported prevalence of infection with different acanthocephalan species of 2-75% (Dezfuli et al. 2001) and from Kilise stream in Turkey (Murat River) with reported prevalence of *Echinorhynchus baeri* infections of 84.5% (Amin et al. 2016).

Total Tl concentrations in different bioindicator organisms

Thallium accumulation was the highest in acanthocephalans compared to gammarids and fish intestine and muscle in all sites and seasons. Thallium concentrations in fish intestinal tissue and gammarids were similar, while the lowest accumulation was observed in fish muscle (Fig. 4a-d). Spatial differences in Tl accumulation were significant in the fish intestine and muscle and in gammarids, with higher concentrations observed in organisms from the reference site (Fig. 4b-d). Temporal differences were significant only in gammarids, showing higher Tl concentrations in spring (Fig. 4b).

As for acanthocephalans, significant differences were not observed, although average values were evidently higher at the reference compared to the contaminated site in both seasons and in spring at both locations (Fig. 4a). However, as in many other studies, high variability in metal levels was observed among acanthocephalan individuals, probably as a reflection of host mobility, different parasite age and thus different exposure time or differences in metal uptake rate among parasite individuals (Filipović Marijić et al. 2013; Sures et al. 1999). Average Tl concentrations in acanthocephalans from the Krka River were higher than the average Tl concentration in *Acanthocephalus lucii* from the lake Mondsee in Austria (300 µg kg^{-1}) (Sures et al. 1999) and in *Pomphorhynchus laevis* from the Danube River near Budapest (200 µg kg^{-1}) (Thielen et al. 2004).

In gammarids, Tl concentrations ranged from 15.2 to 50.4 μg kg^{-1} w.w., with significantly higher levels at the reference site in both investigated seasons, and in spring at both sites (Fig. 4b). The same trend of significantly higher Tl concentrations at the reference site was confirmed by the fish samples; the fish intestine in both seasons and fish muscle in autumn (Fig. 4c-d). Thallium was less accumulated in the fish muscle than in the intestine (Fig. 4c-d), which was expected knowing the importance of the intestinal tissue in diet borne metal uptake (Dallinger et al. 1987; Clearwater et al. 2000). Different ecotoxicological studies have already reported that fish muscle represents one of the tissues with the lowest metal concentrations (Farkas et al. 2002; Nachev and Sures 2016). The average Tl values in brown trout muscle (5-17 μg kg^{-1} w.w.) are in the lower range reported for typical concentrations of Tl in muscle of different fish species (0.74-110 μg kg^{-1} w.w.) (Cunningham et al. 2019; Das et al. 2006; Engström et al. 2004; Karbowska 2016). Jardine et al. (2019) examined concentrations of Tl in a food web of the Slave River in Canada and found that tissue concentrations declined with increasing trophic position. Tl concentrations were measured in large and small fish, invertebrates and periphyton and fish had the lowest concentrations. Average total Tl concentrations in the muscle of pike, walleye and whitefish were 0.005 mg kg^{-1} w.w. which is comparable to our results for the brown trouts from the town of Knin. (Jardine et al. 2019). Other than that, Gantner et al. (2009) reported the range of 0.005-0.017 mg kg^{-1} among years for Arctic char (*Salvelinus alpinus*) from the Lake Hazen in Canada. Average Tl concentration for lake trout (*Salvelinus namacycush*) from Lake Michigan was 0.14 mg kg $^{-1}$ w.w. which is much higher than values obtained in our research (Lin et al. 2001). In the same specimens of brown trout, average Tl concentrations in fish intestine and muscle were lower than concentrations obtained in the liver, which were around 100-200 μg kg^{-1}, depending on the season and location (Dragun et al. 2018). Other than that, in the same fish, trend of higher concentrations in samples from the reference site was proven significant for the total and cytosolic concentrations of Tl and few other metals, Cd, Cs and Mo, in at least one season in intestinal tissue and liver and gammarids (Dragun et al. 2018; Mijošek et al. 2019a, 2019b). However, as levels in water samples were comparable (Fig. 1), the cause of significantly higher Cd, Cs, Mo and Tl concentrations in fish from the reference site cannot be explained by waterborne uptake and it requires further investigation, with special focus on river sediments and food as other metal sources, considering dietary intake as the important uptake route in fish (Clearwater et al. 2000). Diet content has already been shown as an important source of Tl in juvenile fathead minnows by Lapointe and Couture (2009), where diet borne exposure route was suggested as even more important risk of Tl toxicity than waterborne exposure route because aqueous Tl appeared to be better regulated.

Bioconcentration factors (BCF) were calculated to express the relation of Tl concentrations in the intestinal parasites and host tissues and gammarids (Table 4). Following similar Tl concentrations, BCF were mostly comparable between gammarids and fish intestinal tissue and ranged from 20-30 in gammarids and 17-34 in the fish intestine. BCF were much higher in relation to the fish muscle (49-112) (Table 4). Evidently higher accumulation in acanthocephalans than in fish tissues and intermediate hosts is probably based on the parasite dependence on host micronutrients since they lack a gastrointestinal system (Sures 2002). Although there is not much data on BCF regarding Tl concentrations, we could make comparison with the research of Sures et al. (1999), who reported the BCF of Tl in *Acanthocephalus lucii,* with respect to different organs of perch as host and zebra mussels, of 30 for both fish intestine and muscle, which is in accordance with our results for the intestine, but not for the muscle. Another research conducted by Thielen et al. (2004) reported higher BCF than in our study, which were 60 for the Tl analyzed in *Pomphorhynchus laevis* in relation to the intestine of barbel.

BCF provide information on the duration of metal exposure (Siddall and Sures 1998) in the environment as parasites accumulate metals more rapidly due to their short life span of 50-140 days (Kennedy 1985) in comparison to the average life span of fish (10-15 years) (Kottelat and Freyhof 2007). Therefore, BCF provide a possibility of comparing short term and long term metal exposure, so high ratio between concentrations in parasite and concentration in the host tissues serves as an indication of the recent increase in metal exposure, while low ratio with generally high metal levels indicates a longer, continuous exposure (Siddall and Sures 1998; Sures et al. 1999). Our results might be considered as an indication of continuous metal exposure in the Krka River, with comparable levels at both locations, with exception of BCF for acanthocephalan/muscle ratio at river source in spring (Table 4). Such results confirm previous findings on higher Tl concentrations in fish, gammarids and parasites from the Krka River source and require further investigation, with special focus on river sediments and food as possible metal sources. However, as metal levels can be influenced by different factors as age, size or fish physiological condition, season and breeding time, changes in BCF can also reflect all of these parameters, and not only the environmental exposure so they should be interpreted with caution.

Conclusions

Dissolved Tl concentrations were rather low and comparable in the water of the karst Krka River and lowland Ilova River in Croatia (average 5-13 ng L^{-1}). Salmonid and cyprinid fish species from the two investigated rivers showed different Tl accumulation capacity. In addition, in the Krka River, higher Tl concentrations were observed in all bioindicator organisms from the reference site compared to the site influenced by the wastewater impact. All of this might reflect other Tl sources besides water (sediment and/or food) and possibly some more significant natural origin of Tl in the area of the Krka River source, but this phenomenon needs further investigation.

Thallium concentrations and BCF values showed the most effective Tl accumulation in acanthocephalans in comparison to the fish intestine and muscle and gammarids, which confirmed the potential of fish parasites to be applied as bioindicator organisms of metal exposure in the aquatic ecosystems. However, due to large variations in metal accumulation in acanthocephalans, results should be interpreted with caution and the topic needs further research.

Comparison of Tl distribution in the intestine of fish from the Krka and Ilova River revealed that on average, Tl is present in the metabolically available fraction (cytosol), which could potentially be toxic, from 45 to 70% in brown trout and from 30 to 50% in Prussian carp. Tl concentrations were also much higher in salmonid than cyprinid fish, which could be associated with different ecology of the species and different Tl sources in the two rivers. Hence, Tl accumulation showed both species- and site-specific differences. The obtained Tl proportions in the cytosolic cellular fraction, i.e. metabolically available, are in accordance with the available literature data and since cytosols include heat-stable proteins, part of the metal will probably still be detoxified in the organisms.

To our knowledge, these are the first data on the distribution of Tl in the intestinal fish tissue, so presented results can serve as preliminary data on total and cytosolic Tl concentrations in intestine of salmonid and cyprinid fish. Further research should be focused, besides on total and cytosolic Tl content, on the detailed distribution in subcellular intestinal fractions to increase our knowledge on this technological critical element and Tl impact on aquatic organisms.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Amin OM, Heckmann RA, Evans RP, Tepe Y (2016). A description of *Echinorhynchus baeri* Kostylew, 1928 (Acanthocephala: Echinorhynchidae) from *Salmo trutta* in Turkey, with notes on synonymy, geographical origins, geological history, molecular profile, and X-ray microanalysis. *Parasite* **23**, 56-69. https://doi.org/10.1051/parasite/2016067

Andres S, Ribeyre F, Tourencq JN, Boudou A (2000). Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). *Science of the Total Environment* **248**, 11-25. https://doi.org/10.1016/S0048-9697(99)00477-5

Barst BD, Rasabal M, Campbel PGC, Muir DGC, Wang X, Köck G, Drevnick PE (2016). Subcellular distribution of trace elements and liver histology of landlocked Arctic char (*Salvelinus alpinus*) sampled along a mercury contamination gradient. *Environmental Pollution* **212**, 574–583. https://doi.org/10.1016/j.envpol.2016.03.003

Belzile N, Chen YW (2017). Thallium in the environment: a critical review focused on natural waters, soils, sediments and airborne particles. *Applied Geochemistry* **84**, 218-243. https://doi.org/10.1016/j.apgeochem.2017.06.013

Bonneris E, Giguère A, Perceval O, Buronfosse T, Masson S, Hare L, Campbell PGC (2005). Sub-cellular partitioning of metals (Cd, Cu, Zn) in the gills of a freshwater bivalve, *Pyganodon grandis*: role of calcium concretions in metal sequestration. *Aquatic Toxicology* **71**, 319–334. https://doi.org/10.1016/j.aquatox.2004.11.025

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997). Parasitology meets ecology on its own terms: Margolis et al. revisited. *Journal of Parasitology* **83**, 575−583. https://doi.org/10.2307/3284227

Canadian Council of Ministers of the Environment (CCME) (2003). Summary of existing Canadian environmental quality guidelines. http://ceqg-rcqe.ccme.ca/download/en/215

Cheam V, Lechner J, Desrosiers R, Sekerka I, Lawson G, Mudroch A (1995). Dissolved and total thallium in Great-Lakes waters. *Journal of Great Lakes Research* **21**, 384-394.

Clearwater SJ, Baskin SJ, Wood CM, McDonald DG (2000). Gastrointestinal uptake and distribution of copper in rainbow trout. *Journal of Experimental Biology* **203**, 2455-2466.

Cobelo-García A, Filella M., Croot P, Frazzoli C, Du Laing G, Ospina-Alvarez N, Rauch S, Salaun P, Schäfer J, Zimmermann S (2015) COST action TD1407: network on technology-critical elements (NOTICE)—from environmental processes to human health threats. *Environmental Science and Pollution Research* **22,** 15188-15194.

Commission of the European Communities (2006). Proposal for a Directive of the European Parliament and of the Council on Environmental Quality Standards in the Field of Water Policy and Amending Directive 2000/60/EC. COD, Brussels (2006/0129)

Couture P, Rajotte JW (2003). Morphometric and metabolic indicators of metal stress in wild yellow perch (*Perca flavescens*) from Sudbury, Ontario: a review. *Journal of Environmental Monitoring* **5**, 216−221. https://doi.org/10.1039/B210338A

Couture P, Fortin C, Hare L, Lapointe D, Pitre D (2011). Critical Review of Thallium in Aquatic Ecosystems. Report R-1272, Environment Canada. 36 pp.

Cunningham PA, Sullivan EE, Everett KH, Kovach SS, Rajan A, Barber MC (2019). Assessment of metal contamination in Arabian/Persian Gulf fish: A review. Marine Pollution Bulletin **143**, 264-283. https://doi.org/10.1016/j.marpolbul.2019.04.007

Dallinger R, Kautzky H (1985). The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdneri*): a field study. *Oecologia* **67**, 82−89. https://doi.org/10.1007/BF00378455

Dallinger R, Prosi F, Senger H, Back H (1987). Contaminated Food and Uptake of Heavy Metals by Fish (a Review and Proposal for Further Research). *Oecologia (Berlin)* **73**, 91-98.

Das AK, Chakraborty R, Cervera ML, Guardia M (2006). Determination of thallium in biological samples. *Analytical and Bioanalytical Chemistry* **385**, 665–670. https://doi.org/10.1007/s00216-006-0411-8

Dezfuli BS, Giari L, De Biaggi S, Poulin R (2001). Associations and interactions among intestinal helminths of the brown trout, *Salmo trutta*, in northern Italy. *Journal of Helminthology* **75**, 1–6. https://doi.org/10.1017/S0022149X01000518

Dragun Z, Filipović Marijić V, Krasnići N, Ivanković D, Valić D, Žunić J, Kapetanović D, Vardić Smrzlić I, Redžović Z, Grgić I, Erk, M (2018). Total and cytosolic concentrations of twenty metals/metalloids in the liver of brown trout *Salmo trutta* (Linnaeus, 1758) from the karstic Croatian river Krka. *Ecotoxicology and Environmental Safety* **147**, 537–549. https://doi.org/10.1016/j.ecoenv.2017.09.005.

Dumas J, Hare L (2008). The internal distribution of nickel and thallium in two freshwater invertebrates and its relevance to trophic transfer. *Environmental Science and Technology* **42**, 5144-5149. https://doi.org/10.1021/es800378j

Engström E, Stenberg A, Senioukh S, Edelbro R, Baxter, DC, Rodushkin I (2004). Multi-elemental characterization of soft biological tissues by inductively coupled plasma sector field mass spectrometry. *Analytica Chimica Acta* **521**, 123–135. https://doi.org/10.1016/j.aca.2004.06.030

EPA (2012). United States Environmental Protection Agency: priority pollutants. http://water.epa.gov/scitech/methods/cwa/pollutants.cfm.

Escudero LB, García CB, da Silva SM, Barón JH (2015). An eco-friendly cellular phase extraction technique based on the use of green microalgal cells for trace thallium species determination in natural water samples. *Analytical Methods* **7**, 7480-7487. https://doi.org/10.1039/C5AY01667C

European Union (2010). Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* No. L 276/33 (20. 10. 2010).

Farkas A, Salánki J, Spacziár A (2002). Relation between growth and heavy metal concentration in organs of bream *Abramis brama* L. populating lake Balaton. *Archives of Environmental Contamination and Toxicology* **43**, 236–243. https://doi.org/10.1007/s00244-002-1123-5

Filipović Marijić V, Raspor B (2010). The impact of the fish spawning on metal and protein levels in gastrointestinal cytosol of indigenous European chub. *Comparative Biochemistry and Physiology* **708** C, 133–138. https://doi.org/10.1016/j.cbpc.2010.03.010

Filipović Marijić V, Raspor B (2012). Site-specific gastrointestinal metal variability in relation to the gut content and fish age of indigenous European chub from the Sava River. *Water, Air & Soil Pollution* **223**, 4769– 4783. https://doi.org/10.1007/s11270-012-1233-2

Filipović Marijić V, Vardić Smrzlić I, Raspor B (2013). Effect of acanthocephalan infectionon metal, total protein and metallothionein concentrations in European chub from a Sava River section with low metal contamination. *Science of the Total Environment* **463–464**, 772–780. https://doi.org/10.1016/j.scitotenv.2013.06.041

Filipović Marijić V, Raspor B (2014). Relevance of biotic parameters in assessment of the spatial distribution of gastrointestinal metal and protein levels during spawning period of

European chub (*Squalius cephalus* L.). *Environmental Science and Pollution Research* **21**, 7596–7606. https://doi.org/10.1007/s11356-014-2666-5

Filipović Marijić V, Vardić Smrzlić I, Raspor B (2014). Does fish reproduction and metabolic activity influence metal levels in fish intestinal parasites, acanthocephalans, during fish spawning and post-spawning period? *Chemosphere* **112**, 449-455. https://doi.org/10.1016/j.chemosphere.2014.04.086

Filipović Marijić V, Kapetanović D, Dragun Z, Valić D, Krasnići N, Redžović Z, Grgić I, Žunić J, Kružlicová D, Nemeček P, Ivanković D, Vardić Smrzlić I, Erk M (2018). Influence of technological and municipal wastewaters on vulnerable karst riverine system, Krka River in Croatia. *Environmental Science and Pollution Research* **25**, 4715–4727. https://doi.org/10.1016/10.1007/s11356-017-0789-1

Gantner N, Power M, Babaluk JA, Reist JD, Kock G, Lockhart LW, Solomon KR, Muir DCG (2009). Temporal trends of mercury, cesium, potassium, selenium and thallium in arctic char (*Salvelinus alpinus*) from lake Hazen, Nunavut, Canada: Effects of trophic position, size, and age. *Environmental Toxicology and Chemistry* **28**, 254-263. https://doi.org/10.1897/08-054.1.

Geffard A, Sartelet H, Garric J, Biagianti-Risbourg S, Delahaut L, Geffard O (2010). Subcellular compartmentalization of cadmium, nickel, and lead in *Gammarus fossarum*: Comparison of methods. *Chemosphere* **78**, 822-829. https://doi.org/10.1016/j.chemosphere.2009.11.051

Giguère A, Campbell PGC, Hare L, McDonald DG, Rasmussen JB (2004). Influence of lake chemistry and fish age on cadmium, copper, and zinc concentrations in various organs of indigenous yellow perch (*Perca flavescens*). *Canadian Journal of Fisheries and Aquatic Sciences* **61**, 1702-1716. https://doi.org/10.1139/f04-100

Giguère A, Campbell PGC, Hare L, Couture P (2006). Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient. *Aquatic Toxicology* **77**, 178-189. https://doi.org/10.1016/j.aquatox.2005.12.001

Heidinger RC, Crawford SD (1977). Effect of temperature and feeding rate on the liver somatic index of largemouth bass, *Micropterus salmoides*. *Journal of the Fisheries Research Board of Canada* **34**, 633–638.

HRN EN 14011 (2005). Fish Sampling by Electric Power (in Croatian). Croatian Standard Institute, Zagreb.

Jardine TD, Doig LE, Jones PD, Bharadwaj L, Carr M, Tendler B, Lindenschmidt KE (2019). Vanadium and thallium exhibit biodilution in a northern river food web. *Chemosphere* **233**, 381-386. https://doi.org/10.1016/j.chemosphere.2019.05.282.

Jarić I, Višnjić-Jeftić Ž, Cvijanović G, Gačić Z, Jovanović LJ, Skorić S, Lenhardt M (2011). Determination of differential heavy metal and trace element accumulation in liver, gills, intestine and muscle of sterlet (*Acipenser ruthenus*) from the Danube River in Serbia by ICP-OES. *Microchemical Journal* **98**, 77−81. https://doi.org/10.1016/j.microc.2010.11.008

Jenkins JA (2004). Fish Bioindicators of Ecosystem Condition at the Caleasieu Estuary, Louisiana. National wetlands research center USGS, Lafayette.

Karbowska B (2016). Presence of thallium in the environment: sources of contaminations, distribution and monitoring methods. *Environmental Monitoring and Assessment* **188**, 640-659. https://doi.org/10.1007/s10661-016-5647-y

Kennedy CR (1985). Regulation and dynamics of acanthocephalan population. In: Biology of the Acanthocephala. (Eds. DWT Crompton, BB Nickol) pp. 385−416 Cambridge University Press, Cambridge.

Kottelat M, Freyhof J (2007). Handbook of European Freshwater Fishes. Kottelat, Cornol, Switzerland and Freyhof, Berlin.

Krasnići N, Dragun Z, Erk M, Ramani S, Jordanova M, Rebok K, Kostov V (2018). Size-exclusion HPLC analysis of trace element distributions in hepatic and gill cytosol of Vardar chub (*Squalius vardarensis* Karaman) from mining impacted rivers in north-eastern Macedonia. *Science of the Total Environment* **613/614**, 1055-1068. https://doi.org/10.1016/j.scitotenv.2017.09.160

Lambert Y, Dutil J-D (1997). Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of cod (*Gadus morhua*)? *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 104−112. https://doi.org/10.1139/cjfas-54-S1-104

Lan CH, Lin TS (2005). Acute toxicity of trivalent thallium compounds to *Daphnia magna*. *Ecotoxicology and Environmental Safety* **61**, 432-435.

Langston WJ, Bebiano MJ (1998) Metal metabolism in aquatic environments. (Chapman and Hall: London)

Lapointe D, Couture P (2009). Influence of the route of exposure on the accumulation and subcellular distribution of nickel and thallium in juvenile fathead minnows (*Pimephales promelas*). *Archives of Environmental Contamination and Toxicology* **57**, 571–580. https://doi.org/10.1007/s00244-009-9298-7

Lapointe D, Gentes S, Ponton DE, Hare L, Couture P (2009). Influence of prey type on nickel and thallium assimilation, subcellular distribution and effects in juvenile fathead minnows (*Pimephales promelas*). *Environmental Science and Technology* **43**, 8665–8670. https://doi.org/10.1021/es901929m

Law S, Turner A (2011). Thallium in the hydrosphere of south west England. *Environmental Pollution* **159**, 3484-3489. https://doi.org/10.1016/j.envpol.2011.08.029

Lin TS, Nriagu JO, Wang XQ (2001) Thallium concentration in lake trout from Lake Michigan. *Bulletin of Environmental Contamination and Toxicology* **67**, 921–925. https://doi.org/10.1007/s001280209.

MacKenzie K (1999). Parasites as pollution indicators in marine ecosystems: a proposed early warning sytem. *Marine Pollution Bulletin* **38**, 955-959. https://doi.org/10.1016/S0025-326X(99)00100-9

Marcogliese DJ (2004). Parasites: Small players with crucial roles in the ecological theater. *EcoHealth* **1,** 151-164. https://doi.org/10.1007/s10393-004-0028-3

Mijošek T, Filipović Marijić V, Dragun Z, Ivanković D, Krasnići N, Erk M, Gottstein S, Lajtner J, Sertić Perić M, Matoničkin Kepčija R (2019a). Comparison of electrochemically determined metallothionein concentrations in wild freshwater salmon fish and gammarids and their relation to total and cytosolic metal levels. *Ecological Indicators* **105**, 188-198. https://doi.org/10.1016/j.ecolind.2019.05.069

Mijošek T, Filipović Marijić V, Dragun Z, Krasnići N, Ivanković D, Erk M (2019b). Evaluation of multi-biomarker response in fish intestine as an initial indication of anthropogenic impact in the aquatic karst environment. *Science of the Total Environment* **660**, 1079-1090. https://doi.org/10.1016/j.scitotenv.2019.01.045

Moddock DM, Burton MPM (1999). Gross and histological observations of ovarian development and related condition changes in American plaice. *Journal of Fish Biology* **53**, 928–944. https://doi.org/10.1111/j.1095-8649.1998.tb00454.x

Mrakovčić M, Brigić A, Buj I, Ćaleta M, Mustafić P, Zanella D (2006). Red book of freshwater fish of Croatia.Ministry of Culture, State Institute for Nature Protection. Republic of, Croatia (253 pp).

Nachev M, Sures B (2016). Seasonal profile of metal accumulation in the acanthocephalan *Pomphorhynchus laevis*: a valuable tool to study infection dynamics and implications for metal monitoring. *Parasites & Vectors* **9**, 300-308. https://doi.org/10.1186/s13071-016-1576-4

Nielsen SG, Rehkämper M, Porcell D, Andersson P, Halliday AN, Swarzenski PW, Latkoczy C, Günter D (2005). Thallium isotope composition of the upper continental crust and rivers – An investigation of the continental sources of dissolved marine thallium. *Geochimica Cosmochimica Acta* **19**, 2007-2019. https://doi.org/10.1016/j.gca.2004.10.025

Ospina-Alvarez N, Burakiewicz P, Sadowska M, Krasnodebska-Ostrega B (2015) Tl^{I} and Tl^{III} presence in suspended particulate matter: speciation analysis of thallium in wastewater. *Environmental Chemistry* **12**, 374-379. https://doi.org/10.1071/EN14181

Paggi L, Orecchia P, Del Marro M, Iori A, Manilla G (1978). Parasites of *Salmo trutta* from the Tirino River II. Host-Parasite interactions of helminth species. *Parassitologia* **20**, 161–168.

Peter ALV, Viraraghavan T (2005). Thallium: a review of public health and environmental concerns. *Environment International* **31**, 493-501. https://doi.org/10.1016/j.envint.2004.09.003

Pickard J, Yang R, Duncan B, McDevitt CA, Eickhoff C (2001) Acute and sublethal toxicity of thallium to aquatic organisms. Bulletin *of Environmental Contamination and Toxicology* **66**, 94–101. https://doi.org/10.1007/s0012800210.

Radić S, Gregorović G, Stipaničev D, Cvjetko P, Šrut M, Vujčić V, Oreščanin V, Klobučar GIV (2013). Assessment of surface water in the vicinity of fertilizer factory using fish and plants. *Ecotoxicology and Environmental Safety* **96**, 32-40. https://doi.org/10.1016/j.ecoenv.2013.06.023

Ralph L, Twiss MR (2002). Comparative toxicity of thallium(I), thallium(III), and cadmium(II) to the unicellular alga *Chlorella* isolated from Lake Erie. *Bulletin of Environmental Contamination and Toxicology* **68**, 261-268. https://doi.org/10.1007/s001280247

Ricker WE (1975). Computation and interpretation of biological statistics of fish populations. Bulletin of the Fisheries Research Board of Canada 191, 1–382.

Rosabal M, Pierron F, Couture P, Baudrimont M, Hare L, Campbell PG (2015). Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, Tl) in livers of American (*Anguilla rostrata*) and European (*Anguilla anguilla*) yellow eels. *Aquatic Toxicology* **160**, 128–141. https://doi.org/10.1016/j.aquatox.2015.01.011

Sasi H (2008). The length and weight relations of some reproduction characteristics of Prussian carp, *Carassius gibelio* (Bloch, 1782) in the South Aegean Region (Aydın-Turkey). *Turkish Journal of Fisheries and Aquatic Sciences* **8**, 87-92.

Sertić Perić M, Matoničkin Kepčija R, Miliša M, Gottstein S, Lajtner J, Dragun Z, Filipović Marijić V, Krasnići N, Ivanković D, Erk M (2018). Benthos-drift relationships as proxies for the detection of the most suitable bioindicator taxa in flowing waters – a pilot-study within a Mediterranean karst river. *Ecotoxicology and Environmental Safety* **163**, 125–135. https://doi.org/10.1016/j.ecoenv.2018.07.068

Siddall R, Sures B (1998). Uptake of lead by *Pomphorhynchus laevis* cystacanths in *Gammarus pulex* and immature worms in chub (*Leuciscus cephalus*). *Parasitology Research* **84**, 573-577. https://doi.org/10.1007/s004360050451

Staniskiene B, Matusevicius P, Budreckiene R, Skibniewska KA (2006). Distribution of heavy metals in tissues of freshwater fish in Lithuania. *Polish Journal of Environmental Studies* **15**, 585−591.

Sures B, Siddall R, Taraschewski H (1999). Parasites as accumulation indicators of heavy metal pollution. *Parasitology Today* **15**, 16−21. https://doi.org/10.1016/S0169-4758(98)01358-1

Sures B (2001). The use of fish parasites as bioindicators of heavy metals in aquatic ecosystems: a review. *Aquatic Ecology* **35**, 245-255. https://doi.org/10.1023/A:1011422310314

Sures B (2002). Competition for minerals between *Acanthocephalus lucii* and its definitive host perch (*Perca fluviatilis*). *International Journal for Parasitology* **32**, 1117−1122. https://doi.org/10.1016/S0020-7519(02)00083-8

Tatsi K, Turner A (2014). Distributions and concentrations of thallium in surface waters of region impacted by historical mining (Cornwall, UK). *Science of the Total Environment* **473**, 139-146. https://doi.org/10.1016/j.scitotenv.2013.12.003

Thielen F, Zimmermann S, Baska F, Taraschewski H, Sures B (2004). The intestinal parasite *Pomphorhynchus laevis* (Acanthocephala) from barbel as a bioindicator for metal pollution in the Danube River near Budapest, Hungary. *Environmental Pollution* **129**, 421-429. https://doi.org/10.1016/j.envpol.2003.11.011

Urien N, Cooper S, Caron A, Sonnenberg H, Rozon-Ramilo L, Campbell PCG (2018). Subcellular partitioning of metals and metalloids (As, Cd, Cu, Se and Zn) in liver and gonads of wild white suckers (*Catostomus commersonii*) collected downstream from a mining operation. *Aquatic Toxicology* **202**, 105–116. https://doi.org/10.1016/j.aquatox.2018.07.001

Vardić Smrzlić I, Valić D, Kapetanović D, Dragun Z, Gjurčević E, Ćetković H, Teskeredžić E (2013). Molecular characterisation and infection dynamics of *Dentitruncus truttae* from trout (*Salmo trutta* and *Oncorhynchus mykiss*) in Krka River, Croatia. *Veterinary Parasitology* **197**, 604-613. https://doi.org/10.1016/j.vetpar.2013.07.014

Wallace WG, Lee B-G, Luoma SN (2003). Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Marine Ecology Progress Series* **249**, 183–197. https://doi.org/10.3354/meps249183

Wootton RJ (1990). Ecology of teleost fishes. Chapman and Hall, Fish and Fisheries Series 1. London, New York.

Xiao T, Guha J, Boyle D, Liu CQ, Zheng B, Wilson GC, Rouleau A, Chen J (2004). Naturally occurring thallium: a hidden geoenvironmental health hazard? *Environment International* **30**, 501–507. https://doi.org/10.1016/j.envint.2003.10.004

Yeltekin AC, Sağlamer E (2019). Toxic and trace element levels in *Salmo trutta macrostigma* and *Oncorhynchus mykiss* trout raised in different environments. *Polish Journal of Environmental Studies* **28**, 1613−1621. https://doi.org/10.15244/pjoes/90620

Zhelev ZhM, Tsonev SV, Boyadziev PS (2018). Significant changes in morpho-physiological and haematological parameters of *Carassius gibelio* (Bloch, 1782) (Actinopterygii: Cyprinidae) as response to sporadic effusions of industrial wastewater into the Sazliyka River, Southern Bulgaria. *Acta Zoologia Bulgarica* **70**, 547−556.

Zitko V (1975). Toxicity and pollution potential of thallium. *Science of the Total Environment* **4**, 185-192. https://doi.org/10.1016/0048-9697(75)90039-X

Figure captions:

Fig 1. Thallium concentrations in water samples from the Krka and Ilova River from two sampling sites (reference and contaminated site) and two sampling campaigns (autumn and spring). Statistically significant differences (Mann-Whitney U test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and solid line, and between two sampling sites within the same season with different superscript letters (A and B).

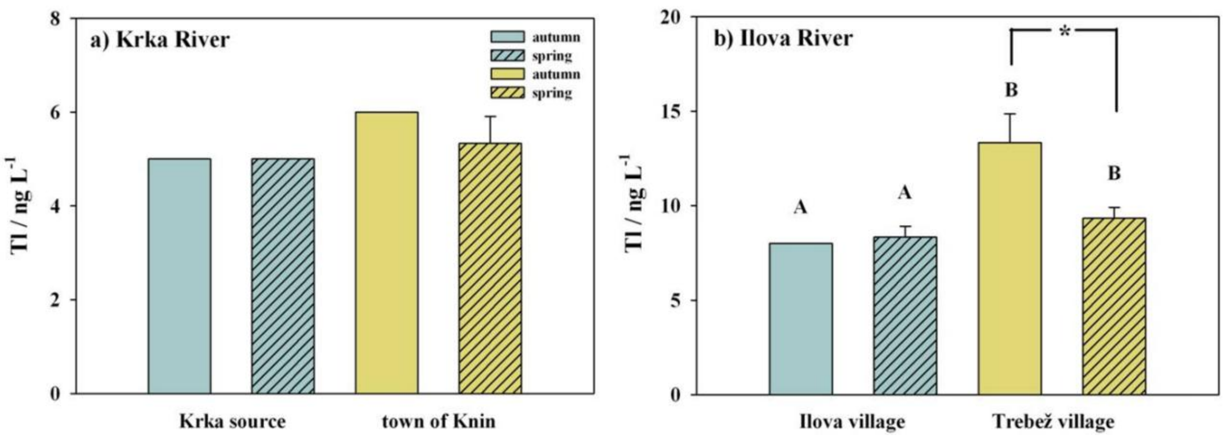

Fig 2. Thallium concentrations (mean±S.D., μg kg^{-1} w.w.) in different fractions of the intestinal tissue of *S. trutta* from the Krka River at two sampling sites (reference and contaminated site) in two sampling campaigns (autumn and spring). Statistically significant differences (Mann-Whitney U test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and solid line, and between two sampling sites within the same season with different superscript letters (A and B). Site legend: green – Krka River source, autumn season; dashed-green – Krka River source, spring season; yellow - town of Knin, autumn season; dashed-yellow - town of Knin, spring season.

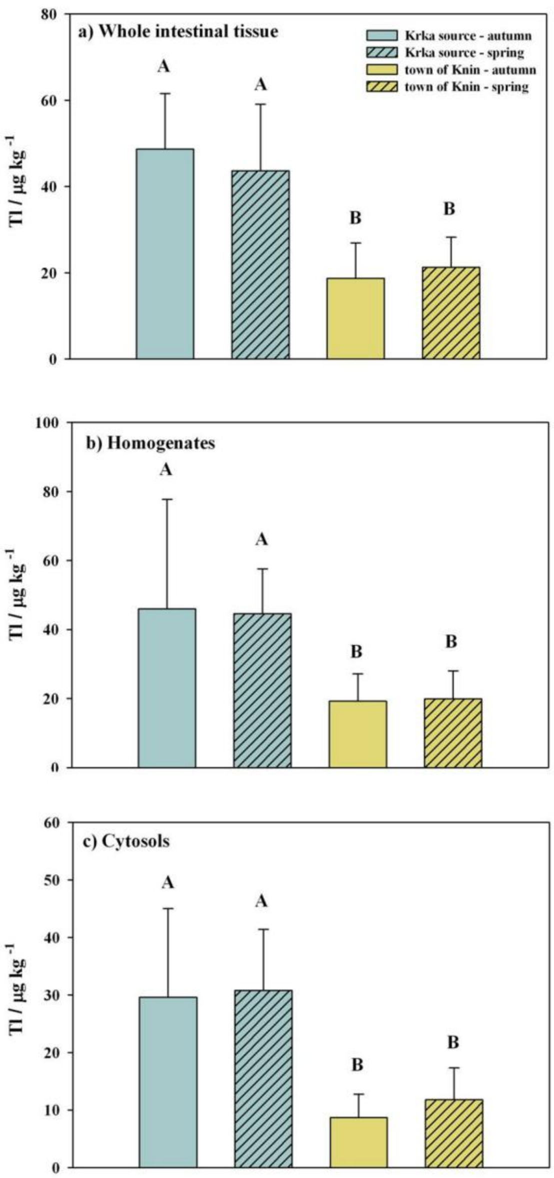

Fig 3. Thallium concentrations (mean±S.D., μg kg^{-1} w.w.) in different fractions of the intestinal tissue of *C. gibelio* from the Ilova River at two sampling sites (reference and contaminated site) in two sampling campaigns (autumn and spring). Statistically significant differences (Mann-Whitney U test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and solid line, and between two sampling sites within the same season with different superscript letters (A and B). Site legend: green – Ilova village, autumn season; dashed-green – Ilova village, spring season; yellow - Trebež village, autumn season; dashed-yellow - Trebež village, spring season.

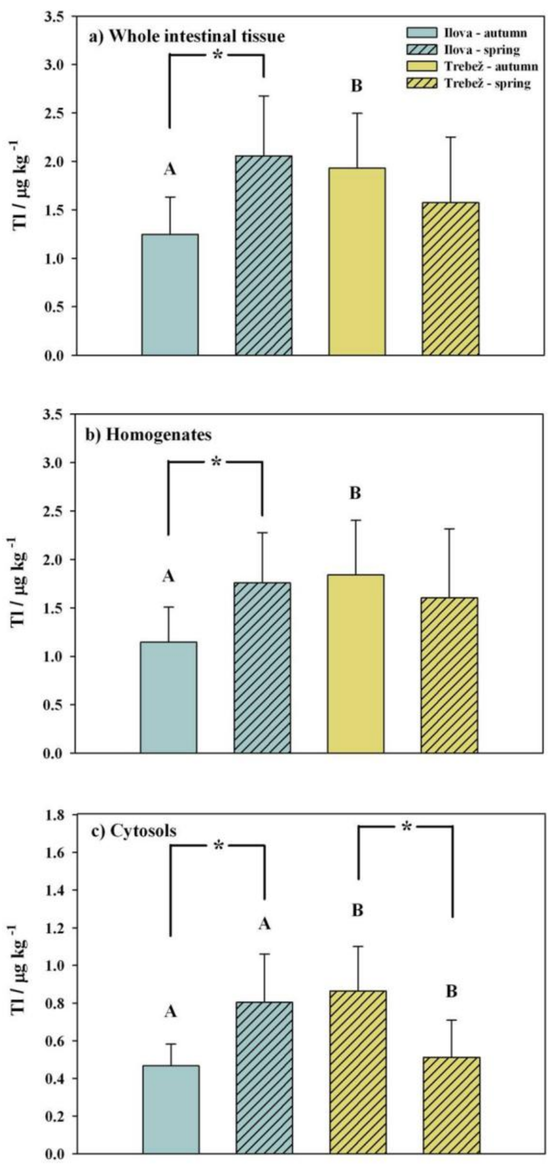

Fig 4. Thallium concentrations (mean±S.D., μg kg^{-1} w.w.) in different bioindicator organisms from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka downstream of town of Knin) in two sampling campaigns (autumn and spring). Statistically significant differences (Mann-Whitney U test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and solid line, and between two sampling sites within the same season with different superscript letters (A and B).

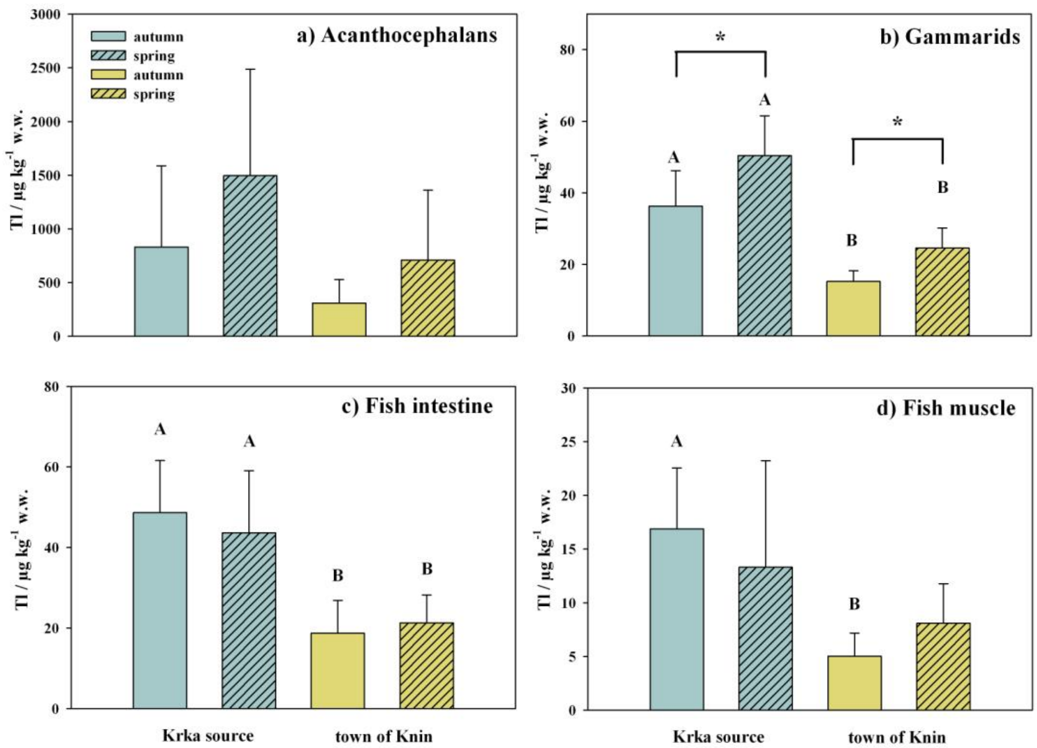

Table 1. Biometric parameters (mean ± S.D.) of fish caught in the Krka River and the Ilova River at the reference (Krka River source and Ilova village) and contaminated site (Krka downstream of Knin and Trebež village) in two sampling campaigns (autumn and spring). Statistically significant differences (Mann-Whitney U test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and between two sampling sites within the same season are assigned with different superscript letters (A and B).

Species	Location	Season	Total length (cm)	Body mass (g)	FCI (g cm^{-3}*100)	GSI (%)	HSI (%)
Salmo trutta	**Krka River source**	**Autumn 2015** n = 16	24.15±4.29*	152.71±78.64*	1.00±0.08^{A}	3.72±2.49*	0.92±0.25*
		Spring 2016 n = 16	18.36±1.94*	66.09±19.64*	1.04±0.06^{A}	0.40±0.33*$^{, A}$	1.27±0.30*
	Krka downstream of Knin	**Autumn 2015** n = 20	23.16±5.49*	165.45±108.96*	1.12±0.10*$^{, B}$	2.30±2.61*	0.97±0.12*
		Spring 2016 n = 16	19.64±3.19*	96.01±45.49*	1.19±0.09*$^{, B}$	0.15±0.06*$^{, B}$	1.50±0.47*
Carassius gibelio	**Ilova village**	**Autumn 2017** n =20	16.19±1.62^{A}	69.82±23.17*$^{, A}$	1.59±0.09*$^{, A}$	3.11±1.44	5.87±1.78*
		Spring 2018 n =23	15.90±2.16	54.57±21.43*$^{, A}$	1.31±0.10*$^{, A}$	5.25±3.60^{A}	1.44±0.53*
	Trebež village	**Autumn 2017** n =20	18.83±2.91^{B}	122.34±58.13^{B}	1.70±0.12^{B}	4.67±2.68*	5.44±1.52*
		Spring 2018 n =20	17.53±3.86	103.03±83.00^{B}	1.67±0.15^{B}	7.63±4.67*$^{, B}$	2.36±0.77*

FCI – Fulton condition index; GSI –gonadosomatic index; HSI –hepatosomatic index

Table 2. The proportions of Tl amount present in the cytosolic fractions of intestinal tissue of *S. trutta* and *C. gibelio* at two sampling sites in two sampling campaigns (expressed as percentage %). The ratio on total metal levels obtained by two different procedures is given as well (Homogenate/whole tissue). All fractions (cytosol, homogenate, whole tissue) were digested using HNO_3 and H_2O_2.

Species	Location and season	Cytosol/ homogenate (%)	Cytosol/ whole tissue (%)	Homogenate/ whole tissue (%)
Salmo trutta	**Krka source - autumn**	**64**	**61**	**94**
	Krka source - spring	**69**	**71**	**102**
	town of Knin - autumn	**45**	**46**	**103**
	town of Knin - spring	**59**	**55**	**94**
Carassius gibelio	**Ilova - autumn**	**41**	**38**	**92**
	Ilova - spring	**45**	**39**	**85**
	Trebež - autumn	**47**	**45**	**95**
	Trebež - spring	**32**	**33**	**103**

Table 3. Characteristics of acanthocephalans hosted in *S. trutta*: prevalence (number and percentage of infected fish), mean intensity of infection (mean ± S.D.) and total number of parasite individuals.

	Krka River source		**town of Knin**	
Season and sample number	**Autumn 2015 n=6**	**Spring 2016 n=6**	**Autumn 2015 n=6**	**Spring 2016 n=6**
Prevalence (number and % of trouts infected with parasites)	6, 100%	5, 83%	6, 100%	6, 100%
Mean intensity of infection (mean ± S.D.)	35.3±19.7	25.7±5.4	35.2±43.3	6.2±7.6
Total number of parasite individuals in sampled fish	212	154	211	37

Table 4. Bioconcentration factors ($\mathbf{C_{[parasite]}/C_{[host\ intestine]}}$) for *D. truttae* in gammarids and fish from the Krka River.

BCF	Bioindicator organisms or organs	Krka River source		town of Knin	
		Autumn 2015	**Spring 2016**	**Autumn 2015**	**Spring 2016**
Tl	**Acanthocephala/ gammarids**	22.88	29.68	20.34	28.85
	Acanthocephala/ intestine	17.09	34.31	16.53	33.31
	Acanthocephala/ muscle	49.15	112.49	61.82	87.60