Metal bioaccumulation in stygophilous amphipod *Synurella ambulans* in the hyporheic zone: The influence of environmental factors

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Abstract

Given the increasing need to protect vulnerable freshwater ecosystems and make them more resilient to human use and climate change, biomonitoring of the hyporheic zone (HZ), which plays a critical role in pollution attenuation, is essential. The aim of the present study was to assess the potential of the amphipod species *Synurella ambulans* as a bioindicator of metal contamination in the HZ of the Sava River (Croatia). Amphipods were collected during the four seasons at two sampling sites (average sampling depth 55 cm) differing in type (agricultural and urban) and intensity (diffuse and point source contamination) of anthropogenic influence, one located upstream (Medsave), and the other downstream (Jarun) of the wastewater treatment plant discharge. Concentrations of Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Rb, Sn, Zn, Ca, K, Mg and Na were measured in the interstitial water, sediments and specimens of *S. ambulans* by HR ICP-MS. Physicochemical parameters (temperature, DO, O_2 saturation, pH, conductivity, alkalinity, total water hardness, COD_{KMnO4}, nutrients) were measured in the interstitial water, while organic carbon was measured in the interstitial water and sediments. Metal concentrations in interstitial water and sediments were below thresholds set by environmental quality standards. Metal concentrations in *S. ambulans* were classified as follows: higher at the Jarun site (Al, Cr, Fe, Ni, Pb, Sn), higher at the Medsave site (Cd, Cu, Rb) and mostly comparable at both sites (Co, Mn, Zn). Bioaccumulation factors were generally higher at Jarun, with average values ranging from 322 to 143,278 L kg^{-1}. Bioaccumulation of metals in *S. ambulans* depended on various environmental factors, with metal exposure level and dissolved macro elements showing the strongest association with metals accumulated in *S. ambulans*. The findings provided the first evidence on the suitability of *S. ambulans* as a good bioindicator of chronic metal contamination in the HZ.

Key words: The Sava River, Croatia, crustaceans, bioindicator, groundwater connected ecosystems, anthropogenic impact

1. Introduction

The hyporheic zone (HZ) is a unique, dynamic ecotonal interface between terrestrial and aquatic ecosystems, groundwater and surface water, and riparian vegetation (Orghidan, 1955; Gibert et al., 1990; Käser, 2010; Lewandowski et al., 2019). As a key component of fluvial ecosystems, the HZ plays a crucial role in ecohydrology, biogeochemical cycling, and nutrient fluxes (e.g., nutrient turnover, retention and (bio)transformation of trace organic compounds, remineralisation of organic matter through microbial activity, retention of fine particulate matter and pollutant attenuation), and as a refuge for aquatic organisms (Käser, 2010; Boano et al., 2010, 2014; Lewandowski et al., 2019; Tewari et al., 2022). The HZ is an important habitat for a diverse community – the hyporheos (Williams and Hynes, 1974; Brunke and Gonser, 1997; Boulton, 2000) thanks to the large surface area of the sedimentary matrix (Lewandowski et al., 2019). Important components of these hyporheic communities are macroinvertebrates and meiofauna due to their bioturbation and bioirrigation activities, resulting in improved sediment permeability, increased respiration of freshwater sediments and effect on water biogeochemistry (Peralta-Maraver et al., 2018; Tewari et al., 2022). The HZ is a part of the groundwater-connected ecosystems (GWCEs), which are characterized as ecosystems that require access to groundwater as a key element for the functioning of animal and plant communities (Richardson et al., 2011). Research and protection of GWCEs is a globally crucial issue because changes in groundwater supply and their interactions with surface waters can lead to changes in ecosystem structure, and species composition and diversity (Eamus et al., 2006).

Increasing anthropogenic activities are altering hydro-biogeochemical processes and nutrient fluxes in the HZ, threatening hyporheic communities and food webs. The HZ is adversely affected by wastewater treatment plants (WWTPs), whose discharges contain many pollutants, such as metals, pesticides, and nutrients (nitrates, phosphates, organic carbon) (Carey and Migliaccio, 2009; Tewari et al., 2022). Among the various contaminant categories, such as chlorinated and aromatic hydrocarbons, mineral oils, pesticides, and fertilizers, metals pose the greatest threat to groundwater ecosystems (Panagos et al., 2013). Although metals and metalloids are found naturally in the Earth's crust, their presence in the environment has increased significantly due to anthropogenic activities, such as mining, smelting, foundries, traffic (fossil fuel combustion), domestic effluents and agricultural uses (pesticides, fertilisers

etc.) (Briffa et al., 2020). Metals are persistent in the environment for a very long time because they are not biodegradable, (Masindi and Muedi, 2018) and therefore pose a serious threat to aquatic organisms. However, in the HZ metals of natural or anthropogenic origin can be immobilized by and/or adsorbed on the sediment particles or sequestered by microbial communities that mediate the transport and fate of metals (Herzog et al., 2016; Lewandowski et al., 2019; Tewari et al., 2022). Metals accumulated by microbiota at the bottom of the food chain may be transferred further up, which is evident by biofilm grazing activities of protists, meio- and macroinvertebrates inhabiting the HZ (Marsden and Rainbow, 2004; Lewandowski et al., 2019).

As the HZ is a vulnerable GWCE, it is crucial to identify living organisms as bioindicators that reflect groundwater quality conditions and the current state of the ecosystem. Unlike chemical analysis of water, bioindicators provide information on the long-term presence and bioavailability of metals in the environment (Morillo et al., 2005). The use of hyporheic species as bioindicators has already been recognized as very useful for monitoring the effects of pollution sources because of their direct exposure to changes in nutrient concentrations and food webs (Pacioglu, 2010). However, very little is known about the metal bioaccumulation in amphipods inhabiting GWCEs and the use of hyporheic amphipods as bioindicators of metal contamination. To date, only a few authors have investigated metal/metalloid accumulation in subterranean amphipods from the interstitial zone (Plénet, 1995, 1999; Canivet et al., 2001; Coppellotti Krupa and Guidolin, 2003). Two subterranean amphipods (*Niphargus* cf. *rhenorhodanensis* and *Niphargopsis casparyi*) accumulated far more Zn and Cu than the epigean *Gammarus fossarum* at sites exposed to different levels of pollution (Plénet, 1995). The previous studies on groundwater amphipods are useful for comparison because of the strong spatial connectivity between the underlying phreatic (groundwater zone) and the HZ. More data are available on metal toxicity and accumulation in epigean amphipods (Martin and Holdich, 1986; Ugolini et al., 2004). It has been previously shown that amphipods (Talitroidae) can be used as biomonitors for the bioavailability of trace metals (Cu, Zn, Cd, Fe, Mn, Pb, Ni) (Rainbow et al., 1993; Fialkowski et al., 2003).

Assessing variations in metal concentrations in amphipods and consequently differences in metal bioaccumulation is important for their implementation in biomonitoring programmes. Various environmental factors (e.g., changes in oxygen concentration and salinity, interactions between metals,

physicochemical properties of sediment, pH) and species traits (e.g. gender, body size, brooding, behaviour, moulting and growth) can affect metal uptake (Marsden and Rainbow, 2004). Seasonal variation in a number of physicochemical parameters, such as temperature, pH, dissolved oxygen saturation, conductivity, dissolved organic carbon (DOC), nutrient concentration, and dissolved macro element concentrations, also affect accumulated trace metal concentrations (Fialkowski et al., 2003). In a study by Amyot et al. (1994), seasonal changes in metal concentrations (Fe, Ni, Pb) were found in the amphipod *Gammarus fasciatus*. Moreover, the influence of seasons on the bioaccumulation of essential metals (Co, Mn and Zn) was shown in *Gammarus pulex* and *G. fossarum*, where water temperature was the main environmental parameter controlling metal content in the organisms (Lebrun et al., 2015).

Therefore, the main motivation for our study was to investigate the influence of anthropogenic contamination of the Sava River with special focus on its HZ and metal/metalloid accumulation in the stygophilous crustacean *Synurella ambulans* (Amphipoda, Crangonyctidae) as a potential bioindicator species. Despite being a major drainage basin of Southeast Europe and an important corridor connecting five countries, the Sava River lacks comprehensive data that would reveal coupled physical, chemical, and biological processes in its HZ. The middle and lower courses of the Sava River are polluted by wastewater treatment discharge from settlements and agricultural run-offs (Kapetanović et al., 2015). In particular, domestic and industrial wastewater outlets of the cities of Zagreb and Zaprešić are the main sources of pollution in the study area. Concentrations of metals (As, Cd, Cr, Co, Cu, Pb, Ni and Zn), nutrients, and total organic carbon (TOC) in the sediment of the study area upstream and downstream of the discharge of insufficiently treated effluents from pharmaceutical industry were recently quantified by Milaković et al. (2019). Their results showed that metal concentrations were slightly higher at the discharge and downstream sites than at the upstream site. Additionally, in that same study area, concentrations of potentially toxic elements (Cd, Cr, Cu, Ni, Pb, and Zn) were determined in the sediment (bulk and fine-grained fraction) of the Sava River (Vdović et al., 2021), and for all of these elements, except for Ni, the values were below the Predicted No Effects Concentration (PNEC) (Bakke et al., 2010).

However, no data on metal bioaccumulation in amphipod crustaceans in the HZ of the Sava River exist so far. Therefore, the following trace metals were included in our current study: Al, Cd, Co, Cr, Cu, Fe,

Mn, Ni, Pb, Rb, Sn and Zn. They involve both essential elements (Mn, Zn, Cu etc.) and elements with no known biological function (non-essential elements) such as Cd, Pb and Sn (Rainbow, 1995). These metals were selected for the research because they are commonly found in pharmaceutical wastewater (Ramola and Singh, 2013), municipal water, fertilizers, and pesticides used in agricultural industry (Hariprasad and Dayananda, 2013). Also, agricultural land was shown to be polluted with heavy metals Mn, Cd, Pb and Co coming from anthropogenic sources (Sungur et al., 2014; Sungur et al., 2019). Higher mobility levels of those metals may indicate environmental pollution. All of these trace metals are potentially toxic to aquatic organisms above a certain threshold (Rainbow et al., 1990). When in excess, trace metals may interfere with normal metabolic functioning (Rainbow, 2007) and eventually have lethal effect. Metals can disrupt the gill function of crustaceans and lead to hypoxia, inhibit enzymes, alter mitochondrial function and delay mitosis or damage neurosecretory cells in the brain (Spicer and Weber, 1991; Fingerman et al., 1996). Organisms need to spend energy on excretion and/or detoxification of metals, which can negatively affect their growth and reproduction (Marsden and Rainbow, 2004). Besides trace elements, macro elements (Ca, K, Mg, Na) were also measured. These are essential elements, involved in physiological processes and crucial for the proper development of organisms. They are present in crustaceans in mg g^{-1} of tissue wet weight (w.w.). Calcium (i.e., $CaCO_3$) is one of the major components of exoskeleton in crustaceans.

For these reasons, the aim of this study was to determine whether *S. ambulans* could be used as a bioindicator of the contamination level of the HZ with trace metals of concern and to examine the importance of seasonal variations of metal concentrations in amphipods. In the aqueous compartment, the following factors that could affect metal bioaccumulation were selected: metal concentrations in interstitial water, (i.e., exposure level), dissolved macro element concentrations (Na, Mg, K, Ca), conductivity, dissolved oxygen saturation, concentrations of hydrogen ions (pH), and dissolved organic carbon (DOC). In the sediments, the factors considered were concentrations of metals bound in the sediment and total organic carbon (TOC). Since the interstitial habitats and their fauna are understudied this paper tends to improve research gaps and highlights the need to protect the groundwater connected ecosystems. Also, it contributes to the understanding of the complex interactions between organisms

161 living in the HZ, anthropogenic pollution, hyporheic biogeochemistry, and physicochemical conditions
162 in the HZ.
163
164 **2. Materials and methods**
165 2.1 Study area
166 This study was carried out in a section of the Sava River in the north-west of the Republic of Croatia
167 (Fig. 1). With a total catchment area of 97,713 km^2, it is the largest tributary of the Danube River. The
168 Sava River catchment belongs to three different regions: the Alpine region (the Alps and the Dinarides),
169 mountainous region, and the lowland region of the peri-Pannonian basin (Simić et al., 2015).
170 Considering the land use, a great part of the Sava River basin is covered by agricultural surfaces (42.4
171 %) (ISRBC, 2009). The study area is located in the lower stretch of the Upper Sava River Plain which
172 belongs to the water body type HR-R_5b as very large lowland river in the Pannonian ecoregion
173 (Directive on Water Quality Standard of the Government of the Republic of Croatia, GRC, 2019; EEA
174 2009; Tomas et al., 2017). The environmental quality standards for surface water of specified water
175 body types and groundwater are set out in the GRC (2019).
176 Two sampling sites on gravel bars of the Sava River were chosen at different distances from pollution
177 sources. The first sampling site near the village of Medsave (45°50′04″N, 15°46'32″E), in the rural zone
178 with agricultural land that is located approximately 3 km upstream from the wastewater outlet from the
179 WWTP of the city of Zaprešić. The second sampling site near the city district of Jarun (45°46′24″N,
180 15°55′56″E), in an urban area of the city of Zagreb, is located about 13 km downstream from this
181 wastewater outlet. WWTP in the city of Zaprešić receives treated wastewaters from the pharmaceutical
182 industry, baker's yeast production and the wastewaters from local municipality, which are then treated
183 and discharged into the Sava River (Ferina, 2015). A previous study at the effluent discharge site
184 revealed elevated concentrations of heavy metals in sediments of the Sava River and their accumulation,
185 especially Cu and Zn, compared to the lower concentrations at the upstream reference site, suggesting
186 metal pollution from incoming industrial waste (Milaković et al., 2019).
187
188 2.2 Field sampling

189 2.2.1 Amphipod sampling

190 Amphipods were sampled by Bou-Rouch pumping method (Bou and Rouch, 1967) in the HZ of the
191 Sava River gravel bars once per season in winter (December 9 – 10, 2018), spring (April 2 – 3, 2019),
192 summer (July 23 – 24, 2019) and autumn (October 22 – 23, 2019) at both sampling sites. At least three
193 samples were taken at each sampling site. A mobile steel pipe (Ø 50 mm, 110 cm long) with a perforated
194 distal end (thirty-five 5 mm diameter holes) was embedded into the gravel bar. For each sample, 50 L
195 of interstitial water were pumped from an average depth of 55 cm below the surface of the gravel bar
196 and this procedure was repeated in at least three points (along the gravel bar) per sampling site. At each
197 sampling point, a mixture of water, sediment and invertebrates was extracted by pumping. The sampling
198 was done using a set of fine-meshed hand nets (100, 200 and 500 μm). Only amphipod specimens
199 retained on the two largest nets (200 and 500 μm) were stored in polyvinyl chloride bottles and
200 transported in portable refrigerator to the laboratory for processing. The specimens were identified
201 under stereomicroscope (Zeiss Stemi 2000 C) based on the morphological characters for *S. ambulans*
202 as described in Karaman (1993), sorted, counted, snap-frozen in liquid nitrogen and then stored at -80
203 °C until further analyses.
204
205 2.2.2 Interstitial water sampling
206 Samples of the interstitial water were collected at the same time when we sampled the amphipods using
207 the same Bou-Rouch pump. Physicochemical water parameters, concentration and saturation of
208 dissolved oxygen (DO), water temperature (T), conductivity (Cond), and pH were measured in each
209 sample at both sampling sites immediately after collection using a portable multiparameter water quality
210 monitoring instruments calibrated before each survey (oximeter OXI 96, WTW GmbH, Weilheim,
211 Germany, for T and DO; pH-meter 330i, WTW GmbH, Weilheim, Germany, for pH and conductometer
212 Sension 5; Hach, Loveland, CO, USA, for Cond). Alkalinity was also determined *in situ* by acid titration
213 (0.1 mol L^{-1} HCl) with methyl orange as an indicator of the endpoint colour change.
214 For the analyses of dissolved trace- and macro element concentrations in interstitial water from the HZ,
215 samples were collected in triplicate at each sampling site by pumping the interstitial water into
216 polyethylene plastic bottles (volume 250 mL). Bottles were pre-cleaned with metal-free acid (v/v 10%

HNO3, p.a., Merck, Germany). Immediately after sampling, the water sample was filtered at the collection site through a cellulose acetate filter (0.45 μm pore diameter) (Sartorius, Germany) mounted on syringe, to obtain only the dissolved fraction of trace- and macro elements. Aliquots of 20 mL of filtered samples for metal/metalloid analyses were transferred into acid pre-cleaned polyethylene bottles, acidified with 400 μL of concentrated nitric acid (HNO_3) (Rotipuran® Supra 69%, Carl Roth, Germany) and stored at 4 °C until metal measurements. For the subsequent analysis of physicochemical parameters in the laboratory, an additional water sample of 1 L was collected and stored at 4 °C. Samples for the determination of dissolved organic carbon (DOC) and particulate organic carbon (POC) were collected in dark, glass bottles (1 L) pre-cleaned with chromosulfuric acid (2-5% $Na_2Cr_2O_7$ in ≈90% H_2SO_4, Kemika, Croatia) and rinsed well with organic-free Milli-Q water (Millipore) and ambient water.

2.2.3 Sediment sampling

The sampling of the sediments from the HZ was performed as follows. As the steel pipe has 5-mm diameter holes the large gravel particles cannot pass into the pump. At least three subsamples were taken at each sampling site and mixed to obtain the composite sample representative of the sampling site. The polypropylene container (15 L) with the sediment suspension was transported to the laboratory and left at 10 °C for at least 48 hours to precipitate the sediment by gravity. The overlying water was carefully decanted and the sediment sample was frozen at -80 °C and then dried by lyophilisation (FreeZone® 2.5 L, Labconco Corporation, USA). The fine fraction of the sediment (containing < 63 μm particles) was obtained by sieving the sediment sample through a stainless steel sieve with a mesh size of 63 μm (Retsch, Germany) and grounding it to a fine powder using a ball-mill (Pulverisette 7; Fritsch, Germany). The obtained sediment samples were stored in plastic bags and subsequently used for the determination of metals and TOC.

2.3 Laboratory analyses

2.3.1 Determination of physicochemical parameters in water

Concentrations of nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), orthophosphate ($P\text{-}PO_4^{3-}$) and total water hardness (TWH) were determined in the laboratory according to the standard methods of APHA (1985), whereas total chemical oxygen demand (COD_{KMnO4}) was measured by standardized acidic potassium permanganate titrimetric method (Deutsches Institut für Normung, 1986). Total water hardness was determined complexometrically using a standard ethylenediaminetetraacetic acid (EDTA) solution (Kompleksal® III, Kemika, Croatia; 1 mL=10 mg CaO/ L by titrating 100 mL of water) and the respective indicator buffer tablets (Kompleksin®, Kemika, Croatia) (APHA, 1985). Nitrite, nitrate and orthophosphate were all determined spectrophotometrically, applying the diazotization method (using sulfanilic acid and 1-naphthylamine) to determine nitrites, the sodium salicylate method for nitrates, and the molybdenum blue phosphorus method for orthophosphates (APHA, 1985). Nitrite ion concentration was determined by measuring the absorbance of the azo complex at λ=520 nm; nitrate ion concentration by measuring the absorbance of the yellow nitrosalicylic acid complex at λ=420 nm; and orthophosphate ion concentration by measuring the absorbance of the molybdenum blue complex at λ=690 nm. The absorbance of each sample was measured using the respective reagent blanks as reference solutions for each ion (nitrite, nitrate, orthophosphate). Calibration curves were constructed for each ion from a series of eight standard solutions within the nitrite, nitrate and orthophosphate ranges (0.001 – 0.1 $N\text{-}NO_2^-$/mg L^{-1}; 0.05 – 1 $N\text{-}NO_3^-$/mg L^{-1}, 0.01 – 0.15 $P\text{-}PO_4^{3-}$/mg L^{-1}). The concentration of each ion was determined using the absorbance readings of each sample in conjunction with the calibration curves.

2.3.2 Determination of organic carbon concentration in water and sediment samples

DOC and POC were measured in the interstitial water and total organic carbon (TOC) was measured in the sediment samples. Organic carbon was measured since it is known to complex trace metal ions and influences metal levels in amphipods (Amyot et al., 1994). Water samples were filtered through precombusted glass fiber filters (Whatman, GF/F, pore size 0.7 μm). Glass filtration system (Millipore, USA) connected to a vacuum pump (GAST Manufacturing Inc., USA) was used. All glassware used for the sample filtration was washed with chromosulfuric acid (2-5% $Na_2Cr_2O_7$ in ≈90% H_2SO_4, Kemika, Croatia) and rinsed well with organic-free Milli-Q water (Millipore) and water samples.

Filtered samples were collected in glass vials, preserved with mercury chloride ($HgCl_2$; 10 mg L^{-1}) and stored at 4 °C in the dark until analysis. After collection of the DOC subsamples GF/F filters were rinsed with Milli-Q water to remove salts from the filter, transferred in liquid nitrogen (-80 °C) and stored at -50 °C until POC analysis. Dissolved organic carbon concentrations were analysed in triplicates using the sensitive High-Temperature Catalytic Oxidation (HTCO) method at 680 °C (Dafner and Wangersky, 2002; Dautović et al., 2017). For DOC measurements, Shimadzu total organic carbon analyser TOC-V_{CPH} with platinum/silica catalyst (Elemental microanalysis, UK) and Non-Dispersive Infrared (NDIR) detector for CO_2 measurements were used. DOC concentrations were corrected for system and Milli-Q blanks. The average instrument blank and Milli-Q blank corresponded to 0.07 mg C L^{-1} (n=30) and the reproducibility was high (1.9%). After filtration, particulate organic carbon (POC) was determined from the material remaining on the GF/F filters. The filters were acidified with HCl (2 mol L^{-1}) to remove the inorganic carbonate fraction, dried over night to remove excess HCl and burned in a stream of oxygen at 900 °C. POC was analysed using a solid sample module SSM-5000A in conjunction with a TOC-V_{CPH} total organic carbon analyser (Shimadzu), calibrated with glucose. POC concentrations were corrected based on blank filter measurements. The average filter blank value including the instrument blank corresponded to 0.005 mg C L^{-1}. The reproducibility obtained for the glucose standard was high (3.0%). Total organic carbon (TOC) content was determined from lyophilised, well homogenized sediment samples, using a Shimadzu SSM-5000A connected to a TOC-V_{CPH} analyser, by high temperature (900 °C) catalytic (mixture of Pt/silica and CoO) oxidation method with IR detection of CO_2 and calibrated with D(+)-glucose (Merck, Germany) (Dautović et al., 2012). Methods for organic carbon determination (DOC, POC, TOC) are accredited according to HRN EN ISO/IEC 17025:2017.

2.3.3 Determination of metal concentrations in amphipods, water and sediments

Prior to metal quantification, all amphipod samples were digested in a laboratory drying oven (FN 055, Nuve, Turkey) at 85 °C for 3.5 h by adding an oxidation mixture containing concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and H_2O_2 (Suprapur®, 30% Merck, Germany) in a volume ratio of 100:1. After digestion, solutions of amphipods were left to cool, and afterwards, they

were stored at 4 °C. Amphipods were pooled due to their small size and mass (30 - 40 specimens per sample, mass of a sample: 0.03 - 0.06 g; Table S1). The number of pooled samples per sampling site was seven (n = 7). The oxidation mixture was used as a blank sample and digested in the same way as the samples. The efficiency of acid digestion was verified by digesting certified reference materials for trace metals: Oyster tissue (SRM 1566a, National Institute of Standards and Technology, NIST, USA) and Scallop tissue (RM IAEA-452, International Atomic Energy Agency, Austria). The average recovery (n = 8) of the trace elements analysed in amphipods was as follows (%): Cd (99.4), Co (84.0), Cu (110.7), Fe (86.2), Mn (90.4), Ni (110.6), Pb (99.7), Rb (103.4) and Zn (85.2).

Prior to the trace and macro elements determination in sediments, portions of around 50 mg of ground subsamples were digested using a microwave oven (Multiwave 3000, Anton Paar, Austria) in a two-step digestion procedure: 1) using a mixture of nitric acid (HNO_3, 65%; p.a., Kemika, Croatia), hydrochloric acid (HCl, 37%; p.a., Kemika, Croatia) and hydrofluoric acid (HF, 48%; p.a., Kemika, Croatia) and 2) using boric acid (40 g L^{-1} H_3BO_3; Fluka, Switzerland). Procedure of sediment digestion is described in detail by Fiket et al. (2017). 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 were in the range from 90 to 102%, depending on the element measured (Fiket et al., 2017). The limit of detection (LOD) of the method, calculated as three times the standard deviation of ten consecutive measurements of the procedural blank, varied between 0.03 to 1.5 mg kg^{-1} for the trace elements, and from 10 to 45 mg kg^{-1} for major elements (Al, Ca, Fe, K, Mg, Na) (Fiket et al., 2017).

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) was used for element analyses. Depending on the element, the measurement was operated in low (m/Δm is about 300 - ^{85}Rb, ^{111}Cd, ^{120}Sn and ^{208}Pb), in medium (m/Δm is about 4000 - ^{23}Na, ^{24}Mg, ^{27}Al, ^{42}Ca, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu and ^{66}Zn) and in high resolution mode (m/Δm is about 10,000 - ^{39}K). Indium (1 μg L^{-1}, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added as an internal standard to all samples and solutions. Two external calibrations were performed using standard solutions prepared in 1.3% HNO_3 (Rotipuran Supra 69%, Carl Roth, Germany). For macro elements,

multi-element stock standard solution containing Ca (2.0 g L^{-1}), Mg (0.4 g L^{-1}), Na (1.0 g L^{-1}), and K (2.0 g L^{-1}) (Fluka, Germany) was used for preparation of calibration standards. For trace elements, calibration solution was prepared by dilution of multi-element standard solution (100 mg L^{-1}; Analytika, Czech Republic) with added standard solutions of Rb (1 g L^{-1}; Sigma-Aldrich, Germany) and Sn (1 g L^{-1}; Analytika, Czech Republic). In addition to the interstitial water, amphipod and sediment samples, blank samples were prepared and treated the same way and used for the measurement of elements. The accuracy and the precision of HR ICP-MS measurements was verified by analysing the quality control sample for macro elements (QC for Minerals, Catalog number 8052, UNEP GEMS, Burlington, ON, Canada) and for trace elements (QC for trace metals, Catalog number 8072, UNEP GEMS, Burlington, ON, Canada). A good agreement was observed between certified values and our measurements in interstitial water, resulting in the following average recoveries (%): Na (101.2 ± 1.8), Mg (98.0 ± 3.5), Ca (101.6 ± 1.7), K (100.1 ± 3.4), Al (97.3 ± 3.1), Cd (98.7 ± 1.7), Co (98.8 ± 3.0), Cr (97.0 ± 1.7), Cu (97.5 ± 2.4), Fe (96.5 ± 2.2), Mn (97.4 ± 3.1), Ni (99.1 ± 2.5), Pb (99.1 ± 2.5), Sn (99.6 ± 2.3) and Zn (94.5 ± 2.2). Obtained LOD for the measured trace elements in the water were (μg L^{-1}) as follows: Al (0.4), Cd (0.002), Co (0.019), Cr (0.06), Cu (0.40), Fe (0.6), Mn (0.05), Ni (0.10), Pb (0.02), Rb (0.003), Sn (0.012) and Zn (0.100), whereas for the macro elements, they were (mg L^{-1}) Ca (0.02), K (0.002), Mg (0.006) and Na (0.006).

2.4 Statistical analyses

Main statistical analyses were carried out using SigmaPlot 11.0 (Systat Software, USA). Considering that the assumptions of normality and homogeneity of variance were not always met, the Mann-Whitney U test was applied to test the significance of differences in metal concentrations in *S. ambulans* (n = 7, number of replicates per sampling site in each sampling season), the interstitial water (n = 3), and the fine fraction of sediment (n = 1) between two sampling sites. The significance of variability in metal concentrations between sampling seasons was tested by Kruskal-Wallis one way analysis of variance on ranks, separately for each sampling site. Differences were regarded as significant at $p < 0.05$. Correlation coefficients (for linear dependence) and their *p* values between concentrations of metals in amphipods and environmental parameters (metal concentrations in interstitial water, metal

concentrations in fine fraction of sediments, dissolved macro element concentrations (Na, Mg, K, Ca) in interstitial water, conductivity, oxygen saturation in interstitial water, concentrations of hydrogen ions (pH), DOC in interstitial water, and TOC in sediments) were determined in Python 3 (Van Rossum and Drake, 2009), using the Pearson correlation formula. Furthermore, logarithmic equation was used to determine further associations between the data above (logarithmic associations are indicated in the Table 4 in italic), since not all relations between the tested parameters are likely to be linear. Additionally, multiple linear regression (MLR) models were constructed in order to examine the extent to which observed trace metal levels in *S. ambulans* could be explained by a limited set of powerful predictors. The environmental parameters used as predictor variables for the MLR modelling of metal accumulation in *S. ambulans* were the same as for the simple correlation analysis explained above. With this set of predictor variables, all possible subset regression models were computed using Python 3. The percentage of total variance explained by the MLR model was reported as the adjusted R^2 value. The results of MLR statistics for models with $p < 0.05$ are presented in Supplementary material (Microsoft Excel file). The level of significance for all correlation and regression analyses was set to $\alpha = 0.05$.

3. Results

Since the HZ is the habitat of the studied amphipod *S. ambulans*, the comprehensive description of its characteristics in terms of basic water physicochemistry and organic carbon contents as well as in terms of contamination with metals in interstitial water and sediments was essential in providing a framework for studying the bioaccumulation of metals in these organisms. Therefore, the following three subsections are devoted to this purpose, especially as these data present new information on the characteristics of the HZ. The information on metal accumulation in *S. ambulans* and metal bioavailability addresses the potential risk of affecting human health since it inhabits groundwater-connected ecosystems, and groundwater is the only source of drinking water for the population inhabiting the investigated area.

3.1 Physicochemical parameters in interstitial water

Since the HZ is the interface between groundwater and surface water, the physicochemical parameters in the interstitial water were compared with both the surface water quality standards (WQS) and the

groundwater quality standards (GQS) established in the Croatian Directive on Water Quality Standards (GRC, 2019) (Table 1). According to the GQS, nitrates, nitrites, and phosphates in interstitial water at the Medsave and Jarun sites mostly met the established threshold values in all seasons (Table 1). According to the WQS, concentrations of phosphates and nitrates indicated less than good water quality status in certain seasons (Table 1).

All COD_{KMnO4} values measured met the threshold value for surface waters of good quality for the river type HR-R_5b (Table 1) (GRC, 2019). The values of COD_{KMnO4} of interstitial water samples from Medsave village were higher than in Jarun in winter, summer, and autumn (Table 1), indicating higher amount of dissolved organic matter in the water.

In this study, temperature varied seasonally, with maximum values in summer (Medsave: 23.3 °C; Jarun: 16.5 °C) and minimum in winter (Medsave: 10.4 °C) and spring (Jarun: 12.7 °C) (Table 1). Comparison between temperature in the HZ and surface water showed that in the HZ in winter temperature was 10.4 °C at Medsave and 13.1 °C at Jarun (Table 1), while the surface water temperature was 9.0 °C at Medsave and 7.5 °C at Jarun. In summer, the water temperature in the HZ at the Jarun site was 16.5 °C and in the surface water 22 °C.

The concentrations of dissolved oxygen in the interstitial water at the Jarun site ranged from 4.40 in summer to 7.35 mg O_2 L^{-1} in autumn (Table 1), compared to the concentrations of dissolved oxygen in the surface water of the Sava River, which ranged from 8.60 in summer to 11.49 mg O_2 L^{-1} in spring (data not shown). At the Medsave site, dissolved oxygen concentrations in the interstitial water were also lower than in the surface water of the river. In summer, the low concentrations of dissolved oxygen (Table 1) in the HZ of the Sava River coincided with low flow. Hydrological data from the Croatian Meteorological and Hydrological Service show that, on average, lower water levels (-249 cm) and discharge (122 m^3/s) were recorded in summer and autumn at both sites (Fig. S1), compared to spring and winter (-214 cm; 179 m^3/s).

At the Medsave site, pH of the interstitial water from the HZ indicated a slightly alkaline environment with average value of 8.30 and higher values during all seasons compared to the Jarun site (Table 1). At the Jarun site, pH had an average of 7.61 (Table 1).

411 Conductivity can serve as a useful measure of dissolved inorganic materials, and it was higher during
412 all seasons at the Jarun site compared to the Medsave site (Table 1).
413
414 3.2 Organic carbon concentrations in interstitial water and sediment samples
415 Concentrations of DOC in the interstitial water were higher at the Medsave site than at the Jarun site in
416 all seasons, with the highest values measured at the Medsave site in spring and summer (1.37 mg L^{-1})
417 (Table 1). Particulate organic carbon (POC) in interstitial water was higher at the Jarun site than at the
418 Medsave site in all seasons, with the highest values observed in summer and autumn at both sampling
419 sites (Table 1). Total organic carbon (TOC) in sediment was higher at the Medsave site than at Jarun in
420 most seasons (spring, summer, and autumn), with a maximal value of 2.97% in summer (Table 1).
421
422 3.3 Metal concentrations in interstitial water and sediments from HZ
423 In terms of differences between sampling sites, concentrations of dissolved macro elements (Ca, K, Mg,
424 Na) and Ni in interstitial water from HZ were statistically higher at the Jarun site than at the upstream
425 Medsave sampling site, while Al and Fe were statistically higher at the Medsave site (Table 2).
426 Concentrations of Cr did not differ between the two sampling sites (Table 2). Although the statistical
427 data analyses showed significant differences between the sampling sites for most of the studied elements
428 in at least one season, their concentrations were in a relatively narrow range (Table 2). Specifically, in
429 the interstitial water, the concentration ranges of metals were: < 0.1 µg L^{-1} (Cd, Co, Pb, Sn), < 1 µg L^{-1}
430 (Cr), 1 - 5 µg L^{-1} (Al, Cu, Ni, Rb, Zn), and 1 - 10 µg L^{-1} (Fe, Mn).
431 The environmental quality standards (EQS) set by the European Directive on Environmental Quality
432 Standards in the Field of Water Policy (EPCEU, 2008/105/EC) and the related Croatian Directive on
433 Water Quality Standards (GRC, 2019) are defined for Cd, Pb, Hg, and Ni, while the EQS set by the
434 European Groundwater Directive (EPCEU, 2006/118/EC) are defined for As, Cd, Pb, and Hg. The EQS
435 values of the EU Directive and the Croatian Directive are identical. The comparison with the EQS
436 revealed that the concentrations of dissolved Cd, Ni and Pb in our study were below these values (Table
437 2).

438 In relation to seasonal differences in dissolved metal concentrations in interstitial water, statistically
439 significant values were observed for Al, Cd, Co, Cr, Cu, Fe, Ni, Mg and Na at both sampling sites
440 (Table 2). The highest concentrations of most of the metals analysed were detected in summer and
441 autumn at both sampling sites (Table 2).
442 Element composition of the fine sediment fraction showed no significant difference between the
443 Medsave and Jarun sampling sites in terms of metal concentrations within the same season (Table 3).
444 Although seasonal differences in metal concentrations in the fine fraction of sediment were observed,
445 they were not statistically significant (Table 3).
446 The obtained values of metal concentrations in the fine fraction of sediments (Table 3) were also
447 compared with the US EPA-Sediment Quality Guidelines (2000), Canadian Sediment Quality
448 Standards (2001), and Norwegian Sediment Quality Standard (Bakke et al., 2010) for metals in the
449 sediment. Concentrations of Cd, Cr, Cu, Pb, Ni, Zn in the sediment fine fraction at both sampling sites
450 in all studied seasons were lower than the probable effect concentrations (PECs; i.e. concentrations
451 above which harmful effects are likely to be observed) for sediments (PECs for Cd, Cr, Cu, Pb, Ni, Zn:
452 3.5, 90, 197, 91.3, 315 mg kg^{-1} DW, respectively), according to US EPA (2000) and Canadian Sediment
453 Quality Standards (2001), which are identical. Concentrations of Cd, Cr, Cu, Pb, Ni and Zn (Table 3)
454 were lower than the PNEC value for chronic exposure ($PNEC_{chronic}$; 2.6, 560, 51, 83, 46, 360 mg kg^{-1},
455 respectively) (Bakke et al., 2010). In addition, metal concentrations in the sediment fine fraction were
456 compared to threshold effect concentrations (TEC), i.e., concentrations of sediment-associated
457 contaminants below which no harmful effects on sediment-dwelling organisms are expected, defined
458 by MacDonald et al. (2000). In our study, concentrations of Cd, Cr, Cu, Pb, Ni, and Zn in the sediment
459 fine fraction (Table 3) were not higher than TEC (0.99, 43.4, 31.6, 35.8, 22.7, and 121 mg kg^{-1} DW,
460 respectively) at both sampling sites in all seasons studied.
461
462 3.4 Metal concentrations in *S. ambulans*
463 The results on metal/metalloid concentrations in *S. ambulans* represent the first data for this
464 stygophilous amphipod in real environmental conditions. In general, a similar increasing order of total
465 metal/metalloid concentrations in amphipods was observed in all sampling campaigns at both sampling

sites. Specifically, in winter and spring at the Medsave site (Figs. 2 and 3) this increasing order was as follows: Cd < Sn < Co < Pb < Ni < Cr < Rb < Mn < Zn < Cu < Al < Fe. In summer and autumn, the order was the same as in winter and spring, except for of Sn, which was lower than Cd. Total trace metal/metalloid concentrations in amphipods at Jarun (Figs. 2 and 3) showed an increasing order in winter: Cd < Sn < Co < Pb < Ni < Cr < Rb < Cu < Zn < Mn < Al < Fe. In spring and summer this order was the same as in winter, except that Mn was lower than Zn, while in autumn it was the same as in spring and summer, except that Sn was lower than Cd (Figs. 2 and 3). Macro elements – Mg < Na< K < Ca – had the same increasing order of concentrations in *S. ambulans* in all seasons at both sampling sites (Fig. S2).

In addition, the results showed different spatial and temporal patterns in the measured metals/metalloids, so they were presented in three groups: i) metals with higher concentrations at the Jarun site (Fig. 2), ii) metals with higher concentrations at the Medsave site (Figs. 3a – 3c), and iii) metals with mostly comparable concentrations at both sampling sites (Figs. 3d – 3f). In general, higher metal accumulation was observed for Al, Cr, Fe, Ni, Pb, and Sn in *S. ambulans* specimens from the Jarun site compared to the upstream Medsave site in all studied seasons (Fig. 2). However, these differences were significant for Al, Cr, Fe, Ni, and Pb in winter, for Cr and Pb in spring, and for Cr, Ni, Pb, and Sn in autumn (Fig. 2). On the other hand, concentrations were significantly higher in amphipods at Medsave compared to the downstream site Jarun, in all four seasons for Rb (Fig. 3c), in all seasons except autumn for Cu (Fig. 3b), and in winter and summer for Cd (Fig. 3a). Also, Cu was higher in autumn at Medsave, but not significantly (Fig. 3b). There was no significant difference between two sampling sites within the same season for Co, Mn, and Zn (Fig. 3 d–f), except for Co in summer (Fig. 3d).

Significant seasonal differences in metal concentrations in *S. ambulans* were observed for Cd, Co, Mn, Rb, Sn, and Zn at both sampling sites (Figs. 2 and 3). No significant seasonal differences in metal concentrations in *S. ambulans* were observed for Cr, Fe, Ni, and Pb at either sampling site, for Cu at Jarun, and for Al at the Medsave site (Figs. 2 and 3). The macro elements – Mg, Na, K, and Ca – showed the same increasing order of concentrations in *S. ambulans* at both sampling sites in all seasons.

4. Discussion

4.1. Factors affecting metal bioaccumulation in *S. ambulans*

The physiology of the amphipod itself and the physicochemical conditions in the environment undoubtedly play an important role in metal uptake and accumulation in *S. ambulans*. Since *S. ambulans* is a stygophilous amphipod inhabiting the hyporheic ecotone, its metal exposure is probably mostly influenced by the physicochemical conditions in the interstitial water and sediment of the HZ. In this case, metal contents in the fine fraction of the sediment could be more important than the total sediment elemental composition because of its binding properties of metal ions and its possible role in the diet of *S. ambulans*. Therefore, the following factors in the interstitial water that may affect the bioaccumulation of metals in *S. ambulans* were considered: exposure level, concentrations of dissolved macro elements (Na, Mg, K, Ca), conductivity, O_2 saturation, pH, and DOC. In sediments, total concentrations of metals contained in the fine fraction of the sediment and TOC were considered. In addition, other biological factors and amphipod physiology that might influence metal accumulation were discussed.

The statistical results showed that the concentrations of all studied metals in *S. ambulans* were strongly correlated with the level of metal exposure in the interstitial water, revealing the predominant influence of the waterborne route of exposure on metal bioaccumulation (Table 4). Significant positive correlations with dissolved metals in the interstitial water were found for all metals (Table 4), similarly as previously demonstrated for Cd, Co, Cu, Mn, Ni, Pb, and Zn in gammarid species in the surface waters (Amyot et al., 1994; Lebrun et al., 2014).

It has already been shown that bioaccumulation of metals by amphipods cannot be explained solely by contamination levels in water, but also by environmental factors such as water physicochemistry, especially the macro element (Ca, K, Mg, Na) concentrations in the freshwater (Lebrun et al., 2014). In our study, concentrations of most trace metals in *S. ambulans* exhibited significant negative association with all dissolved macro elements (Table 4), which could indicate the competition between cations of macro and trace elements for binding sites important for transfer of metal cations from the environment into the organism. This was in line with the finding for *G. pulex*, which indicated that an increase in the concentration of Ca in water reduced Ni uptake in the organism (Lebrun et al., 2011). It was suggested

522 that Ni behaves similarly to Ca, competing with Ca binding sites (Lebrun et al., 2011). In addition,
523 increased Ca concentration in water suppressed the uptake of Cd, Ni, Pb, and Zn in *Daphnia magna*
524 (Komjarova and Blust, 2009).
525 Conductivity was another factor that could influence the bioaccumulation of metals in *S. ambulans*. It
526 was observed that concentrations of Cr, Ni, Pb, and Sn accumulated by *S. ambulans* correlated
527 significantly positively with conductivity in the interstitial water (Table 4). One of the possible
528 explanations for this correlation could be the increased availability of these elements in interstitial
529 water. It has been shown that high conductivity (in the range of 144.2 – 252.2 μS cm^{-1}) increases the
530 bioavailability of heavy metals by releasing them from the sediments due to the competition of ions for
531 adsorption sites (Miao et al., 2021). Since the adsorption force of metals in the carbonate-bound fraction
532 is relatively weak, conductivity has a large impact on metals in this form (Chakraborty et al., 2016).
533 Therefore, high conductivity of the interstitial water samples in our study (Table 1) could favour the
534 desorption of Cr, Ni, Pb and Sn from the sediment, as carbonates comprise a significant portion of the
535 suspended particulate matter in the samples in the Sava River in Zagreb (Lučić et al., 2019).
536 A significant negative association of oxygen saturation with Al, Cr, Fe, Ni, Pb, and Sn was observed in
537 *S. ambulans* (Table 4). A possible explanation here can also be related to the characteristic geochemical
538 properties of a particular metal. Specifically, dissolved oxygen affects the redox conditions in river
539 water and the transformation of heavy metals in sediments (Miao et al., 2021). The redox conditions
540 and concentrations of different binding ligands in the sediments determine the bioavailability of metals
541 and toxicity to biota (Calmano et al., 1993). Sediment in the HZ is usually characterized by low oxygen
542 concentrations and changes in redox potential relative to surface water. Consequently, such reductive
543 conditions can lead to the release of trace metal contaminants adsorbed on or precipitated with Fe and
544 Mn oxides (Benner et al., 1995; Harrison, 2018). Therefore, we could suppose that in our study, redox
545 conditions (i.e. low oxygen saturation) possibly affected the behaviour of Al, Cr, Fe, Ni, Pb, and Sn,
546 and favoured their release from sediments, and consequently, increased their bioaccumulation in *S.*
547 *ambulans*.
548 Additionally, dissolved oxygen can influence not only the physicochemical forms of metals, but also
549 the physiology of organisms. Amphipods living in adverse environmental conditions such as hypoxia

may develop a strategy of metabolic depression, displayed as decreased metabolic rate, resulting in lower metal uptake and accumulation (Jakob et al., 2017). In our study, the opposite pattern was observed since significant negative correlation of metals with oxygen saturation was recorded in *S. ambulans*. Accordingly, metabolic depression may not be the strategy adopted by *S. ambulans* under hypoxic conditions, as this species is stygophilous and can adapt to living in a habitat with decreased oxygen concentrations. To date, little is known about this subject, so more research is needed.

Next two factors considered to affect metal bioaccumulation in *S. ambulans* were pH and DOC. In our study, a positive correlation of Cu and Rb concentrations accumulated by *S. ambulans* was observed with pH and DOC in the interstitial water from the HZ (Table 4). Variations in pH may affect the oxidizable metal fraction bound to organic matter in the following way. Organic matter tends to degrade in alkaline conditions, increasing the bioavailability of metals in the oxidizable fraction (Wen et al., 2015). Besides, natural complexing ligands such as DOC and dissolved organic matter (DOM) can influence the transport and bioavailability of metals in water in both ways, eventually increasing or decreasing bioaccumulation and toxicity that is metal specific. On the other hand, a negative correlation of pH and DOC concentration in the interstitial water from the HZ with metal concentration in *S. ambulans* was observed for Cr, Ni and Pb (Table 4). There are several studies about DOC and pH effects on Pb, Ni and Cr bioaccumulation in crustaceans that support our observation. Similarly to our study, a significant negative correlation of DOC with Pb was observed in gammarid species (Lebrun et al., 2020). It is known that the bioavailability of Cr, Ni, and Pb may be limited due to their complexation with DOC (Richard and Bourg, 1991; Niyogi and Wood, 2004; Custer et al., 2016). The suppressive effect of pH on Ni uptake and bioaccumulation under neutral and basic conditions has been described in *Daphnia* (Komjarova and Blust, 2009). The uptake of Pb from water and sediments in isopod *Asellus communis* also increased with decreasing pH (Lewis and Mcintosh, 1986). The complex behaviour of Cr in aquatic systems and surface sediments depends on both abiotic and biotic reactions, including complexation/decomplexation, sorption, precipitation/dissolution, redox and respiration reactions. In general, organic matter and higher pH favour the reduction of more soluble Cr(VI) to Cr(III), which is less soluble and forms precipitates (Gorny et al., 2016). Therefore, such behaviour could result in lower

bioavailability and consequently it could be a possible explanation for observed significant negative correlation between Cr concentrations in *S. ambulans* and DOC and pH in interstitial water (Table 4). A significant positive correlation between metal concentrations in amphipods and in the fine fraction of sediments (< 63 μm) was observed only for Cd and Zn (Table 4), indicating that bioaccumulation of these metals in *S. ambulans* can be determined by metal concentration in sediments. Therefore, it is likely that the uptake of Cd and Zn in *S. ambulans* takes place by ingesting the fine fraction of sediments. Previous studies in this area have reported high concentrations of Cd and Zn in sediment (fraction <2 mm) (Milaković et al., 2019), with values 4-9 times higher than in our study. Therefore, the possibility of using *S. ambulans* as a bioindicator of Cd and Zn exposure from sediment in the HZ would be of great importance for the protection of the Sava River.

Considering the above discussed factors that can affect metal bioaccumulation, Cu showed distinctly different behaviour compared to all other studied metals. Copper concentrations accumulated in *S. ambulans* showed significant negative correlation with concentrations of dissolved Cu in the interstitial water and conductivity, while positive correlations were observed with concentrations of dissolved macro elements, pH and DOC in the interstitial water from the HZ (Table 4). It has been shown that Cu speciation in natural waters is primarily governed by complexation with natural organic ligands such as fulvic acids, which stabilize the dissolved Cu^{+} ion and thus reduce its bioavailability (Breault et al., 1996), which supports our finding. Also, unlike most trace metals studied in *S. ambulans*, a significant positive association was observed between Cu and Ca in the interstitial water (Table 4), which was in agreement with a previous finding in gammarid species (Lebrun et al., 2020). High conductivity can favour metal desorption from the sediment, although this is not the case for Cu, which is more strongly bound to organic ligands present in the organic coating on fine particles of sediment (Miao et al., 2021). Consequently, this could result in lower availability of Cu in the interstitial water and lower bioaccumulation in *S. ambulans* and explain the significant negative correlation between conductivity and Cu concentration accumulated in *S. ambulans* (Table 4). Additionally, Cu is known for its affinity for organic ligands (Breault et al., 1996) and can be ingested by an organism by dietary uptake route. Thus, *S. ambulans* feeding on organic matter, could simultaneously take up and accumulate Cu, which

604 can explain the significant positive correlation between DOC concentration in interstitial water and Cu
605 concentration accumulated in *S. ambulans* (Table 4).
606 In addition, biological factors may also influence metal accumulation in *S. ambulans*. It has been shown
607 that the gill surface area is relatively large in small amphipods compared to larger amphipods (Moore
608 and Taylor, 1984). Since *S. ambulans* is a small amphipod species, the gills may play an important role
609 in metal uptake. Crustaceans exposed to waterborne Cd have been shown to accumulate the metal
610 primarily in the gills and hepatopancreas, causing alterations in their structure (Soegianto et al., 1999).
611 In aquatic invertebrates, feeding behaviour and ecological preferences have been identified as important
612 characteristics for assessing the accumulation and toxicity of sediment-bound metals (De Jonge et al.,
613 2010).
614 In addition to physicochemical conditions in the environment, the physiology of amphipods may also
615 influence metal uptake and accumulation. The uptake rate of trace metals in amphipods can be
616 controlled by Ca pumps during transport across epithelial membranes, through which metals with
617 similar ionic radius and same charge as Ca can enter (Rainbow, 1995; Ahearn et al., 2004). The
618 proportion of trace metals taken up via the Ca route may be affected during the moulting process in
619 crustaceans, which involves Ca regulatory mechanisms. For example, Cd^{2+} accumulation in freshwater
620 *Gammarus pulex* has been associated with greater activity of Ca pump, which can be attributed to the
621 freshwater, i.e. low-calcium environment (Wright, 1980). It is also known that Cu^{2+} ions can use
622 mitochondrial Ca transport proteins to enter the organelle and thus interact with Ca regulatory processes
623 (Ahearn et al., 2004). The moulting cycle of *S. ambulans* has not yet been investigated, but for
624 comparison with another amphipod species, the moult cycle of *Gammarus fossarum* is known to last
625 approximately 30 days under laboratory conditions (Geffard et al., 2010). Future studies on this topic
626 are needed to elucidate the influence of moulting on trace metal uptake *in S. ambulans*.
627
628 4.2. Metal bioaccumulation in *S. ambulans* and its potential as bioindicator species
629 Our findings on metal accumulation in *S. ambulans* represent the cumulative concentration from the
630 combined exposure route because, in field studies like this one, we cannot distinguish between water
631 and dietary exposure. Nevertheless, we discussed the bioaccumulation of several most relevant

accumulated metals, which are known as environmental pollutants (Cd, Cr, Ni, Pb) and essential metals (Cu, Zn, Mn) in relation to both water and sediment concentrations. Furthermore, as there are no literature data on metal accumulation in *S. ambulans* we compared our results with several recent studies on other amphipod species inhabiting both surface waters (epigean species) and groundwaters (hypogean species).

First, we compared metal concentrations in interstitial water and in the specimens of *S. ambulans*. Total dissolved metal concentrations in water (consisting of hydrated or free metal ions, colloids, and complexes with organic and inorganic ligands) were taken into account since they are more bioavailable to aquatic organisms than particulate metal fraction (Elder, 1988). Previous study on the Sava River water identified sources of dissolved trace metals and classified the Sava River as moderately polluted river, with almost one-third of the metals present coming from anthropogenic sources, where Cd, Mo, Pb, Ti, and Zn originated from industrial effluents, pesticides and fertilisers from agriculture (Lučić et al., 2022). According to our study maximal concentrations of Cd and Cu in the interstitial water at the Jarun site and of Cu at the Medsave site (Table 2) were consistent with maximal concentrations in *S. ambulans* (Fig. 3), suggesting efficient accumulation of these metals in the amphipod specimens, although their concentrations in water were relatively low (Cd < 0.1 µg L^{-1} and Cu 1 - 5 µg L^{-1}, Table 2). The potential of amphipod crustaceans living in surface and interstitial ecosystems as bioindicators of metal pollution has already been emphasized in the studies on the relationships between metal levels (Zn, Cu) in the environment (water, sediment) and these organisms in the Rhône and Ain rivers (France) (Plénet, 1995). The average concentration of Zn in *S. ambulans* in our study was much lower (12.5 µg g^{-1}, both sites and all seasons) compared to the Zn concentration in the hypogean amphipod *Niphargus* cf. *rhenorhodanensis* (170.0 µg g^{-1}) from the Rhône River in France (Plénet, 1995). Also, Cu concentration in *S. ambulans* was on average 12.0 µg g^{-1} compared to 152.0 µg g^{-1} in *N.* cf. *rhenorhodanensis* (Plénet, 1995). Increasing metal bioaccumulation with exposure levels was also shown in gammarids. Concentrations of essential metals (Cu, Zn, Mn) in the amphipod *Gammarus pulex* increased with the contamination level of the Seine River (Lebrun et al., 2014). Compared to our study, the minimal concentrations of Cu and Zn in *G. pulex* (Lebrun et al., 2014) were 3 and 5 times higher, respectively, than in *S. ambulans* (Cu 6.83 µg g^{-1}; Zn 10.52 µg g^{-1}; Figs. 3b and 3f). Copper

660 bioaccumulation in *G. pulex* correlated with total metal concentrations in the river water (Lebrun et al.,
661 2014). In another study on caged *G. pulex* and *G. fossarum*, concentrations of Ag, Co, Mn, Pb, and Zn
662 in amphipods correlated with total metals in the river water (Lebrun et al., 2015). These species were
663 also shown to significantly accumulate Ni and Pb at environmental concentrations (Urien et al., 2017),
664 indicating their ability to be good bioindicators. In contrast to these extensive studies on epigean
665 gammarids, hypogean amphipods such as *S. ambulans* have yet to be studied to support their use as
666 bioindicator organisms capable of detecting changes in metal exposure in the environment.
667 Also, we compared metal concentrations in the fine fraction of sediment (< 63 µm) and in *S. ambulans*.
668 Sediment is a major sink and source of metal contaminants and contains many toxic metals that pose
669 serious threats to the aquatic environment (Zhang et al., 2014). Therefore, the assessment of metal
670 concentrations in sediment in anthropogenically impacted areas is important because it can provide
671 evidence of the effect on aquatic ecosystems and biota. In particular, the fine fraction of sediment is
672 mainly responsible for the binding of trace/toxic metals and the distribution of contaminants in river
673 environments (Vdović et al., 2021) due to its large surface area and special geochemical composition
674 (Stone and Droppo, 1994). Analysis of suspended particulate matter (originating from resuspension of
675 sediment material) in the Sava River in the same area as in our study indicated anthropogenic input of
676 ecotoxic elements (Ni, As, Cr, Pb, Bi, Cd, Zn, Sb) into the environment (Lučić et al., 2019). In addition,
677 soil fertilization in the watersheds of the Krapina River (tributary of the Sava River with the confluence
678 upstream of the Jarun site) were shown to contribute to the input of Cu, Zn, and Pb into the sediments
679 (Oreščanin et al., 2004). In our study maximal concentrations of Cd, Cr, Ni, and Pb in sediment at the
680 Medsave site and of Sn at the Jarun site (Table 3) were consistent with maximal concentrations in *S.*
681 *ambulans* (Figs. 2 and 3), suggesting efficient accumulation of these metals in amphipod specimens.
682 Moreover, Cu concentrations in *S. ambulans* were higher (Fig. 3b) than in the sediment fine fraction at
683 both sites and in all seasons (Table 3). Plénet (1995) observed higher concentrations of Cu in the epigean
684 amphipod *Gammarus fossarum* than in the sediment, which was also the case in *S. ambulans*.
685 Concentrations of Cd in autumn (Fig. 3a) and of Zn in winter (Fig. 3f) were higher in *S. ambulans* than
686 in sediment at the Jarun site (Table 3).

687 According to Mösslacher (2000), existing groundwater quality criteria are insufficient to protect
688 groundwater biota since groundwater crustaceans are more sensitive to chemical stressors than closely
689 related epigean species. In the last few decades, epigean amphipods, such as *Gammarus fasciatus*, *G.*
690 *pulex*, *G. fossarum* and *Echinogammarus berilloni* have been recognized as useful organisms in
691 biomonitoring of metal pollution because they respond to low metal exposures in the surrounding
692 environment (Amyot et al., 1994; Lebrun et al., 2015; Lebrun et al., 2020). Moreover, the epigean
693 freshwater amphipod *Crangonyx pseudogracilis*, which belongs to the Crangonyctidae family, same as
694 *S. ambulans*, has been suggested as a potential new indicator species in biomonitoring programmes
695 because its behaviour changes upon exposure to toxicants (Kirkpatrick et al., 2006). However, there are
696 only few hypogean amphipods which have been suggested as bioindicators of pollution. For example,
697 the groundwater amphipod *Niphargus montellianus* has been proposed as a biological indicator for
698 monitoring heavy metal pollution (Coppellotti Krupa and Guidolin, 2003). Thus, it is important to
699 recognize *S. ambulans* as a potential hypogean bioindicator species in HZ in order to protect this
700 particularly vulnerable ecotone and sensitive component of fluvial ecosystems.
701
702 4.3. Bioaccumulation factors (BAFs) in *S. ambulans*
703 BAFs are commonly used to represent the partitioning of a chemical between water and aquatic
704 organisms (Karlsson et al., 2002) and are often used as the simplest empirical model for hazard
705 identification. In this study, BAFs were calculated for each season and sampling site as the ratio of the
706 metal concentration in total amphipod body (µg $kg^{-1}_{w.w.}$) to the metal concentration in the interstitial
707 water (µg L^{-1}) from the HZ of the Sava River, expressed in L $kg^{-1}_{w.w.}$.
708 The essential trace metals were concentrated in amphipods in this order: Co < Mn < Zn < Cu < Fe with
709 average BAFs of 1,965, 4,256, 8,509, 14,873 and 78,811 L $kg^{-1}_{w.w.}$, respectively. Specimens of *S.*
710 *ambulans* also concentrated non-essential metals Ni, Sn, Rb, Cr, Cd, Pb, and Al with average BAFs of
711 322, 1,470, 1,723, 1,999, 2,783, 6,772 and 143,278 L $kg^{-1}_{w.w.}$, respectively. In general, BAFs were
712 higher at the Jarun site than at the Medsave site, being highest at both sampling sites mostly in winter.
713 In contrast to non-essential metals, bioaccumulation of essential metals (Fe, Cu, Zn) is naturally high
714 in *S. ambulans* due to their vital role in the structure of proteins and enzymes. Moreover, Cu is a

functional part of the respiratory protein hemocyanin in crustaceans (Rainbow, 2007). However, the BAFs of Pb and Al in *S. ambulans* are also high, indicating high site-specific water contamination. The highest BAF in *S. ambulans* was recorded for Al. It is known that many crustaceans consume their shed cuticle after moulting as a source of Ca for exoskeleton mineralization, thereby simultaneously ingesting adsorbed metals. Therefore, it is possible that the high concentrations of accumulated Al in *S. ambulans* are derived from Al adsorbed onto the exoskeleton. In freshwater crustacean *Asellus aquaticus* it was shown that the polysaccharide-producing bacteria on the cuticle could enhance the adsorption of Al on the exoskeleton (Elangovan et al., 1999).

Significant negative relationship between the BAFs of Zn and Cu and exposure levels in populations of *G. pulex* was linked to the presence of mechanisms for active regulation and decreased uptake of these essential metals at high exposures (Lebrun et al., 2014). Thus, if there is a significant relationship between BAF and exposure level, it would be unsuitable to use this BAF in assessment models as a pollution-scale independent parameter. Examination of the log-log plots of BAFs versus metal concentrations in the interstitial water of the Sava River revealed a significant negative relationship between the BAFs of Al, Co, Fe, Mn, Sn, and Zn and exposure levels (R^2 = 0.97, 0.88, 0.93, 0.98, 0.81, and 0.89, respectively). In *S. ambulans*, the use of BAFs for hazard identification would be appropriate in the case of these six metals (Cd, Cr, Cu, Ni, Pb and Rb), which are known environmental pollutants that cause toxic effects in biota (Eriksson and Weeks, 1994; Canivet et al., 2001; Besser et al., 2005; Felten et al., 2008) and for which no significant relationship between BAFs and metal concentrations in interstitial water was found.

Although BAFs are used as the simplest empirical model to identify hazards due to exposure to pollutants, bioaccumulation processes are very complex and challenging to interpret. For example, it has been shown that preexposure to one metal can cause changes in the bioaccumulation kinetics of another metal (Urien et al., 2017). In addition, bioaccumulation patterns vary from one species to another and are specific to each metal (Lebrun et al., 2014).

5. Conclusions

In the present study, the use of hyporheic amphipod *S. ambulans* as an indicator of metal contamination in the HZ, as a complex and important site of pollutant attenuation, was evaluated. Concentrations of metals, phosphates and nitrates in the interstitial water from the HZ may be considered moderately increased probably due to agricultural activities and wastewater treatment discharge. *S. ambulans* displayed high BAFs in general, demonstrating its strong capacity to concentrate metals from the environment, even when metal concentrations in interstitial water and sediments did not exceed the EQS. Nevertheless, difference in BAFs between sampled *S. ambulance* populations was observed with higher BAF values in the population downstream of the point pollution source (WWTP discharge). The use of BAFs for hazard identification would be appropriate for Cd, Cr, Cu, Ni, Pb, and Rb. The metals accumulated in *S. ambulans* exhibited the strongest association with metal exposure levels in interstitial water, making it suitable as a bioindicator of chronic metal contamination in the HZ. This study shows that *S. ambulans* is a species capable of metal bioaccumulation. Moreover, macro element concentrations (Ca, K, Mg, Na) in the interstitial water were strongly associated with metals accumulated in *S. ambulans*, which highlights its impact on metal bioavailability. The study revealed that bioaccumulation processes take place also in the hyporheic zone, affecting sensitive stygophilic species such as *S. ambulans*.

CRediT authorship contribution statement

Zuzana Redžović: Conceptualization, Formal analysis, Investigation, Resources, Writing - Original Draft, Visualization. **Marijana Erk:** Conceptualization, Formal analysis, Investigation, Resources, Writing – Original Draft, Visualization, Project administration, Funding acquisition. **Sanja Gottstein:** Conceptualization, Investigation, Resources, Writing – Review & Editing, Project administration. **Mirela Sertić Perić:** Investigation, Writing – Review & Editing. **Jelena Dautović:** Investigation, Writing – Review & Editing. **Željka Fiket:** Investigation, Writing – Review & Editing. **Antun Lovro Brkić:** Software, Formal analysis. **Mario Cindrić:** Writing – Review & Editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Figure captions

Figure 1.

Map of the study area, i.e., upper section of the Sava River in Croatia with sampling locations – Medsave (ME) and Jarun (JA). Location of outlet of wasterwater treatment plant from the town of Zaprešić is indicated as well.

Figure 2.

Concentrations (mean and standard deviations; μg g^{-1} on wet weight basis) of six metals in *Synurella ambulans* sampled at two sites in the Sava River (Medsave – upstream from the wastewater outlet of the town of Zaprešić; Jarun – downstream from this wastewater outlet) once per season, and characterized by higher values at Jarun site: a) Al, b) Cr, c) Fe, d) Ni, e) Pb, f) Sn. Differences between sites in the same season are indicated with asterisk (Mann-Whitney U test, $p < 0.05$) and differences among seasons with different letters (small letters in Jarun sampling site and capital letters in Medsave, Kruskal-Wallis one way ANOVA on ranks, $p < 0.05$).

Figure 3.

Concentrations (mean and standard deviations; μg g^{-1} on wet weight basis) of metals in *Synurella ambulans* sampled at two sites in the Sava River (Medsave – upstream from the wastewater outlet of the town of Zaprešić; Jarun – downstream from this wastewater outlet) once per season, and characterized by higher values at Medsave site: a) Cd, b) Cu, c) Rb and with comparable values at both sampling sites: d) Co, e) Mn, f) Zn. Differences between sites in the same season are indicated with asterisk (Mann-Whitney U test, $p < 0.05$) and differences among seasons with different letters (small letters in Jarun sampling site and capital letters in Medsave, Kruskal-Wallis one way ANOVA on ranks, $p < 0.05$).

Supplementary data

Figure S1. Daily discharge (m^3 s^{-1}) in the Sava River, measured at the Zagreb gauging station (45°47'11.2"N, 15°57'20.2"E, near the Jarun sampling site) during four sampling periods (period from 1st October 2018 to 31st October 2019 is shown). Data are provided from Meteorological and Hydrological Service of Croatia (DHMZ). Arrows point to the seasonal sampling campaigns.

Figure S2. Concentrations (mean and standard deviations; mg g^{-1} on wet weight basis) of four macro elements in *Synurella ambulans* sampled at two sites in the Sava River (Medsave – upstream from the wastewater outlet of the town of Zaprešić; Jarun – downstream from this wastewater outlet) once per season: a) Ca, b) K, c) Mg, d) Na. Differences between sites in the same season are indicated with asterisk (Mann-Whitney U test, $p < 0.05$) and differences among seasons with different letters (small letters in Jarun sampling site and capital letters in Medsave, Kruskal-Wallis one way ANOVA on ranks, $p < 0.05$).

1165 Figure 1

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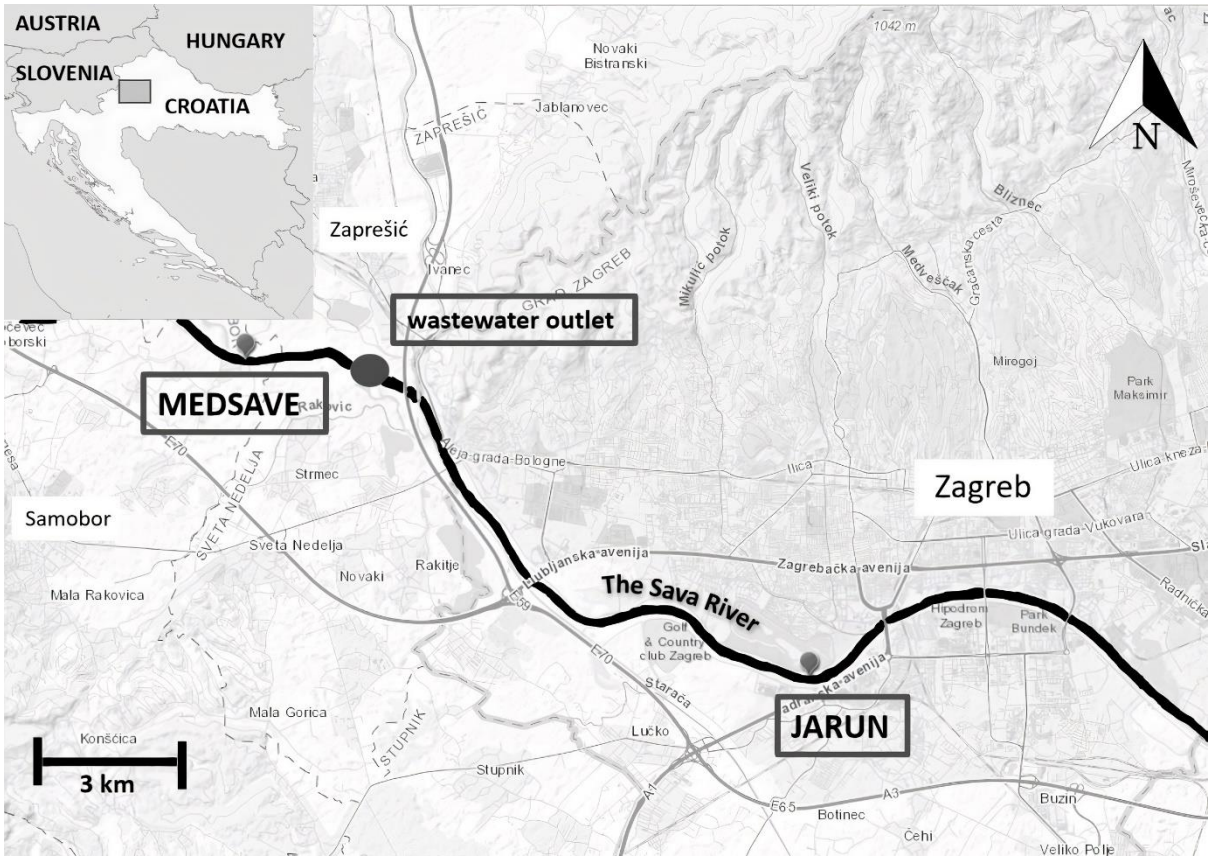

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1170 Figure 2

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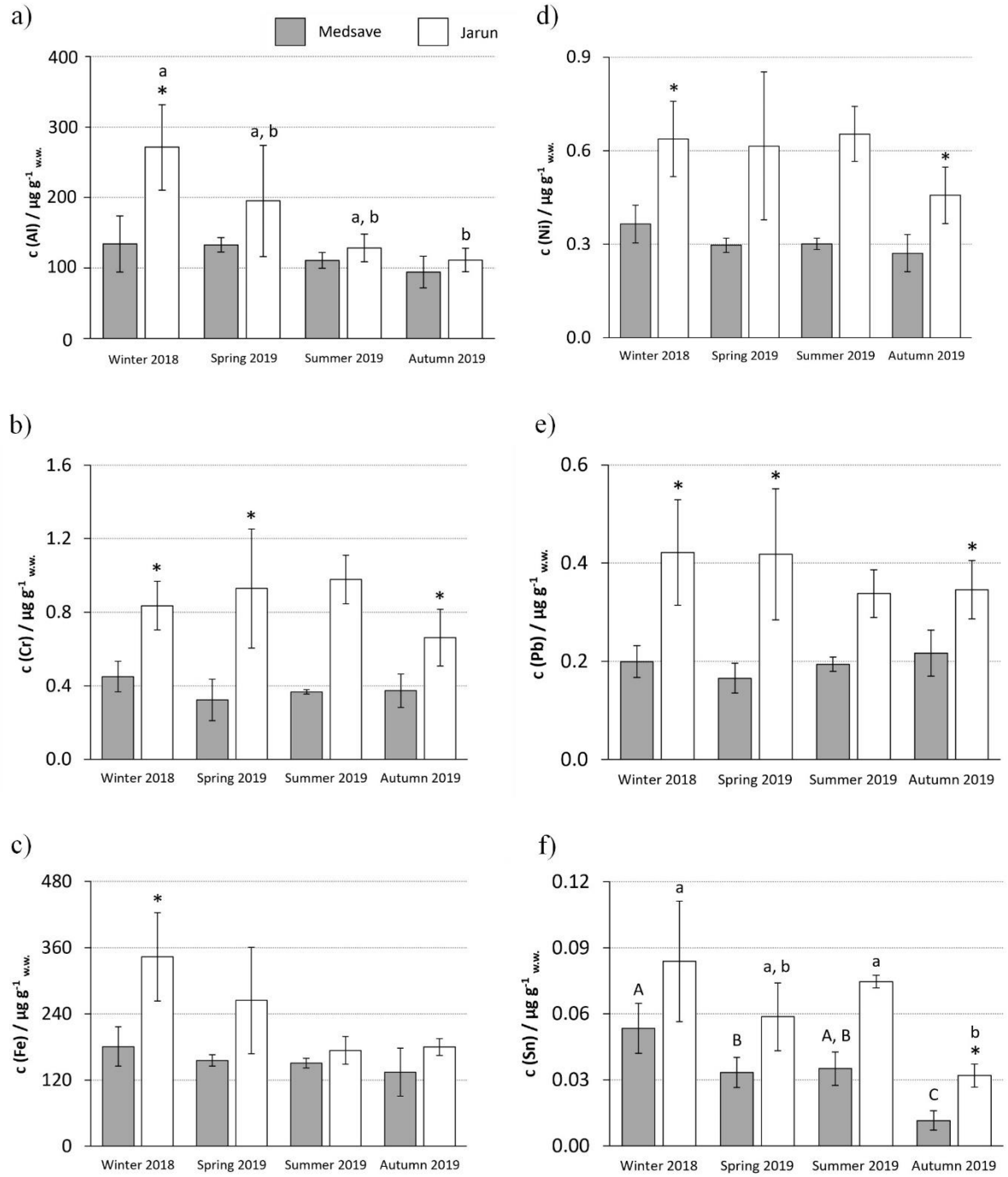

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1175 Figure 3

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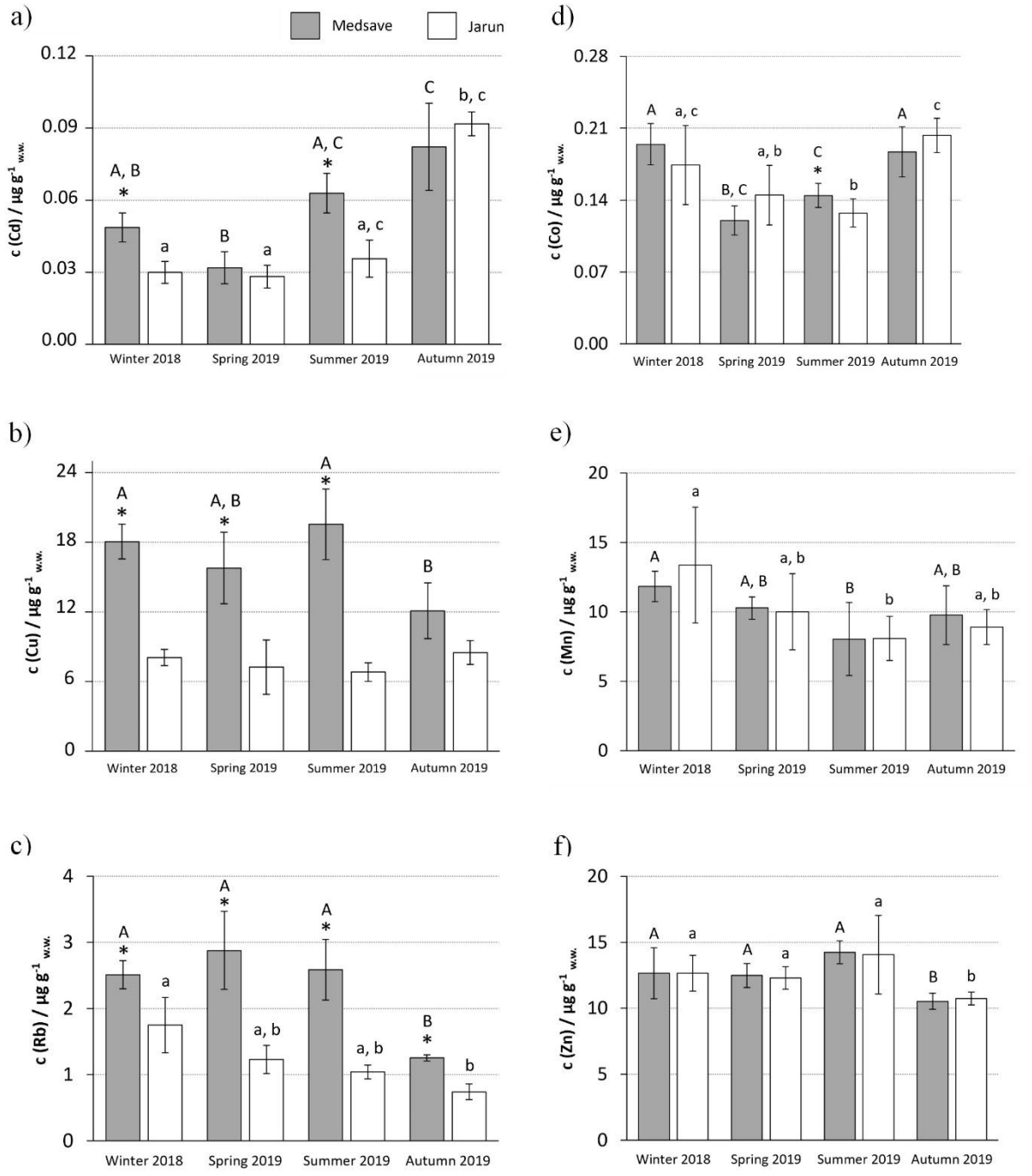

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Table 1. Physicochemical parameters in interstitial water of the Sava River and organic carbon concentrations from two sampling sites (Medsave and Jarun) in four sampling campaigns and threshold values for surface water and groundwater according to Croatian Directive on Water Quality Standards (GRC 2019). Values in bold indicate less than good water quality status of groundwater and of surface water (italic) according to classification in Croatian Directive on Water Quality Standards (GRC 2019).

	Medsave				Jarun			
	Winter 2018	Spring 2019	Summer 2019	Autumn 2019	Winter 2018	Spring 2019	Summer 2019	Autumn 2019
Water temperature (°C)	10.4	12.1	23.3	14.5	13.1	12.7	16.5	16.1
DO (mg O_2 L^{-1})	8.67	7.95	6.10	8.94	5.99	5.87	4.40	7.35
Saturation (% O_2)	78.4	79.0	65.0	88.0	42.0	56.6	44.0	74.3
pH	8.31	8.34	8.55	7.98	8.04	7.46	7.43	7.51
Conductivity (µS cm^{-1})	449	420	432	439	544	580	583	522
Alkalinity (mg $CaCO_3$ L^{-1})	160	158	55	190	190	275	185	145
Total water hardness (mg $CaCO_3$ L^{-1})	242	244	137	267	226	278	271	267
P-PO_4^{3-} (mg L^{-1})	0.06	0.10	0.04	0.10	0.08	***0.24***	*0.17*	*0.20*
N-NO_2^- (mg L^{-1})	0.14	0.06	0.09	0.16	0.11	0.18	0.07	0.15
N-NO_3^- (mg L^{-1})	0.46	*2.31*	1.48	*2.72*	0.70	*1.96*	0.24	*2.34*
COD_{KMnO4} (mg O_2 L^{-1})	1.9	3.8	5.1	4.5	1.8	5.3	2.6	3.5
DOC in water (mg L^{-1})	1.36	1.37	1.37	0.94	0.62	0.82	0.70	0.90
POC in water (mg L^{-1})	0.13	0.52	0.62	0.69	0.69	0.59	0.91	2.63
TOC in sediment (%)	1.07	2.68	2.97	2.83	2.32	1.77	1.92	1.73

N.E. – threshold values not established

Threshold values for good quality status of surface water (river type HR-R_5b): 0.1 (P-PO_4^{3-}); 1.8 (N-NO_3^-) and groundwater: 0.2 (P-PO_4^{3-}); 50 mg L^{-1} (N-NO_3^-)

Table 2. Dissolved trace and macro element concentrations (mean ± S.D.; < LOD values; n = 3) in the interstitial water from the hyporheic zone of the Sava River at two sampling sites (Medsave and Jarun) from four sampling campaigns. Statistically significant differences (Kruskal-Wallis one way ANOVA on ranks) at $p < 0.05$ level among four seasons at Medsave site are assigned with superscript letters A, B and C, D, and at Jarun site with a, b, c and d (the same letter in any of the combination denotes there is no significant difference); while significant differences (Mann-Whitney U test) between two sampling sites at each season are marked with asterisk (*).

	Medsave				Jarun			
	Winter 2018	Spring 2019	Summer 2019	Autumn 2019	Winter 2018	Spring 2019	Summer 2019	Autumn 2019
Trace elements								
Al (µg L^{-1})	2.11 ± 0.55*,A	1.96 ± 0.19A,B	3.94 ± 0.09*,C	3.46 ± 0.80*,B,C	0.38 ± 0.12a	1.79 ± 0.38b,c	1.69 ± 0.24c,d	1.73 ± 0.35b,d
Cd (µg L^{-1})	0.013 ± 0.001A	0.020 ± 0.001*,B	0.030 ± 0.003*,C	0.021 ± 0.001B	0.013 ± 0.003	0.017 ± 0.001	0.012 ± 0.001	0.023 ± 0.001*
Co (µg L^{-1})	0.054 ± 0.005A	0.086 ± 0.005B	0.192 ± 0.004*,C	0.097 ± 0.004D	0.056 ± 0.004a	0.095 ± 0.007b	0.108 ± 0.010b,c	0.091 ± 0.013b,d
Cr (µg L^{-1})	< 0.060 A	0.396 ± 0.026B	0.298 ± 0.037C	0.421 ± 0.030 B,D	< 0.060a	0.327 ± 0.071b, c	0.272 ± 0.041c	0.419 ± 0.051b, d
Cu (µg L^{-1})	0.696 ± 0.057A	0.648 ± 0.037A	1.46 ± 0.101*,B	0.838 ± 0.082C	0.606 ± 0.173a	1.17 ± 0.018*,b	0.476 ± 0.039a	1.21 ± 0.147*,b,c
Fe (µg L^{-1})	2.23 ± 0.15*,A	8.09 ± 0.30*,B	3.21 ± 0.45A,B	4.41 ± 0.85*,A,B	1.31 ± 0.38a	2.81 ± 0.38a,b	8.59 ± 0.30*,b	2.39 ± 0.86a,b
Mn (µg L^{-1})	1.80 ± 0.54	1.36 ± 0.13	2.36 ± 0.16	1.81 ± 0.05	4.28 ± 1.73*,a	8.56 ± 2.46*,b	37.3 ± 2.29c	3.07 ± 0.14a,b
Ni (µg L^{-1})	0.80 ± 0.08A	1.13 ± 0.04B	1.61 ± 0.11C	1.06 ± 0.17B,D	1.12 ± 0.04*,a	2.03 ± 0.14*,a,b	1.88 ± 0.10*,a,b	2.39 ± 0.32*,b
Pb (µg L^{-1})	0.024 ± 0.004	0.043 ± 0.004	0.043 ± 0.003	0.050 ± 0.006	0.046 ± 0.010*	0.050 ± 0.017	0.049 ± 0.013	0.039 ± 0.008
Rb (µg L^{-1})	0.910 ± 0.116	0.983 ± 0.008	1.25 ± 0.016*	0.923 ± 0.031*	1.02 ± 0.112a,b	1.17 ± 0.015*,a	1.05 ± 0.007a,b	0.816 ± 0.024b
Sn (µg L^{-1})	< 0.012	< 0.012	0.012 ± 0.003	0.184 ± 0.041	< 0.012	0.040 ± 0.012	0.029 ± 0.003	0.061 ± 0.010
Zn (µg L^{-1})	2.75 ± 0.66*,a	1.64 ± 0.16a,b	2.20 ± 0.26*,b	1.16 ± 0.12a	1.23 ± 0.32a	1.43 ± 0.19a,b	1.13 ± 0.16b	1.20 ± 0.20a,b
Macro elements								
Ca (mg L^{-1})	69.2 ± 9.77	60.6 ± 1.41	60.5 ± 1.43	69.7 ± 0.38	77.2 ± 2.05a	79.0 ± 1.03*,a	83.5 ± 1.15*,b	83.4 ± 0.29*,b,c
K (mg L^{-1})	1.59 ± 0.29	1.35 ± 0.17	1.81 ± 0.08	1.97 ± 0.02	2.47 ± 0.37*	2.30 ± 0.10*	2.37 ± 0.07*	2.48 ± 0.10*
Mg (mg L^{-1})	14.4 ± 1.38	14.0 ± 0.39	16.4 ± 0.38	16.8 ± 0.16	18.0 ± 0.91*,a	19.1 ± 0.37*,a,c	21.2 ± 0.45*,b	20.2 ± 0.18*,b,c
Na (mg L^{-1})	5.76 ± 0.36	7.62 ± 0.13	8.38 ± 0.20	8.68 ± 0.07	10.8 ± 1.40*,a	12.5 ± 0.09*,a,b	15.1 ± 0.21*,b	13.0 ± 0.18*,a,b

Annual average EQS (GRC, 2019): 0.25^{+} (Cd); 4 (Ni); 1.2 µg L^{-1} (Pb)
Groundwater threshold values: 5 (Cd); 10 µg L^{-1} (20^{++}) (Pb)
$^{+}$ EQS value for Cd for water hardness Class 5: ≥ 200 mg $CaCO_3$ L^{-1}
$^{++}$ threshold value for groundwater in the Zagreb area which contains elevated Pb concentrations of geological origin

Table 3. Element concentrations (mean ± S.D.) in the fine fraction of sediment (< 63 μm particles) from the hyporheic zone of the Sava River at two sampling sites (Medsave and Jarun) from four sampling campaigns (winter 2018, spring, summer and autumn 2019).

	Medsave				Jarun			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Al (g kg^{-1})	5.31 ± 0.03	8.30 ± 0.01	8.96 ± 0.04	9.27 ± 0.02	5.09 ± 0.04	8.79 ± 0.29	7.72 ± 0.03	7.66 ± 0.24
Cd (mg kg^{-1})	0.045 ± 0.002	0.063 ± 0.000	0.086 ± 0.001	0.088 ± 0.001	0.037 ± 0.002	0.051 ± 0.002	0.078 ± 0.001	0.070 ± 0.001
Co (mg kg^{-1})	1.06 ± 0.01	1.62 ± 0.06	1.77 ± 0.06	1.80 ± 0.03	1.04 ± 0.01	1.61 ± 0.00	1.52 ± 0.00	1.41 ± 0.03
Cr (mg kg^{-1})	16.2 ± 0.29	14.5 ± 0.28	13.6 ± 0.06	13.1 ± 0.03	19.9 ± 0.06	19.6 ± 0.15	19.0 ± 0.15	14.5 ± 0.41
Cu (mg kg^{-1})	5.11 ± 1.95	5.30 ± 0.11	4.24 ± 0.02	4.07 ± 0.05	4.58 ± 0.07	5.77 ± 0.01	5.42 ± 0.08	4.49 ± 0.16
Fe (g kg^{-1})	2.51 ± 0.05	3.56 ± 0.09	4.33 ± 0.04	4.55 ± 0.07	2.28 ± 0.00	3.52 ± 0.08	3.79 ± 0.04	3.79 ± 0.12
Mn (mg kg^{-1})	71.3 ± 0.62	148 ± 1.27	180 ± 1.15	183 ± 0.54	76.4 ± 0.12	114 ± 2.13	101 ± 0.13	75.3 ± 1.67
Ni (mg kg^{-1})	8.69 ± 0.13	8.14 ± 0.16	7.51 ± 0.08	7.37 ± 0.08	10.4 ± 0.12	11.2 ± 0.15	10.2 ± 0.26	7.50 ± 0.28
Pb (mg kg^{-1})	4.92 ± 0.01	4.04 ± 0.03	4.86 ± 0.08	5.11 ± 0.03	3.77 ± 0.03	4.90 ± 0.03	5.69 ± 0.06	5.28 ± 0.02
Rb (mg kg^{-1})	6.71 ± 0.01	11.8 ± 0.13	14.5 ± 0.06	15.4 ± 0.07	6.51 ± 0.03	12.2 ± 0.06	12.0 ± 0.06	11.9 ± 0.07
Sn (mg kg^{-1})	0.893 ± 0.147	0.905 ± 0.121	0.565 ± 0.020	0.589 ± 0.004	0.968 ± 0.144	0.956 ± 0.129	0.673 ± 0.003	0.631 ± 0.010
Zn (mg kg^{-1})	14.4 ± 0.96	18.6 ± 0.42	18.0 ± 0.03	18.3 ± 0.31	10.4 ± 0.04	16.8 ± 0.31	15.5 ± 0.12	15.8 ± 0.27
K (g kg^{-1})	1.43 ± 0.02	2.17 ± 0.02	2.09 ± 0.05	2.28 ± 0.08	1.37 ± 0.11	2.28 ± 0.11	1.82 ± 0.05	1.92 ± 0.07
Mg (g kg^{-1})	6.41 ± 0.14	3.34 ± 0.03	2.54 ± 0.02	2.42 ± 0.05	5.19 ± 0.11	3.61 ± 0.04	3.22 ± 0.02	3.44 ± 0.02
Na (g kg^{-1})	0.75 ± 0.01	0.64 ± 0.05	0.57 ± 0.03	0.44 ± 0.04	0.82 ± 0.09	0.74 ± 0.09	0.49 ± 0.03	0.42 ± 0.01

Table 4. Relations between environmental parameters in the HZ and metal concentrations in *S. ambulans*. Pearson correlation coefficients (r) are shown in first row and *p* values are below. Significant correlations are marked in bold while significant associations are in bold and italic.

Variables	Al	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Rb	Sn	Zn
Dissolved metals in IW	**0.998** **3×10^{-29}**	**0.579** **3×10^{-3}**	**0.998** **4×10^{-28}**	**0.999** **5×10^{-33}**	**-0.771** **1×10^{-5}**	**0.998** **2×10^{-27}**	**0.967** **2×10^{-14}**	**0.994** **1×10^{-22}**	**1.000** **3×10^{-35}**	**0.999** **4×10^{-30}**	**0.986** **2×10^{-18}**	**0.752** **2×10^{-5}**
Metals in fine fraction of sediment	0.221 3×10^{-1}	**0.566** **4×10^{-3}**	0.160 5×10^{-1}	0.160 5×10^{-1}	-0.025 9×10^{-1}	0.252 2×10^{-1}	0.181 4×10^{-1}	0.196 4×10^{-1}	0.128 6×10^{-1}	0.199 4×10^{-1}	0.217 3×10^{-1}	**0.565** **4×10^{-3}**
Na (in IW)	**-0.945** **4×10^{-12}**	-0.264 2×10^{-1}	**-0.951** **1×10^{-12}**	**-0.959** **1×10^{-13}**	**0.829** **6×10^{-7}**	**-0.944** **5×10^{-12}**	**-0.938** **1×10^{-11}**	**-0.951** **1×10^{-12}**	**-0.959** **1×10^{-13}**	**-0.927** **8×10^{-11}**	**-0.927** **8×10^{-11}**	**-0.566** **4×10^{-3}**
K (in IW)	**-0.616** **1×10^{-3}**	-0.171 4×10^{-1}	**-0.632** **9×10^{-4}**	**-0.763** **1×10^{-5}**	**0.687** **2×10^{-4}**	**-0.630** **1×10^{-3}**	**-0.631** **9×10^{-4}**	**-0.728** **5×10^{-5}**	**-0.681** **3×10^{-4}**	**-0.575** **3×10^{-3}**	**-0.741** **3×10^{-5}**	-0.157 5×10^{-1}
Ca (in IW)	**-0.963** **5×10^{-14}**	-0.203 3×10^{-1}	**-0.969** **7×10^{-15}**	**-0.984** **7×10^{-18}**	**0.679** **3×10^{-4}**	**-0.959** **1×10^{-13}**	**-0.961** **9×10^{-14}**	**-0.978** **2×10^{-16}**	**-0.974** **9×10^{-16}**	**-0.939** **1×10^{-11}**	**-0.934** **3×10^{-11}**	-0.358 9×10^{-2}
Mg (in IW)	**-0.973** **2×10^{-15}**	-0.291 2×10^{-1}	**-0.978** **2×10^{-16}**	**-0.975** **9×10^{-16}**	**0.848** **2×10^{-7}**	**-0.971** **4×10^{-15}**	**-0.962** **7×10^{-14}**	**-0.966** **2×10^{-14}**	**-0.984** **7×10^{-18}**	**-0.958** **2×10^{-13}**	**-0.931** **4×10^{-11}**	**-0.631** **1×10^{-3}**
Conductivity (IW)	0.519 2×10^{-1}	-0.311 5×10^{-1}	-0.032 9×10^{-1}	**0.980** **2×10^{-5}**	**-0.818** **1×10^{-2}**	0.613 1×10^{-1}	0.080 9×10^{-1}	**0.955** **2×10^{-4}**	**0.891** **3×10^{-3}**	-0.658 8×10^{-2}	**0.755** **3×10^{-2}**	0.140 7×10^{-1}
DO (mg O_2 L^{-1}) (in IW)	-0.393 3×10^{-1}	0.460 3×10^{-1}	0.542 2×10^{-1}	**-0.783** **2×10^{-2}**	0.477 2×10^{-1}	-0.412 3×10^{-1}	0.259 5×10^{-1}	**-0.785** **2×10^{-2}**	-0.592 1×10^{-1}	0.274 5×10^{-1}	**-0.785** **2×10^{-2}**	-0.699 5×10^{-2}
Saturation O_2 (in IW)	***-0.698*** ***5×10^{-2}***	0.591 1×10^{-1}	0.366 4×10^{-1}	**-0.830** **1×10^{-2}**	0.525 2×10^{-1}	***-0.704*** ***5×10^{-2}***	-0.158 7×10^{-1}	**-0.886** **3×10^{-3}**	**-0.708** **5×10^{-2}**	0.220 6×10^{-1}	**-0.952** **3×10^{-4}**	-0.612 1×10^{-1}
pH (in IW)	-0.126 8×10^{-1}	-0.053 9×10^{-1}	-0.082 8×10^{-1}	**-0.815** **1×10^{-2}**	**0.896** **3×10^{-3}**	-0.251 5×10^{-1}	0.221 6×10^{-1}	**-0.723** **4×10^{-2}**	**-0.762** **3×10^{-2}**	**0.891** **3×10^{-3}**	-0.295 5×10^{-1}	0.246 6×10^{-1}
DOC (in IW)	-0.524 2×10^{-1}	0.089 8×10^{-1}	-0.123 8×10^{-1}	**-0.841** **9×10^{-3}**	**0.944** **4×10^{-4}**	-0.607 1×10^{-1}	-0.136 7×10^{-1}	**-0.823** **1×10^{-2}**	**-0.885** **3×10^{-3}**	**0.812** **1×10^{-2}**	-0.503 2×10^{-1}	0.173 7×10^{-1}
TOC (in sediment)	-0.139 7×10^{-1}	0.070 9×10^{-1}	-0.352 4×10^{-1}	-0.426 3×10^{-1}	0.235 6×10^{-1}	-0.227 6×10^{-1}	-0.253 5×10^{-1}	-0.410 3×10^{-1}	-0.333 4×10^{-1}	0.246 6×10^{-1}	-0.382 4×10^{-1}	0.063 9×10^{-1}

IW – interstitial water

SUPPLEMENTARY MATERIALS

Metal bioaccumulation in stygophilous amphipod *Synurella ambulans* in the hyporheic zone: The influence of environmental factors

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The following are the supplementary data related to this article.

Multimedia component 1: Table S1

Wet weight of pooled samples of *Synurella ambulans* (mean ± S.D., $n = 7$) analysed per sampling site in each season

Multimedia component 2: Figure S1

Daily discharge (m^3/s) in the Sava River, measured at the Zagreb gauging station (45°47'11.2"N, 15°57'20.2"E, near the Jarun sampling site) during four sampling periods (period from 1st October 2018 to 31st October 2019 is shown). Data are provided from Meteorological and Hydrological Service of Croatia (DHMZ). Arrows point to the seasonal sampling campaigns.

Multimedia component 3: Figure S2

Concentrations (mean and standard deviations; mg^{-1} on wet weight basis) of four macro elements in *Synurella ambulans* sampled at two sites in the Sava River (Medsave – upstream from the wastewater outlet of the town of Zaprešić; Jarun – downstream from this wastewater outlet) once per season: a) Ca, b) K, c) Mg, d) Na. Differences between sites in the same season are indicated with asterisk (Mann-Whitney U test, $p < 0.05$) and differences among seasons with different letters (small letters in Jarun sampling site and capital letters in Medsave, Kruskal-Wallis one way ANOVA on ranks, $p < 0.05$).

Multimedia component 4: Supplementary tables with the results of multiple linear regression models

Table S1: Wet weight of pooled samples of *Synurella ambulans* (mean ± S.D., n = 7) analysed per sampling site in each season.

	Medsave (g)	Jarun (g)
Winter 2018	0.033 ± 0.004	0.042 ± 0.009
Spring 2019	0.060 ± 0.010	0.053 ± 0.010
Summer 2019	0.029 ± 0.005	0.025 ± 0.008
Autumn 2019	0.030 ± 0.004	0.035 ± 0.009

Figure S1

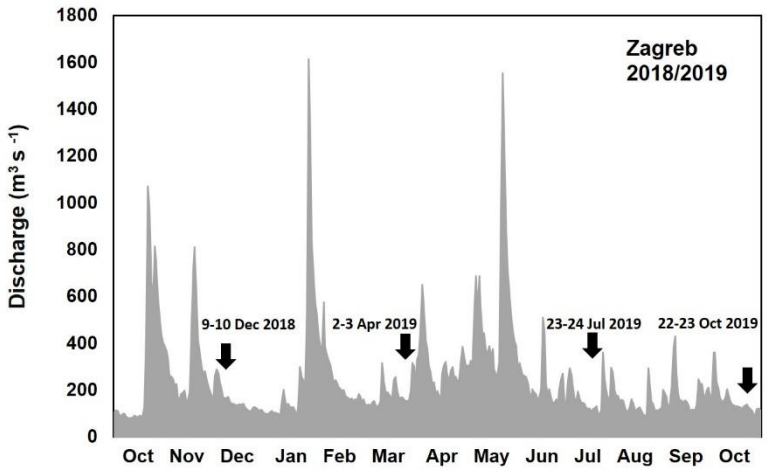
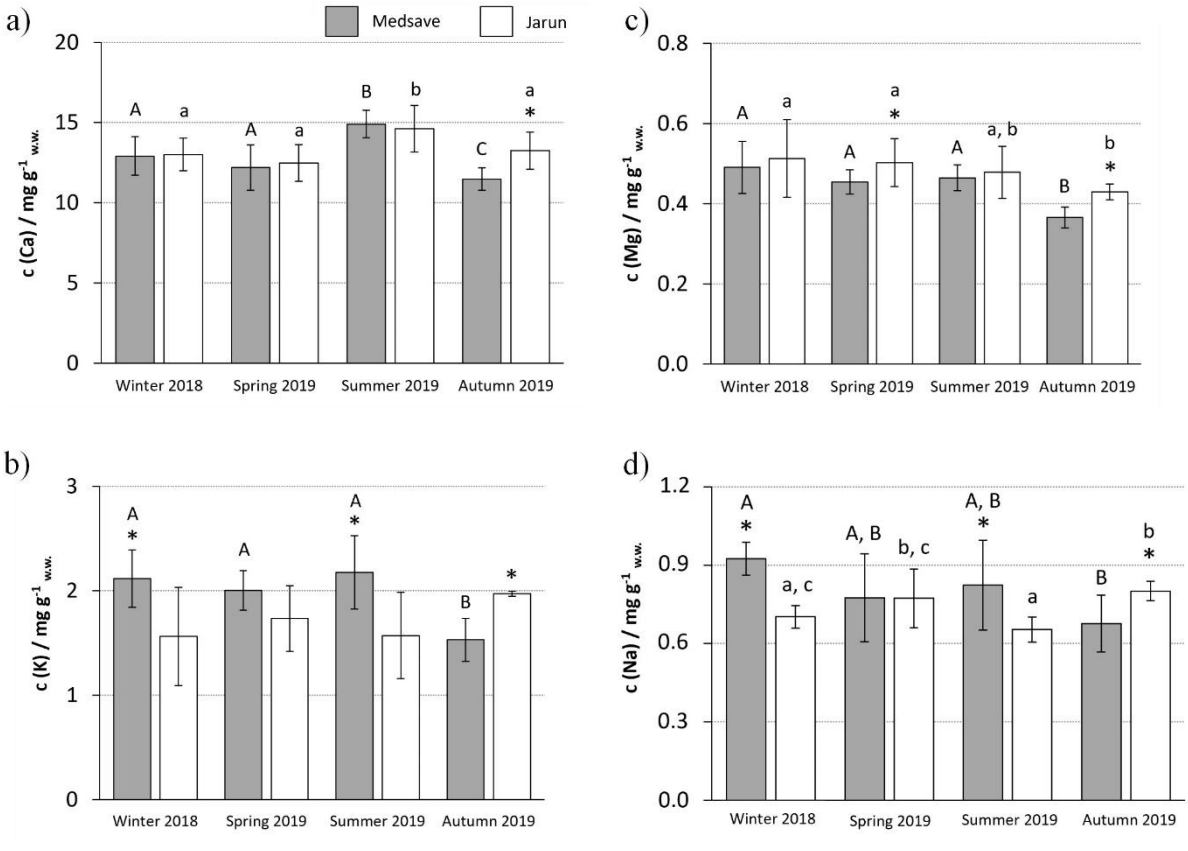
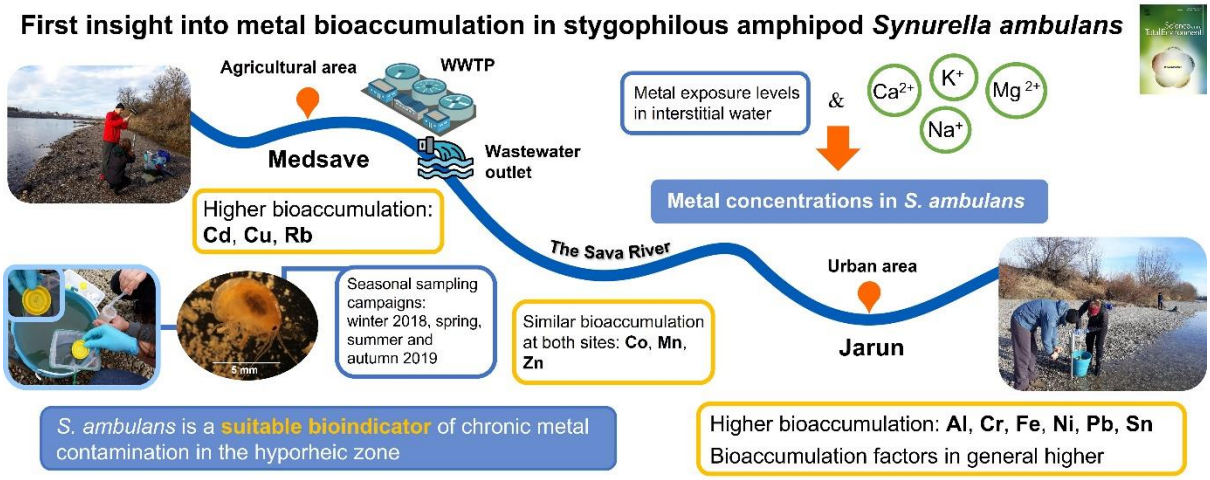


Figure S2



Graphical abstract