

Biološki odgovori u indikatorskim organizmima na izloženost otpadnim vodama u dinaridskim i panonskim rijekama

Mijošek, Tatjana

Doctoral thesis / Disertacija

2021

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Science / Sveučilište u Zagrebu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: <https://um.nsk.hr/um:nbn:hr:217:987611>

Rights / Prava: [In copyright](#)/[Zaštićeno autorskim pravom.](#)

Download date / Datum preuzimanja: **2024-08-06**



Repository / Repozitorij:

[Repository of the Faculty of Science - University of Zagreb](#)





Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET
BIOLOŠKI ODSJEK

Tatjana Mijošek

**BIOLOŠKI ODGOVORI U
INDIKATORSKIM ORGANIZMIMA NA
IZLOŽENOST OTPADNIM VODAMA U
DINARIDSKIM I PANONSKIM RIJEKAMA**

DOKTORSKI RAD

Zagreb, 2021.



University of Zagreb

FACULTY OF SCIENCE
DIVISION OF BIOLOGY

Tatjana Mijošek

**BIOLOGICAL RESPONSE IN
BIOINDICATOR ORGANISMS TO
WASTEWATERS EXPOSURE IN
DINARIC AND PANNONIAN RIVERS**

DOCTORAL THESIS

Zagreb, 2021

Ovaj je doktorski rad izrađen u Laboratoriju za biološke učinke metala Instituta Ruđer Bošković u Zagrebu pod vodstvom dr. sc. Vlatke Filipović Marijić, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Rad je financiran u okviru projekta Hrvatske zaklade za znanost „Akumulacija, unutarstanično mapiranje i učinci metala u tragovima u akvatičkih organizama” koji je vodila dr. sc. Marijana Erk.

INFORMACIJE O MENTORU

Dr. sc. Vlatka Filipović Marijić rođena je 3. studenoga 1975. godine u Sisku. Zaposlena je u Laboratoriju za biološke učinke metala Zavoda za istraživanje mora i okoliša na Institutu Ruđer Bošković u Zagrebu. Diplomirala je 2000. na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu te stekla zvanje dipl. inž. biologije (smjer ekologija), magistrirala iz područja oceanologije, a zatim doktorirala iz područja biologije. Trenutno radi kao viša znanstvena suradnica čije znanstveno istraživanje obuhvaća primjenu različitih bioindikatorskih organizama u procjeni kakvoće okoliša, korištenjem bioloških promjena kao pokazatelja izloženosti različitim zagađivačima. Voditeljica je niza znanstveno-istraživačkih, znanstveno-popularizacijskih i komercijalnih projekata u području ekotoksikologije, biomonitoringa i okolišne parazitologije, koji su rezultirali nizom publikacija, kongresnih sudjelovanja, predavanja te nagrada na domaćoj i međunarodnoj razini. Osim znanstvenog rada, aktivna je dugi niz godina u popularizaciji znanosti poput vođenja Otvorenih dana Instituta Ruđer Bošković, koordinatorica je znanstvenih radionica i predavanja na Institutu Ruđer Bošković, za što je primila već nekoliko godišnjih nagrada Instituta Ruđer Bošković te Državnu nagradu za znanost u području popularizacije znanosti u 2019. godini.

ZAHVALE

Hvala mojoj mentorici dr. sc. Vlatki Filipović Marijić na povjerenju i pruženoj prilici da izradim ovu doktorsku disertaciju u Laboratoriju za biološke učinke metala. Zahvaljujem na mentorstvu, pomoći, usmjeravanju i savjetima tijekom planiranja i provedbe istraživanja te pisanja disertacije i prethodnih znanstvenih radova te na uključivanju na različite projekte.

Zahvaljujem i ostalim bivšim i sadašnjim članovima LBUM-a na susretljivosti, podršci, pomoći u laboratorijskom radu, analizi podataka, korekcijama radova i ugodnom društvu.

Nesrete, uz korak stjecanja doktorata, neizmjereno mi je drago što sam ovdje našla i osobu na koju mogu računati u bilo kojem trenu i koja od samih početaka nije bila samo kolegica, već prijateljica. Učinila si i loše dane boljima i lakšima i neizmjereno ti hvala. ☺

Zahvalu dugujem i kolegama iz drugih laboratorija Instituta Ruđer Bošković (Laboratorij za anorgansku geokemiju okoliša i kemodinamiku nanočestica, Laboratorij za molekularnu ekotoksikologiju, Laboratorij za akvakulturu i patologiju akvatičkih organizama) na pristupačnosti, ustupanju uređaja za mjerenja i pomoći u uzorkovanju. Hvala i dr. sc. Andreasu Ziteku i dr. sc. Thomasu Prohaski na pomoći u analizi kalcificiranih struktura riba.

Hvala svim članicama komisije na konstruktivnim i korisnim ispravcima i prijedlozima.

Hvala prof. dr. sc. Ivani Maguire i izv. prof. dr. sc. Jasni Lajtner na entuzijazmu, podršci, pristupačnosti i lijepim uspomnama iz ranijih faza studiranja zbog kojih sam i poželjela ostati u znanosti.

Također, hvala ekipi „Ručalica“, Mileni i Tomislavu, kao i ostalim prijateljima, koji su bili podrška, ugodno društvo i optimisti i kad ja nisam bila. Izrada ovog rada bila je lakša uz vas!

Na kraju najvažnije, najveće fala cijeloj mojoj obitelji koja je vjerovala u mene, motivirala me i gurala i kad bih ja potonula. Iako znam da i dalje niste pročitali sve moje radove, fala na podršci, brizi, slušanju žalopojki, savjetima, ohrabivanju, maminom putu u Francusku (♥) i plaćanju hotela, svim prijevozima, rješavanju problema i kaj ste me svi uvijek (ne)uspješno nastojali održati samo u dobrom i veselom raspoloženju. Mamita i Tatek, uskoro valjda imate i dva dr. sc. ☺ Dr. Dadach, stiže ti i dr. Tanek! <3 ♥ <3

Borna, hvala kaj si dijelio velik dio ovog puta sa mnom, znam da to i nije uvijek bilo najlakše, ali podjela sreće u dobrim danima, a utjeha, zagrljaj, rame za plakanje i potpora u lošim danima puno su značili. ♥

BIOLOŠKI ODGOVORI U INDIKATORSKIM ORGANIZMIMA NA IZLOŽENOST OTPADNIM VODAMA U DINARIDSKIM I PANONSKIM RIJEKAMA

TATJANA MIJOŠEK

Institut Ruđer Bošković, Bijenička 54, 10 000 Zagreb, Hrvatska

Ciljevi ove doktorske disertacije obuhvatili su posljedice utjecaja otpadnih voda i povišenih razina metala na vodene organizme dinaridske rijeke Krke i panonske rijeke Ilove, s naglaskom na bioakumulaciju i potencijalnu toksičnost metala. Koncentracije većeg broja metala u probavilu riba, rakušcima i kukašima te multibiomarkerski pristup ukazali su na povećanu bioakumulaciju metala te povišene razine oksidacijskog stresa ili indukcije metalotioneina u bioindikatorskim organizmima na onečišćenim u odnosu na referentne postaje, ali i na djelotvoran antioksidacijski sustav na svim lokacijama pokazujući da nije došlo do trajnog oštećenja. Praćenje razina metala u ljuskama i otolitima omogućilo je procjenu dugoročne izloženosti i potvrdilo slične trendove kao i biološki odgovori u mekim tkivima riba. Probavilo i tvrde strukture riba, rakušci i kukaši su se pokazali kao korisni bioindikator u procjeni stanja okoliša, a cjelokupno istraživanje je ukazalo na potrebu kontinuiranog monitoringa i zaštite, osobito bitnog zbog blizine Nacionalnog parka Krka te Parka prirode Lonjsko polje.

(251 stranica, 28 slika, 7 tablica, 430 literaturnih navoda, jezik izvornika hrvatski)

Ključne riječi: onečišćenje metalima, citosol, potočna pastrva, babuška, rakušci, kukaši, probavilo, biomarkeri

Mentor: dr. sc. Vlatka Filipović Marijić, viša znanstvena suradnica

Ocjenjivači: prof. dr. sc. Sanja Gottstein

dr. sc. Zrinka Dragun, viša znanstvena suradnica

dr. sc. Irena Vardić Smrzlić, znanstvena suradnica

Rad je prihvaćen:

University of Zagreb
Faculty of Science
Division of Biology

Doctoral thesis

**BIOLOGICAL RESPONSE IN BIOINDICATOR ORGANISMS TO WASTEWATERS
EXPOSURE IN DINARIC AND PANNONIAN RIVERS**

TATJANA MIJOŠEK

Ruđer Bošković Institute, Bijenička 54, 10 000 Zagreb, Croatia

The objectives of this thesis included the impact of wastewater discharges and elevated metal levels on aquatic organisms of Dinaric Krka and Pannonian Ilova rivers, with an emphasis on bioaccumulation and potential toxicity. Concentrations of many metals in the fish intestine, gammarids and acanthocephalans, as well as multibiomarker approach indicated enhanced metal bioaccumulation and elevated levels of oxidative stress or metallothionein induction in bioindicators at contaminated compared to the reference sites, but also to effective antioxidant system in all locations without permanent damage. Monitoring of metal levels in scales and otoliths enabled the assessment of long-term exposure and confirmed similar trends as biological responses in fish soft tissues. Research results confirmed fish intestine, hard structures, gammarids and acanthocephalans as useful bioindicators in the environmental assessment and the importance of continuous monitoring and protection, especially due to proximity of the Krka National Park and Lonjsko Polje Nature Park.

(251 pages, 28 figures, 7 tables, 430 references, original in Croatian)

Keywords: metal contamination, cytosol, brown trout, Prussian carp, gammarids, acanthocephalans, intestine, biomarkers

Supervisor: Vlatka Filipović Marijić, PhD, Senior Research Associate

Reviewers: Sanja Gottstein, PhD, Full Professor

Zrinka Dragun, PhD, Senior Research Associate

Irena Vardić Smrzlić, PhD, Research Associate

Thesis accepted:

SADRŽAJ

1. UVOD	1
1.1. Ciljevi i svrha rada.....	4
1.2. Hipoteze istraživanja	5
2. LITERATURNI PREGLED	6
2.1. Otpadne vode.....	6
2.2. Metali u slatkovodnim ekosustavima	7
2.2.1. Dinaridske i panonske rijeke.....	8
2.2.1.1. Rijeka Krka	9
2.2.1.2. Rijeka Ilova	11
2.3. Metali u vodenim organizmima.....	14
2.3.1. Unos u organizme i međustanični prijenos	14
2.3.2. Esencijalni i neesencijalni metali.....	16
2.3.3. Toksični učinci metala i mehanizmi detoksikacije	17
2.4. Bioindikatorski organizmi	19
2.4.1. Ribe kao bioindikatorski organizmi.....	21
2.4.1.1. Potočna pastrva (<i>Salmo trutta</i> Linnaeus, 1758) - rijeka Krka.....	22
2.4.1.2. Babuška (<i>Carassius gibelio</i> Bloch, 1782) - rijeka Ilova	24
2.4.1.3. Probavilo kao bioindikatorski organ	25
2.4.1.4. Kalcificirane („tvrde“) strukture (otoliti i ljuske) kao indikatorska tkiva	26
2.4.2. Rakušci kao bioindikatorski organizmi.....	29
2.4.2.1. <i>Gammarus balcanicus</i> Schäferna, 1922 i <i>Echinogammarus acarinatus</i> Karaman, 1931 - rijeka Krka.....	29
2.4.2.2. <i>Gammarus fossarum</i> Koch, 1936 i <i>Gammarus roeselii</i> Gervais, 1835 - rijeka Ilova.....	30
2.4.3. Kukaši	31
2.4.3.1. Tipologija, rasprostranjenost i sistematika kukaša.....	31
2.4.3.2. Vanjska i unutrašnja građa kukaša	32
2.4.3.3. Životni ciklus kukaša	33
2.4.3.4. Kukaši kao bioindikatorski organizmi	35
2.4.3.5. <i>Dentitruncus truttae</i> Sinzar, 1955	36
2.5. Biomonitoring i biomarkeri	37
2.5.1. Biomonitoring	37

2.5.2. Biomarkeri	38
2.5.2.1. Metalotioninini (MT) - biomarkeri izloženosti metalima.....	39
2.5.2.2. Biomarkeri oksidacijskog stresa i antioksidacijskog kapaciteta	41
2.5.2.2.1. Malondialdehid (MDA) – biomarker oksidacijskog stresa	42
2.5.2.2.2. Katalaza (CAT) i ukupni glutation (GSH) – biomarkeri antioksidacijskog kapaciteta	44
2.5.2.3. Acetilkinesteraza (AChE) - biomarker izloženosti organskim zagađivalima i metalima	46
2.5.2.4. Ukupni citosolski proteini (TP) - biomarkeri općeg stresa	47
2.6. Analitičke metode.....	48
2.6.1. Analitičke metode za određivanje koncentracija metala u uzorcima iz okoliša	48
2.6.1.1. Masena spektrometrija s induktivno spregnutom plazmom (ICP-MS).....	48
2.6.1.2. Spektrometrija masa s induktivno spregnutom plazmom i laserskom ablacijom (LA ICP-MS).....	49
2.6.2. Analitičke metode za razdvajanje biomolekula na koje se vežu metali.....	50
2.6.2.1. Tekućinska kromatografija visoke djelotvornosti s isključenjem po veličini (SEC-HPLC)	51
2.6.3. Spektrofotometrijske metode za određivanje koncentracija i/ili aktivnosti biomarkera	52
2.7. Dosadašnja istraživanja u rijeci Krki i Ilovi	53
3. ZNANSTVENI RADOVI	55
4. RASPRAVA	90
4.1. Utjecaj razine onečišćenja, reproduktivnog ciklusa (sezona) na morfometrijske i biološke pokazatelje pastrve i babuške.....	91
4.2. Koncentracije metala/metaloida/nemetala u probavilima potočne pastrve i babuške ...	93
4.2.1. Ukupne koncentracije metala u probavilima potočne pastrve i babuške	93
4.2.2. Citosolske koncentracije metala u probavilima potočne pastrve i babuške.....	99
4.2.3. Raspodjela metala/metaloida/nemetala između topljive i netopljive frakcije u probavilima potočne pastrve i babuške	101
4.3. Raspodjela Cd, Co, Cu, Fe, Mo, Se i Zn među citosolskim biomolekulama različitih molekulskih masa u probavilima potočne pastrve iz rijeke Krke i babuške iz rijeke Ilove	105
4.4. Rakušci kao bioindikatorski organizmi	112
4.4.1. Biološki pokazatelji u rakušaca.....	112
4.4.2. Citosolske koncentracije metala u rakušcima	114
4.5. Usporedba kukaša, rakušaca i probavila potočne pastrve iz rijeke Krke kao bioindikatora izloženosti metalima.....	116

4.5.1. Biološka i epidemiološka obilježja kukaša	117
4.5.2. Akumulacija metala u kukašima, rakušcima i probavilu potočne pastrve	117
4.6. Usporedba akumulacije metala u mekim i tvrdim tkivima riba te u kukašima u proljeće 2015. godine	122
4.7. Biomarkerski odgovori u probavilu riba i rakušcima u procjeni stanja okoliša	125
4.7.1. Biomarkeri u probavilu riba	127
4.7.2. Biomarkeri u rakušaca	132
5. ZAKLJUČCI	134
6. POPIS LITERATURE	138
7. PRILOZI	181
8. ŽIVOTOPIS	248

**U OVOJ DOKTORSKOJ DISERTACIJI OBJEDINJENI SU SLJEDEĆI
OBJAVLJENI ZNANSTVENI RADOVI:**

1. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Erk, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matoničkin Kepčija R, 2019*a*. 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.
2. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Krasnići, N., Ivanković, D., Erk, M., 2019*b*. 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.
3. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Erk, M., 2021. Intestine of invasive fish Prussian carp as a target organ in metal exposure assessment of the wastewater impacted freshwater ecosystem. *Ecological Indicators* 122, 107247.

1. UVOD

Povećano ispuštanje organskih i anorganskih zagađivala kao posljedica ubrzanog tehnološkog razvoja, industrije, poljoprivrede, rudarstva, prometa te komunalnih i industrijskih otpadnih voda predstavlja jedan od vodećih okolišnih problema današnjice, osobito u vodenim ekosustavima. Metali/metaloidi su među značajnijim kemijskim zagađivalima, a u prirodne vodotoke dospijevaju uglavnom putem otpadnih voda koje se često bez prikladnog pročišćavanja unose u vodene ekosustave. Za razliku od organskih zagađivala, metali nisu biorazgradivi te unosom u vodotoke samo mijenjaju svoj kemijski oblik čime postaju manje ili više bioraspoloživi, što utječe na njihovu toksičnost, ali jednom uneseni više se ne mogu ukloniti iz biogeokemijskog ciklusa (Sadiq, 1992). Posljedično, stalna izmjena metala u vodenom stupcu između partikularne i otopljene faze, kao i njihovo taloženje u sedimentima, mogu dovesti do povećane i štetne bioakumulacije metala u različitim vodenim organizmima unosom putem hrane, filtracijom vode, i/ili apsorpcijom kroz kožu (Kraemer i sur., 2006). Naime, s biološkog gledišta, razlikujemo esencijalne metale (npr. Cu, Fe, Mo, Zn), koji u organizmu imaju bitnu fiziološku ulogu kao strukturni dijelovi hormona, enzima i drugih složenih proteina, tzv. metaloproteina, uključenih u hidrolizu kemijskih veza, prijenos elektrona i kisika te redoks procese (Holm i sur., 1996) te neesencijalne metale (npr. Cd, Hg i Pb), koji nemaju poznatu biološku ulogu u organizmima. Iako i esencijalni metali mogu postati toksični pri visokim koncentracijama, toksičnost neesencijalnih metala već pri vrlo niskim koncentracijama uglavnom proizlazi iz njihove sličnosti s esencijalnim metalima, zbog čega mogu zamijeniti esencijalne elemente te istim putovima ući u stanice i vezati se za važne biomolekule, čime remete njihovu strukturu ili aktivnost (Wood i sur., 2012).

Potencijalna toksičnost esencijalnih i neesencijalnih metala za organizme uslijed prelaska optimalnih koncentracija, u monitoring programima uz standardnu proceduru mjerenja koncentracija metala u vodi i sedimentima iziskuje provođenje mjerenja i u bioindikatorskim organizmima čiji biološki odgovori daju pouzdaniju i dugoročniju procjenu utjecaja onečišćenja na cjelokupni vodeni okoliš (Kraemer i sur., 2006; Ovaskainen i sur., 2019). Među najčešće korištene bioindikatorske organizme u vodenom okolišu ubrajaju se ribe, školjkaši i rakovi. U većini dostupnih istraživanja najčešće korišteni indikatorski organi riba su jetra, kao metabolički najaktivnije tkivo i mjesto detoksikacije metala, te škrge koje predstavljaju mjesto unosa metala putem vode (Kraemer i sur., 2006; Dragun i sur., 2018a; Krasnići i sur., 2018). Međutim, u pojedinim slučajevima dokazana je ista ili čak veća važnost unosa metala putem prehrane, odnosno probavnim putem, nego unosa metala vodom

(Clearwater, 2000). Unatoč tome, probavilo riba je izuzetno rijetko korišteno kao bioindikatorsko tkivo, osobito u okolišnim istraživanjima (Filipović Marijić i Raspor, 2010, 2012; Filipović Marijić i sur., 2013) te dosadašnja istraživanja probavila uglavnom daju informaciju samo o ukupnim koncentracijama metala u laboratorijskim uvjetima.

Također, u proteklih 20 godina raste interes za otkrivanje veze između onečišćenja i parazitizma u vodenim ekosustavima, kao i za moguću primjenu nametnika kao bioindikatora kakvoće vode, što je dovelo i do stvaranja novog područja nazvanog “okolišna parazitologija” (Sures, 2001; Sures i sur., 2017), unutar kojeg se proučavaju i kukaši. Kukaši su crijevni nametnici u mnogih kralješnjaka, pa tako i riba, koji nemaju vlastiti probavni sustav i oslanjaju se na apsorpciju esencijalnih hranjivih tvari, uključujući i esencijalne metale, kroz površinski integument (Kennedy, 1985). Pokazali su se kao organizmi koji imaju iznimnu sposobnost akumulacije metala, osobito neesencijalnih, čime potencijalno i štite svoje domadare (Sures, 2004; Filipović Marijić i sur., 2013). Sam mehanizam zaštite domadara, kao i učinkovite akumulacije metala u kukašima, još nije poznat. Iako nametnici općenito mogu imati različite mehanizme kontrole svojih domadara poput smanjivanja stope rasta ili fekunditeta, promjena ponašanja ili povećanja stope smrtnosti, utjecati na fiziologiju organizama, osobito kada su domadari beskralješnjaci (Bojko i sur., 2020), kukaši u svojim krajnjim domadarima ponekad uzrokuju lokalna oštećenja probavnog tkiva koja se manifestiraju u promjenama tkiva i imunološkog odgovora riba (Dezfuli i sur., 2002, 2008; Barišić i sur., 2018), no najčešće ne uzrokuju značajne štete niti smrtne ishode.

Uz meka tkiva riba, koncentracije metala moguće je odrediti i u njihovim tvrdim tkivima, primjerice ljuskama, otolitima, kostima ili očnoj leći. Ipak, takva istraživanja najčešće se provode s ciljem određivanja migracija riba ili veličine i sastava populacija nekog područja, a samo u manjem opsegu su se koristila u procjeni utjecaja onečišćenja na okoliš i organizme (Ranaldi i Gagnon, 2008). Prednost tvrdih tkiva u odnosu na meka je što razine metala u njima daju podatke o dugotrajnoj akumulaciji i izloženosti organizma na koju utječu uglavnom samo okolišni uvjeti, dok na razinu metala u mekim tkivima uz okolišne uvjete utječu i fiziologija organizma, mehanizmi eliminacije, metabolička transformacija i preraspodjela u tkivima te regeneracija tkiva (Campana i sur., 2000; Ranaldi i Gagnon, 2008). Mjerenja metala u kalcificiranim strukturama riba su puno rjeđa nego u mekim tkivima (Campana i sur., 2000; Kalantzi i sur., 2019), dok za ribe slatkovodnih ekosustava Republike Hrvatske takva mjerenja ranije uopće nisu provedena.

Kako bi se procijenila sama toksičnost metala, nije dovoljno izmjeriti ukupne koncentracije u tijelu ili odabranom tkivu organizma jer je samo dio akumuliranih metala

metabolički dostupan, a time i potencijalno toksičan za organizme (Wallace i sur., 2003). Naime, metali se prilikom unosa u organizme raspodjeljuju po njihovim organima, stanicama i različitim unutarstaničnim odjeljcima (npr. citosol, granule, organeli i stanične membrane), pri čemu se toksičnost metala uglavnom odnosi na organele i citosol kao metabolički dostupnu frakciju metala (Wallace i sur., 2003) te je stoga nužno izmjeriti razine metala i u citosolskim frakcijama. Slična istraživanja već su provedena korištenjem različitih tkiva slatkovodnih i morskih vrsta riba poput *Squalius cephalus*, *Liza aurata*, *Mullus barbatus*, *Mullus surmuletus* (Dragun i sur., 2007; Filipović Marijić i Raspor, 2003, 2007). U citosolu metali mogu biti vezani na osjetljive biološki važne molekule, ali dio može biti vezan i na molekule uključene u njihovu detoksikaciju poput glutationa (GSH) i metalotioneina (MT). Kako bi se bolje razumio način vezanja metala u pojedinim stanicama, kao i ekotoksikološki značaj, razvijeno je područje nazvano metalomika s ciljem detaljnijeg opisa procesa u kojima pojedini metali sudjeluju, bilo kao funkcionalni sastavni dijelovi ili kao uzročnici toksičnih učinaka (Szpunar, 2005). Tekućinska kromatografija visoke djelotvornosti s razdvajanjem po veličini (SEC-HPLC) tehnika je koja se često primjenjuje u metalomičkim istraživanjima, a omogućava razdvajanje proteina na frakcije na osnovu njihovih molekulskih masa te se uz primjenu spektrometrije masa visoke razlučivosti s induktivno spregnutom plazmom (HR ICP-MS) provodi mjerenje metala u dobivenim frakcijama, s ciljem detektiranja skupina biomolekula koje vežu metale (Montes-Bayon i sur., 2003; Szpunar, 2005). Takav pristup već je korišten za određivanje citosolske raspodjele metala u različitim tkivima vodenih organizama uključujući školjkaše (Strižak i sur., 2014; Lavradas i sur., 2016), i nekoliko vrsta riba (Goenaga i sur., 2003; Krasnići i sur., 2013, 2014, 2018, 2019; Dragun i sur., 2018b, 2020; Urien i sur., 2018). Međutim, u znanstvenoj literaturi ne postoje podaci o citosolskoj raspodjeli metala među biomolekulama probavila niti jedne vrste riba. Navedena kombinirana primjena kromatografske tehnike i HR ICP MS-a služi kao alat za razlučivanje raspodjele metala među citosolskim biomolekulama različitih veličina i pruža osnovu za točnu identifikaciju molekula određene mase te u konačnici za razvoj potencijalnih novih biomarkera izloženosti metalima i toksičnih učinaka metala.

Vrijedan doprinos u procjeni štetnih učinaka metala i drugih zagađivala daju i analize biomarkera, odnosno promjena u staničnim strukturama i funkcijama koje se javljaju kao posljedica izloženosti zagađivalima, koje služe kao mjerljivi i rani pokazatelji antropogenog učinka na organizme (Huggett i sur., 1992). U okolišnim uvjetima izloženosti različitim skupinama zagađivala nužna je primjena multibiomarkerskog pristupa koji omogućava jasniju procjenu bioloških odgovora organizama na zagađenje te može ukazivati na određenu skupinu

zagađivala (Monseratt i sur., 2007). Analiza seta biomarkera općeg i oksidacijskog stresa, antioksidacijskog kapaciteta te izloženosti i učinaka metala povezana s kemijskim analizama u organizmima, vodi i sedimentima, daje najpouzdaniju procjenu o nepovoljnim učincima onečišćenja metalima i drugim zagađivalima na ekosustav i vodene organizme.

S obzirom na navedeno, bitno je razumjeti moguće posljedice ispuštanja otpadnih voda te povišenih razina metala u vodenom okolišu, s posebnim osvrtom na njihovu potencijalnu toksičnost i štetne učinke na organizme. Za postizanje napretka u ovome području pokazala se potreba za primjenom sveobuhvatnog i interdisciplinarnog pristupa kojim će se istražiti biološki odgovori različitih organizama iz prirodnih populacija na utjecaj otpadnih voda te pronaći poveznice s izvorom onečišćenja i utjecajem antropogenih i prirodnih čimbenika.

1.1. Ciljevi i svrha rada

Osnovni cilj ovog istraživanja bio je doprinos boljem razumijevanju učinaka i toksičnosti metala/metaloida nakon njihove bioakumulacije u odabranim bioindikatorskim organizmima, kukašima (*Dentitruncus truttae* Sinzar, 1955), rakušcima (*Gammarus balcanicus* Schäferna, 1922, *Echinogammarus acarinatus* (Karaman, 1931), *Gammarus fossarum* Koch, 1936 i *Gammarus roeselii* Gervais, 1835) te probavilu riba (potočna pastrva; *Salmo trutta* Linnaeus, 1758 i babuška; *Carassius gibelio* Bloch, 1782) iz dinaridske rijeke Krke i panonske rijeke Ilove u području pod utjecajem komunalnih i industrijskih otpadnih voda i drugih antropogenih aktivnosti te pri različitim razinama izloženosti tih organizama metalima. Sukladno navedenom, specifični ciljevi ovog istraživanja bili su:

- procjena akumulacije metala/metaloida u ribama, rakušcima i kukašima;
- određivanje metabolički raspoložive, a time i potencijalno toksične frakcije metala, u citosolu probavila riba;
- procjena dugoročne izloženosti metalima mjerenjem koncentracija metala u kalcificiranim strukturama riba;
- određivanje bioloških odgovora bioindikatorskih organizama na prisutno onečišćenje u odabranim ekosustavima;
- određivanje raspodjele sedam odabranih elemenata u tragovima među citosolskim biomolekulama različitih molekulskih masa u probavilima potočne pastrve i babuške;
- određivanje prostornih i sezonskih razlika u akumulaciji metala i biološkim odgovorima organizama s obzirom na gradijent onečišćenja i fiziološka svojstva organizama u različitim sezonama;

- ispitivanje mogućnosti primjene probavila riba kao bioindikatorskog tkiva u procjeni unosa metala prehranom, kao i kukaša kao bioindikatorskih organizama u monitoring istraživanjima

Navedeni ciljevi ostvareni su kroz sljedeće aktivnosti:

- mjerenje ukupnih i/ili citosolskih koncentracija metala u probavilu riba, rakušcima i kukašima s referentnih i onečišćenih postaja u dvije sezone (jesen i proljeće) primjenom HR ICP-MS tehnike te određivanje koncentracija odabranih elemenata u ljuskama i otolitima potočne pastrve primjenom laserske ablacije i HR ICP-MS-a;
- primjenu multibiomarkerskog pristupa, korištenjem probavila riba kao bioindikatorskog organa, koji je uključivao biomarkere izloženosti metalima (metalotioneini; MT), oksidacijskog stresa (malondialdehid; MDA), antioksidacijskog kapaciteta (katalaza; CAT, i glutation; GSH) te biomarkere općeg stresa (ukupni protein; TP);
- definiranje raspodjela Cd, Co, Cu, Fe, Mo, Se i Zn među biomolekulama različitih molekulskih masa u probavilu riba ovisno o sezoni i razini izloženosti metalima primjenom SEC-HPLC-a i HR ICP-MS-a;
- izračunavanje biokoncentracijskih faktora (BCF) koji daju informaciju o akumulacijskom potencijalu kukaša i ukazuju na trajanje izloženosti metalima

1.2. Hipoteze istraživanja

U skladu s ciljevima istraživanja definirane su i testirane sljedeće hipoteze:

H1. otpadne vode mijenjaju i narušavaju kakvoću vodenog okoliša u koji se ispuštaju;

H2. razlike u akumulaciji metala i razini biomarkera u bioindikatorskim organizmima javljaju se kao posljedica različitog antropogenog utjecaja i/ili sezonalnosti;

H3. istovremena upotreba mekih i kalcificiranih tkiva riba, kao i kukaša omogućava praćenje dugoročne i kratkoročne izloženosti pojedinim metalima u vodenom okolišu;

H4. raspodjela pojedinih metala među citosolskim biomolekulama ovisi o razinama bioakumuliranih metala, kao i o vrsti organizma i ciljnog organa;

H5. kukaši, na temelju učinkovite akumulacije metala, i probavno tkivo riba, kao mjesto unosa metala putem hrane, predstavljaju značajne i pouzdane bioindikatore izloženosti metalima.

2. LITERATURNI PREGLED

2.1. Otpadne vode

U današnje vrijeme ubrzane urbanizacije i industrijalizacije sve je veća potrošnja vode, te se upotrijebljena voda opterećena mnogobrojnim i organskim i anorganskim tvarima, ispušta u prirodne vodotoke, narušavajući kakvoću i biološku ravnotežu vodenih ekosustava.

Otpadne vode su sve vode koje su svojom namjenom promijenile prvobitni sastav, odnosno svoje fizikalne, i/ili kemijske i/ili biološke značajke. Mogu se podijeliti na: oborinske, komunalne, poljoprivredne i industrijske. Oborinske vode u svojem ciklusu kruženja prolaze kroz atmosferu, ispiruju i prenose određene tvari koje su ispuštene u atmosferu. Komunalne otpadne vode nastaju kao posljedica sanitarnih potreba u kućanskim i javnim prostorima i često su smjesa voda od pranja ulica, javnih objekata, kao i otpadnih voda iz uslužnih djelatnosti. Uslijed poljoprivrednih aktivnosti kao što su mljekarstvo, tovilista, ribarstvo ili svinjogojske farme, kao i ispiranja poljoprivrednih zemljišta na kojima se uzgajaju poljoprivredne kulture, dolazi do nastanka poljoprivrednih otpadnih voda. Industrijske otpadne vode potječu od različitih vrsta postrojenja i nastaju uporabom vode u procesu rada i proizvodnje, u industrijskim i drugim proizvodnim pogonima. Iako sve vrste otpadnih voda predstavljaju potencijalnu opasnost za okoliš, posebno industrijske otpadne vode, ovisno o djelatnosti, mogu sadržavati veće količine otrovnih ili teško razgradivih tvari poput metala, kiselina, naftnih derivata, ulja ili sintetskih kemijskih spojeva.

Prema učinku i prirodi onečišćenja, moguće je razlikovati: fizičko, mikrobiološko i kemijsko onečišćenje voda. Promjena boje, mirisa ili temperature vode ubraja se u fizičko onečišćenje, dok prisutnost brojnih (patogenih) mikroorganizama koji inače nisu prisutni u nekom sustavu predstavlja mikrobiološko onečišćenje. Kemijsko onečišćenje može biti prirodnog ili antropogenog podrijetla i predstavlja značajnu opasnost za vodeni okoliš zbog potencijalne toksičnosti za organizme, akumulacije tvari u ekosustavima i organizmima, gubitka bioraznolikosti te općenito štetnog djelovanja na prirodne vode, vodene organizme, te neposredno i na ljudsko zdravlje (Tušar, 2004).

Uz niz drugih onečišćujućih tvari, otpadne vode su značajan izvor metala koji zbog stabilnosti, toksičnosti i sklonosti akumulaciji u ekosustavu čine važan i aktualan okolišni problem. Ovisno o koncentraciji, mogu dovesti do uništenja flore i faune, akumulacije i toksičnih učinaka kod vodenih organizama poput riba, školjkaša ili rakova, koji posljedično, ako se koriste za prehranu, mogu potencijalno štetno djelovati i na ljude. Neki od metala koji se najčešće nalaze u otpadnim vodama uključuju arsen, olovo, živu, kadmij, bakar, nikal,

srebro i cink, od kojih su mnogi toksični već i u vrlo niskim koncentracijama (Akpor i sur., 2014). Iz svega navedenog, nužno je u procjeni stanja okoliša pod utjecajem otpadnih voda obratiti pozornost na onečišćenje metalima s obzirom da sustavi pročišćavanja otpadnih voda nisu uvijek dovoljno učinkoviti ili čak ni ne postoje.

2.2. Metali u slatkovodnim ekosustavima

Metali su u slatkovodnim ekosustavima uvijek prirodno prisutni i njihova koncentracija je rezultat prirodnih procesa kao što su kemijsko i mehaničko trošenje stijena, erozija tla te suho ili vlažno taloženje iz atmosfere. Međutim, u okoliš dopijevaju i preko antropogenih aktivnosti koje uključuju komunalne i industrijske otpadne vode, promet, rudarstvo, poljoprivredu i pojačanu upotrebu gnojiva te ispiranje zagađenih tala (Gaillardet i sur., 2004). Metali koji se prirodno nalaze u većim koncentracijama i u okolišu i u organizmima, ubrajaju se u makroelemente (Ca, K, Mg, Na), dok su elementi s niskim koncentracijama u prirodi nazvani elementima u tragovima, te njihov dodatni unos remeti ravnotežnu raspodjelu u vodenom ekosustavu (Riley i Chester, 1971).

U vodenim sustavima metali dolaze u raznim anorganskim i organskim oblicima, od hidratiziranih iona pa sve do velikih organskih kompleksa. Općenito i zbog jednostavnosti, metale u vodi dijelimo na otopljenu i partikularnu fazu nakon filtriranja uzoraka vode kroz standardni filter promjera pora 0,45 μm . Frakcija koja prođe kroz filter od 0,45 μm definirana je kao uglavnom otopljena, ali kroz takav filter mogu proći i koloidni oblici metala koji obuhvaćaju čestice veličine 1 nm do 450 nm te mogu biti organski i anorganski. Frakcija koja prolazi kroz filter obuhvaća slobodne ione metala, labilne anorganske i organske komplekse, kao i inertne organske komplekse većih molekulskih masa te koloidne čestice, ali isključuje metale koji su vezani na veće čestice te samim time i manje bioraspoloživi (Dragun i sur., 2009). Frakcija koja zaostaje na filteru od 0,45 μm definirana je kao partikularna.

Specijacija metala, odnosno specifični kemijski oblici u kojima se neki metal pojavljuje u prirodi, ima izravan učinak na bioraspoloživost metala, odnosno na toksičnost metala za organizme (Forstner i Wittmann, 1981). Na bioraspoloživost metala utječu i biologija samih organizama (učinkovitost asimilacije metala, prehrambene navike, veličina, starost ili reproduktivna faza), geokemija metala (raspodjela u vodi i sedimentu i specijacija metala) (Roosa i sur., 2016) te mnogobrojni fizikalno-kemijski čimbenici (temperatura, pH vrijednost, ionska jakost, salinitet, tvrdoća vode ili količina otopljenog kisika) (Mason, 2002; Bonnail i sur., 2016). Talozenje i kompleksiranje metala dovode do njihove imobilizacije i smanjene

toksičnosti. Najčešće otopljene oblike metala čine organski kompleksirani metali i/ili anorganski oblici metala. Zbog veličine, metal-organski kompleksi su inertniji i stabilniji te je metalima onemogućen prolazak kroz stanične membrane čime se smanjuje bioraspoloživost takvih metala (Gaillardet i sur., 2004). U slatkovodnim sustavima najčešći organski ligandi za vezanje metala su slabe organske baze poput oksalata i acetata te fulvinske ili huminske kiseline (Gaillardet i sur., 2004), kao i organski ligandi biološkog podrijetla nastali djelovanjem algi i fitoplanktona. Kompleksi metala s anorganskim ligandima su pak vrlo labilni, brzo disociraju u vodi i uglavnom čine metale brzo i lako raspoloživima organizmima. Najznačajniji anorganski ligandi za kompleksiranje metala u riječnim sustavima su molekule vode, ioni HCO_3^- , CO_3^{2-} , OH^- te rjeđe Cl^- , SO_4^{2-} , F^- i NO_2^- (Gaillardet i sur., 2004).

Budući da je voda dinamičan medij, jasno je da koncentracije metala u vodi ukazuju samo na kratkotrajnu izloženost i trenutno stanje okoliša prilikom uzorkovanja. Stoga je, da bi se dobile informacije o bioraspoloživim koncentracijama metala, a time i njihovim potencijalnim toksičnim učincima, nužno pratiti i bioakumulaciju metala u vodenim organizmima kao pokazatelj dugoročne izloženosti sustava i organizama metalima, kao i ključan korak u prepoznavanju opasnosti za žive organizme u vodi te posljedično i za ljude putem lanca ishrane.

Kao primjer slatkovodnih sustava pod utjecajem antropogenih aktivnosti u vidu ispusta otpadnih voda gradova i tvornica, kao i poljoprivrednih i/ili ribnjačarskih aktivnosti te posljedično umjerenog onečišćenja metalima odabrane su dinaridska rijeka Krka te panonska rijeka Ilova u Hrvatskoj. Ovi osjetljivi ekosustavi su ekološki i strateški bitni za Republiku Hrvatsku zbog zaštićenih blizine područja, Nacionalnog parka Krka (NP Krka) te Parka prirode Lonjsko Polje (PP Lonjsko Polje).

2.2.1. Dinaridske i panonske rijeke

Prema podjeli Europe na limnografske regije, zasnovanoj na vodenoj fauni (Illies, 1978), hidrografski prostor Hrvatske podijeljen je na Panonsku i Dinaridsku ekoregiju, te se na nacionalnoj razini Dinaridska ekoregija dodatno dijeli na tri subekoregije: Dinaridsku kontinentalnu subekoregiju, Dinaridsku primorsku subekoregiju i Dinaridsku primorsku subekoregiju Istru (Plan upravljanja vodnim područjima 2016.-2021.).

Što se tiče vodnih područja, teritorij Republike Hrvatske hidrografski pripada slivu Jadranskog mora i slivu Crnog mora te je podijeljen na dva vodna područja – vodno područje rijeke Dunav i jadransko vodno područje (Izvješće o provedbi planova upravljanja vodnim područjima iz Okvirne direktive o vodama, 2015; Zakon o vodama, 2019). Površina vodnog

područja rijeke Dunav iznosi 35117 km², odnosno obuhvaća 62 % hrvatskog kopnenog teritorija. Okosnice otjecanja s vodnog područja su rijeke Sava i Drava, a područje podsliva Save (PS Sava) zauzima 25764 km² ili 73 % površine vodnoga područja rijeke Dunav, a područje podsliva Drave i Dunava (PS Drava) 9353 km² ili 27 % površine ovog vodnog područja. Vodno područje rijeke Dunav u Republici Hrvatskoj je dio šireg međunarodnog vodnog područja Dunava (Plan upravljanja vodnim područjima 2016.-2021.; Tadić i sur., 2020). Jadransko vodno područje se sastoji od više slivova ili dijelova slivova jadranskih rijeka s pripadajućim podzemnim, prijelaznim i priobalnim vodama. Površina iznosi 35303 km², što je oko 40 % ukupnog teritorija Republike Hrvatske. Jadransko vodno područje u Republici Hrvatskoj pripada širem međunarodnom slivu Jadranskoga mora, a dio voda jadranskog vodnog područja su pogranične ili prekogranične vode međudržavnoga značaja (Plan upravljanja vodnim područjima 2016.-2021.). Vodno područje rijeke Dunav i jadransko vodno područje razlikuju se prema geološkim, pedološkim, litološkim, hidrološkim i klimatskim obilježjima, kao i zemljišnom pokrovu, socio-ekonomskim prilikama te prisutnim vrstama na svakom od područja (Plan upravljanja vodnim područjima 2016.-2021.).

Od dviju istraživanih rijeka, rijeka Krka pripada Dinaridskoj primorskoj subekoregiji te jadranskom vodnom području, dok se rijeka Ilova nalazi unutar Panonske ekoregije te pripada vodnom području rijeke Dunav (Plan upravljanja vodnim područjima 2016.-2021.)

Prema Planu upravljanja vodnim područjima 2016.-2021. došlo je do unaprjeđenja tipologije rijeka te se slijedom gledanih abiotičkih čimbenika mogu razlučiti 24 tipa i 47 podtipova tekućica u Hrvatskoj. Korišteni čimbenici za tipizaciju rijeka bili su pripadajuća ekoregija, veličina sliva, geološka i litološka podloga, nadmorska visina te pojedini izborni čimbenici. Prema navedenoj kategorizaciji rijeka Krka većim dijelom svojeg toka, osobito uzvodnog, pripada ekotipu HR_R_12, odnosno prigorskim srednje velikim i velikim tekućicama u Dinaridskoj primorskoj subekoregiji s nadmorskom visinom 200-500 m, veličinom sliva 10-10000 km² te vapnenačkom podlogom. Rijeka Ilova svrstava se u tip HR_R_4 u Panonskoj ekoregiji, odnosno u nizinske srednje velike i velike tekućice s nadmorskom visinom < 200 m, veličinom sliva 10-10000 km² te silikatno ili silikatno-vapnenačkom podlogom (Registar vodnih tijela prema Planu upravljanja vodnim područjima 2016.-2021.).

2.2.1.1. Rijeka Krka

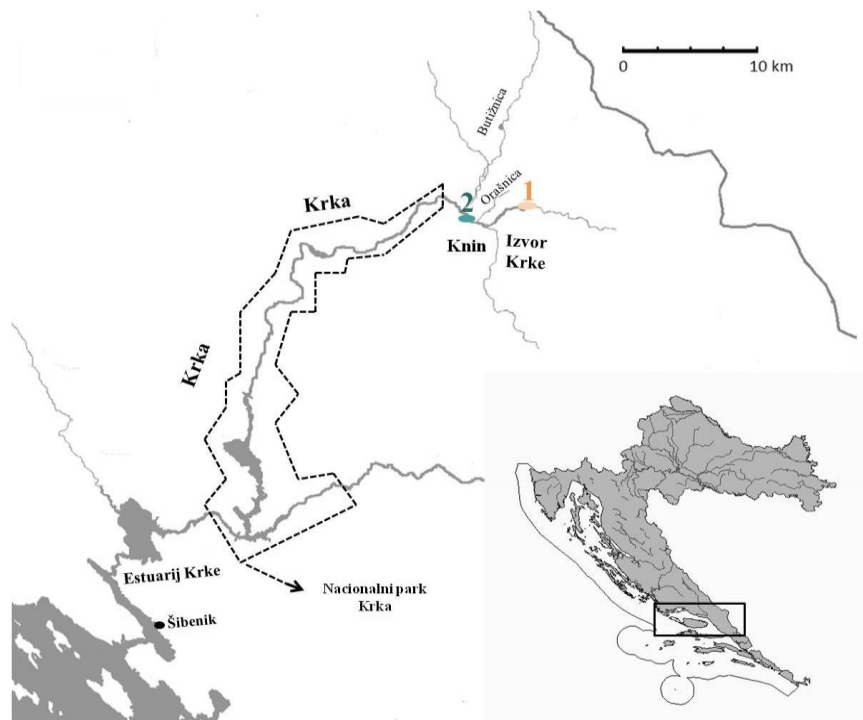
Rijeka Krka je dinaridska krška rijeka u obalnom području južne Hrvatske čija slivna površina iznosi oko 2657 km², dužine je oko 73 km, pri čemu se 49 km odnosi na slatkovodni

dio toka. Pripada slivu Jadranskog mora i izvire u podnožju Dinare podno Topoljskog slapa, udaljenog 3,5 km od grada Knina, a u Jadransko more se ulijeva kod Šibenika (Javna ustanova NP Krka, 2021). U slatkovodnom dijelu toka ima pet pritoka: Krčić, Kosovčicu, Orašnicu, Butižnicu te Čikolu. Zbog prirodnih ljepota, slapova, sedrenih barijera, brzaca i podzemnih izvora područje od 109 km² porječja Krke (dva kilometra nizvodno od Knina do Skradina) i donjeg toka rijeke Čikole proglašeno je 1985. godine Nacionalnim parkom Krka (Javna ustanova NP Krka, 2021).

Iako područje oko rijeke Krke nije gusto naseljeno, rijeka je izložena antropogenim aktivnostima koje predstavljaju potencijalnu opasnost za živi svijet ovog osjetljivog krškog sustava i NP Krka. Naime, komunalne vode grada Knina predstavljaju jednu od potencijalnih opasnosti za onečišćenje Krke jer se bez prikladnog pročišćavanja direktno izlijevaju u rijeku Krku. Nadalje, otpadne vode tvornice vijaka u Kninu ispuštaju se u bazene predviđene za pročišćavanje, ali osobito u vrijeme obilnih oborina te otpadne vode se ulijevaju u rijeku Orašnicu te time indirektno i u samu rijeku Krku zbog čega su ovi bazeni u Planu gospodarenja otpadom Republike Hrvatske za razdoblje 2017.-2022. predstavljeni kao jedna od "crnih točaka" onečišćenja na području Hrvatske. Dodatan problem je što se navedeno područje nalazi svega 2 km uzvodno od granice NP Krka te ugrožava i ovo zaštićeno područje te što zbog uglavnom karbonatne podloge može lako doći do poniranja ovih otpadnih voda u podzemlje čime se ugrožavaju i izvori pitke vode. Uvidom u Registar vodnih tijela prema Planu upravljanja vodnim područjima 2016.-2021., istraživana vodna tijela rijeke Krke uglavnom pokazuju vrlo dobro i/ili dobro ekološko i kemijsko stanje, kao i fizikalno-kemijske čimbenike, hidromorfološke elemente i razinu specifičnih onečišćujućih tvari. Međutim, upravo područje oko grada Knina i nizvodno od Knina, kao i jezero Brljan, pokazuju umjereno, pa čak i loše stanje što se tiče ekološkog i kemijskog stanja i hidromorfoloških elemenata (Izvadak iz Registra vodnih tijela prema Planu upravljanja vodnim područjima 2016. – 2021., klasifikacijska oznaka: 008-02/21-02/74, 2021; Tadić i sur., 2020).

Dva prethodna istraživanja koja navode koncentracije niza metala u vodotoku rijeke Krke uzvodno od početka NP Krka (Ca, Mg, Zn, Cd, Pb i Cu) ukazuju na potencijalne prijetnje nacionalnom parku, osobito u blizini grada Knina (Cukrov i sur., 2008b, 2012). Iz svih navedenih razloga, rijeka je bila predmet sveobuhvatnih istraživanja u razdoblju 2015.-2016. godine s naglaskom na dvije lokacije koje predstavljaju gradijent onečišćenja, referentnoj lokaciji na izvoru rijeke Krke te drugoj postaji 2 km nizvodno od grada Knina i mjesta ispusta komunalnih i industrijskih otpadnih voda (Slika 1) (Filipović Marijić i sur., 2018; Sertić Perić i sur., 2018). Iako su obje lokacije svrstane u kategoriju voda dobre ili vrlo

dobre kakvoće prema Uredbi o standardu kakvoće voda (2019), svi izmjereni fizikalno-kemijski čimbenici vode ukazivali su na lošije okolišne uvjete nizvodno od ispusta otpadnih voda, od kojih su temperatura, vodljivost, ukupne otopljene tvari te tvrdoća vode pokazali statistički značajne razlike između postaja (Sertić Perić i sur., 2018). Iako su koncentracije metala u vodi također relativno niske u čitavom sustavu, što je karakteristično za krške rijeke, zabilježen je značajan porast nekoliko elemenata u blizini grada Knina, osobito Fe, Li, Mn, Mo, Sr, Rb i Ca pri čemu je najviši porast na u odnosu na izvor rijeke Krke zabilježen za Fe i Mn, koji su bili 17, odnosno 38 puta povišeni kod Knina, dok su ostali elementi pokazali prosječni porast od oko 2 puta (Filipović Marijić i sur., 2018; Sertić Perić i sur., 2018). Iako se trenutno onečišćenje pokazalo uglavnom umjerenim, trendovi su ukazali na potencijalne opasnosti za očuvanje ljepota, bogatstva vrsta te specifičnih mikrostaništa unutar samog NP Krka i potrebu trajnog monitoringa i zaštite.



Slika 1. Karta rijeke Krke s označenim mjestima uzorkovanja (izvor Krke kao referentna postaja i lokacija nizvodno od Knina kao onečišćena postaja) (preuzeto i prilagođeno iz Filipović Marijić i sur., 2018)

2.2.1.2. Rijeka Ilova

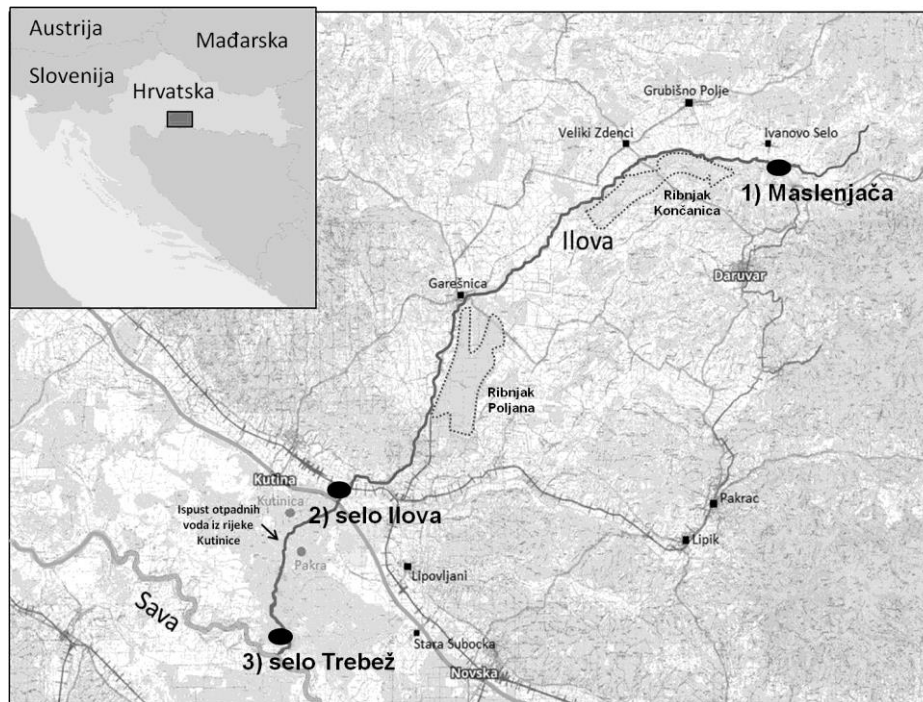
Rijeka Ilova je panonska rijeka u kontinentalnom dijelu središnje Hrvatske čija ukupna slivna površina iznosi oko 1128 km², a dužina oko 96 km (Plan upravljanja vodnim područjima 2016.-2021.), iako su se navedene brojke mijenjale nekoliko puta tijekom godina

zbog niza hidrotehničkih zahvata na porječju rijeke (Plantak i sur., 2016). Izvire na sjevernim obroncima Papuka u naselju Velika Babina Gora na nadmorskoj visini od 205 metara, nastavlja se dolinom koja je nadalje uvjetovana rasjedom između Papuka i južnih obronaka Bilogore te poprima smjer otjecanja od sjeveroistoka prema jugozapadu (Plantak i sur., 2016). Kao lijeva pritoka rijeke Lonje pripada porječju rijeke Save čime ujedno pripada i Crnomorskom slivu. Rijeka Ilova ima puno pritoka pri čemu se kao najveća ističe rijeka Toplica, dok su ostali izdvojeni pritoci Tomašica, Peratovica, Čavlovica, Garešnica, Bršljanica, Šovarnica, Rijeka i Rastovac, Kutinica (Plantak i sur., 2016).

Područje oko rijeke Ilove je izloženo antropogenom pritisku u vidu hidrotehničkih radova koji uključuju pretvaranje močvarnih područja u brojne ribnjake, izgradnju kanala i nasipa, produbljivanja korita rijeka, što je u istraživanju hidromorfološkog stanja tekućica u porječju Ilove rezultiralo time da ukupno 38,5 % duljine vodotoka ne zadovoljava ciljeve prema Okvirnoj direktivi EU-a o vodama jer je dobilo ocjenu umjereno promijenjenog, lošeg ili vrlo lošeg morfološkog stanja (Plantak i sur., 2016). Vodotoci u blizini većih naselja dobili su najlošije, odnosno nezadovoljavajuće ocjene, kao i dionice koje su neposredno povezane s ribnjacima. Uz navedeno, istraživane su i promjene protoka i protočnih režima na rijeci Ilovi te je pokazano da su promjene protočnih režima Ilove u razdoblju od 1961. do 2016. ponajprije uzrokovane promjenom klimatskih elemenata, unatoč već navedenim antropogenim utjecajima u porječju, odnosno provedenim hidrotehničkim izmjenama kao i postojanju nekoliko velikih ribnjaka (Orešić i sur., 2018). Kvalitativni i kvantitativni sastav makrozoobentosa 1991. godine je ukazao na dobro stanje, raznovrsnost i veliku biomasu u gornjem Poilovlju, dok je u nizvodnom smjeru zabilježen puno manji broj vrsta što govori i u prilog lošijem ekološkom statusu (Delić, 1991).

Uz ribnjake, koji su i područja rekreativnog i sportskog turizma te poljoprivrednu aktivnost, srednji i donji dio toka rijeke Ilove ugroženi su ispustom komunalnih voda grada Kutine te neobrađenih industrijskih otpadnih voda tvornice gnojiva (Durgo i sur., 2009). Za razliku od rijeke Krke gdje se antropogeni utjecaj nije toliko očitovao u analizama kakvoće vode i okoliša, uvid u Registar vodnih tijela rijeke Ilove (Izvadak iz Registra vodnih tijela prema Planu upravljanja vodnim područjima 2016. – 2021., klasifikacijska oznaka: 008-02/21-02/74, 2021) ukazuje na loše i vrlo loše stanje većine ispitivanih vodnih tijela ovog ekosustava što se tiče bioloških elemenata kakvoće, ekološkog i kemijskog stanja te hidromorfoloških elemenata, ali uglavnom još uvijek zadovoljavajuće razine specifičnih onečišćujućih tvari.

Unatoč tome, utjecaj svih antropogenih aktivnosti na ovo područje slabo je istražen te je bio predmet samo dva prethodna istraživanja koja su istraživala kemijski sastav vode skupljene u deponiju fosfogipsa iz tvornice gnojiva (Durgo i sur., 2009) te toksični potencijal površinske vode rijeke Ilove (Radić i sur., 2013). Usporedbom tih tvorničkih otpadnih voda na temelju maksimalno dopuštene koncentracije elemenata, prema tada važećem Pravilniku o граниčnim vrijednostima parametara opasnih i drugih tvari u otpadnim vodama (NN 94/08), otkriveno je najveće obogaćenje elementima: F, V, Fe, Cr (VI), Cu i Zn, koji su bili 6-308 puta viši od maksimalno dopuštenih vrijednosti (Durgo i sur., 2009). Kako bi se provelo detaljnije istraživanje cjelokupnog antropogenog utjecaja na živi svijet rijeke Ilove, kao dio HRZZ projekta „AQUAMAPMET” u razdoblju 2017.-2018. godine istraživane su dvije, odnosno tri lokacije prema gradijentu onečišćenja. Prvo mjesto uzorkovanja na rijeci Ilovi smješteno je kod sela Maslenjača i izabrano je kao referentna postaja za uzorke vode i sedimenta jer je pod najslabijim antropogenim utjecajem, odnosno nalazi se u gornjem dijelu njenog toka, uzvodno od svih ribnjaka, industrijaliziranog i poljoprivrednog područja (Slika 2). Druga lokacija smještena je nizvodnije kod sela Ilova i djelomično je pod utjecajem uzgajališta riba (Slika 2). Nadalje, rijeka Ilova oko 1 km nizvodno od grada Kutine prima otpadne vode kroz rijeku Kutinicu u koju se ispuštaju neobrađene otpadne vode tvornice gnojiva i grada Kutine. Dodatnu zabrinutost predstavlja i činjenica da se nizvodno od Kutinice, Ilova ulijeva u rijeku Lonju koja tvori močvarno područje zaštićeno kao Park prirode Lonjsko polje (<http://www.pp-lonjsko-polje.hr/>). Stoga je treća lokacija bila smještena u blizini sela Trebež, koje se nalazi oko 8 km nizvodno od utoka rijeke Kutinice u Ilovu (Slika 2), odnosno unutar zaštićenog područja PP Lonjsko Polje (Mijošek i sur., 2020a). Istraživanje je ukazalo na lošije okolišne uvjete u donjem toku rijeke Ilove kod sela Trebeža gdje su ukupne otopljene soli, količina nitrata te fosfata bili iznad dopuštenih granica, dok je voda kod sela Maslenjače i Ilove uglavnom bila dobre ili vrlo dobre kvalitete. Većina metala je također bila statistički značajno viša u uzorcima vode i sedimenta na onečišćenoj postaji kod sela Trebež, pri čemu je najveći porast u nizvodnom smjeru rijeke zabilježen za elemente poput Al, As, Cd i Ni koji su bili višestruko povišeni u odnosu na dvije uzvodne postaje i ukazali su na značajan antropogeni pritisak u nizvodnom dijelu rijeke Ilove te na potencijalne opasnosti za park prirode (Mijošek i sur., 2020a).



Slika 2. Karta rijeke Ilove s označenim mjestima uzorkovanja (Maslenjača, selo Ilova, selo Trebež) (preuzeto i prilagođeno iz Mijošek i sur., 2020a)

2.3. Metali u vodenim organizmima

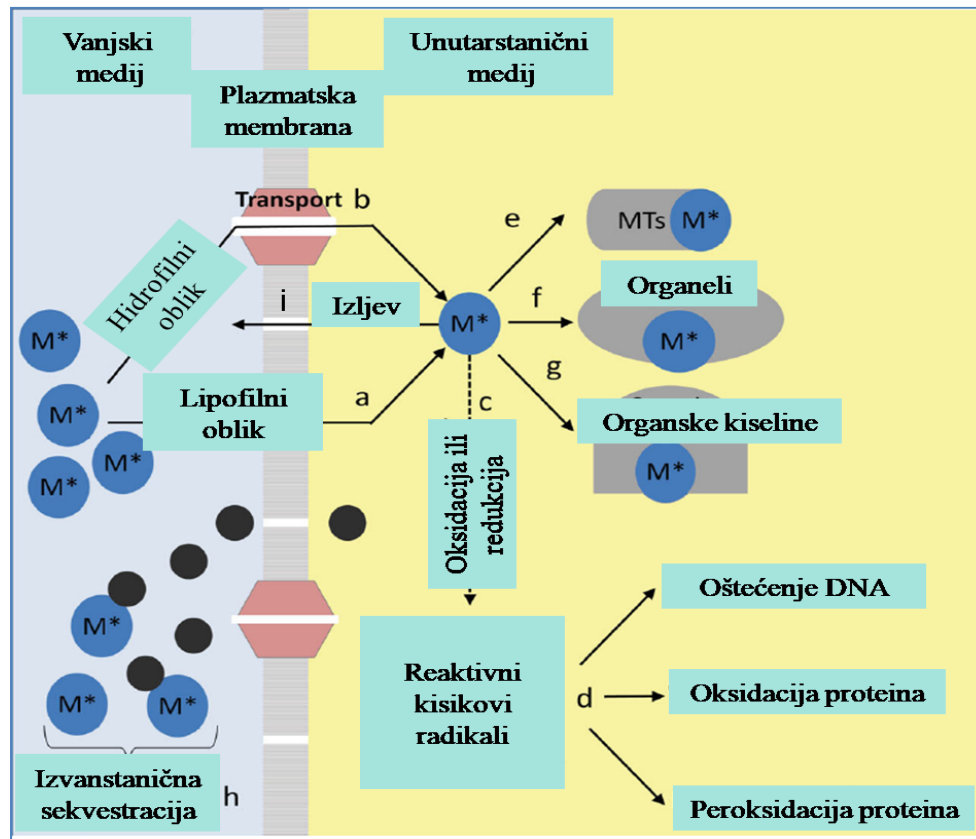
2.3.1. Unos u organizme i međustanični prijenos

Metali u vodene organizme dospijevaju preko vanjske površine tijela, respiratornih organa (škrge), probavnog epitela ili najčešće kombinacijom svih ovih putova (Deb i Fukushima, 1999), pri čemu kao izvor metala za organizme služe i voda i sedimenti i hrana. Unos preko površine tijela dominantan je kod jednostavnijih vodenih organizama, dok u složenijih organizama veću važnost ima izravan unos metala putem škrge i probavila (Deb i Fukushima, 1999). Disanjem, odnosno izmjenom plinova, kroz škrge prolaze i ostale otopljene tvari, uključujući metale, dok unos putem prehrane također predstavlja izravni unos metala u organizam koji često ima i najveći doprinos u akumulaciji metala (Clearwater, 2000). Ukoliko do unosa metala uglavnom dolazi preko vode najčešće govorimo o biokoncentraciji metala u organizmu, a ako se nakupljanje metala odvija putem vode i hrane govori se o bioakumulaciji (Yarsan i Yipel, 2013).

Općenito se metali raspodjeljuju po organima, tkivima i stanicama organizma, kao i različitim unutarstaničnim odjeljcima koji se prema Wallaceu i sur. (2003) dijele na stanične frakcije osjetljive na prisutnost metala (MSF, *metal sensitive fraction*) koje uključuju organele (mitohondriji, lizosomi, mikrosomi) i toplinski nestabilne proteine poput enzima (HDP, *heat-*

denatured proteins), dok biološki detoksicirane frakcije metala (BDF, *biologically detoxified fraction*) uključuju granule bogate metalima (MRG, *metal-rich granules*) i toplinski stabilne proteine poput metalotioneina (HSP, *heat-stable proteins*). Takva je unutarstanična raspodjela metala uvijek specifična za pojedini metal, organ i organizam, ali i dinamična ovisno o uvjetima izloženosti metalima i nizu drugih okolišnih čimbenika (Wang i Rainbow, 2006).

Sam unos metala u stanicu se vrši preko staničnih membrana procesima jednostavne difuzije ili olakšane difuzije, aktivnog transporta i pinocitoze (Simkiss, 1998), a za unos u stanice je najraspoloživiji hidratizirani ionski, odnosno biološki raspoloživ oblik metala u kojem je kation koordiniran sa šest molekula vode. Za organizme je većinom najtoksičniji taj ionski oblik metala, dok se kompleksiranjem s organskim i anorganskim ligandima smanjuje njihova toksičnost, iako postoje i izuzeci poput Hg, Pb, Se i Sn (Florence i Batley, 1977). Ovisno o kemijskom obliku metala, postoje različiti mehanizmi asimilacije i detoksikacije u stanicama organizama. Ukoliko je metal vezan u neki lipofilni kompleks može ući u stanicu direktno pasivnom difuzijom (Slika 3a). Hidrofilni oblici zahtijevaju aktivni oblik prijenosa putem transportnih proteina (Slika 3b). U stanici dolazi do oksidacije ili redukcije metala što može dovesti do povećanja količine reaktivnih kisikovih spojeva (ROS, *reactive oxygen species*), što potom dovodi do oksidacijskog stresa i toksičnih učinaka (Slika 3c,d). Kako bi se izbjegla toksičnost, određeni metali se vežu na termostabilne proteine poput metalotioneina (Slika 3e). Drugi biološki zaštitni mehanizam protiv toksičnosti metala je njihova akumulacija u organelima u formi inertnih granula koje se izlučuju tijekom životnog ciklusa (Slika 3f) (Vijver i sur., 2004). Metali unutar stanice mogu biti vezani i na aminokiseline, proteine i organske kiseline (Slika 3g), kompleksirani već u izvanstaničnom mediju (Slika 3h), a u suvišku mogu se i eliminirati iz organizma (Slika 3i) (de Paiva Magalhaes i sur., 2015).



Slika 3. Različiti mehanizmi asimilacije i detoksikacije metala u stanicama (preuzeto i prilagođeno iz de Paiva Magalhaes i sur., 2015)

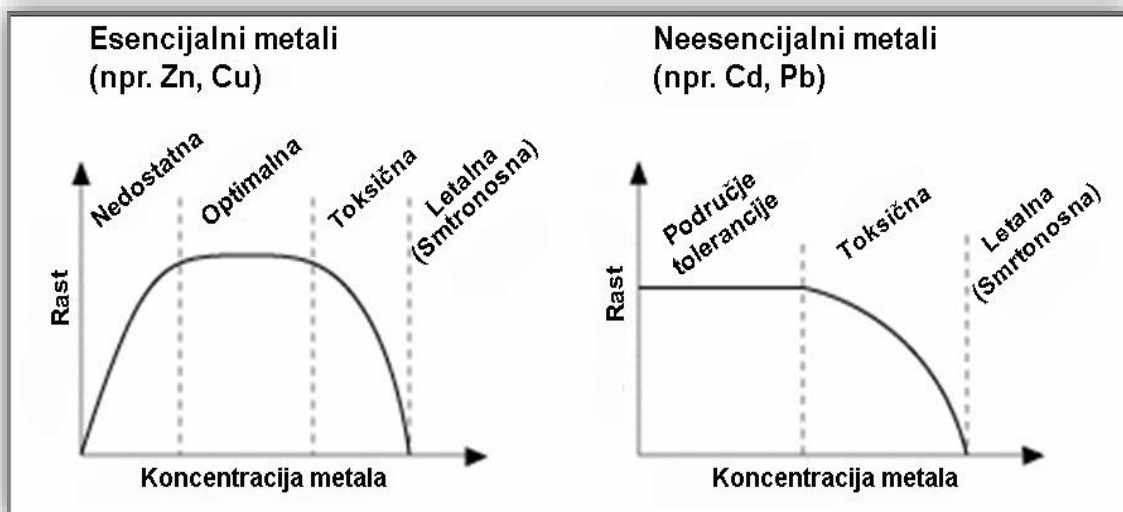
Što se tiče ekskrecije metala, ribe mogu izlučivati štetne tvari, pa tako i metale, iz organizma putem kože, bubrega, škrge i probavila (Olsson i sur., 1998), pri čemu se dominantan način ponovno razlikuje ovisno o elementu.

2.3.2. Esencijalni i neesencijalni metali

S biološke točke gledišta, metali se dijele na esencijalne i neesencijalne. Esencijalni metali su prirodno prisutni u živim bićima te imaju važnu i metaboličku funkciju u organizmima kao dio enzima, hormona ili vitamina, odnosno molekula koje grade stanice i tkiva te njihov nedostatak u organizmu može biti uzrok mnogih štetnih učinaka. U skupinu esencijalnih elemenata se ubrajaju makroelementi Ca, K, Mg i Na te mikroelementi, poput Co, Cu, Fe, Mn, Se ili Zn (Wood i sur., 2012). Iako su ti elementi nužni i neophodni za funkcioniranje organizama, zbog prekomjernog unosa te prelaska optimalnih koncentracija i oni mogu postati toksični. S obzirom na to da esencijalni elementi djeluju na organizme i stimulatивно i potencijalno toksično, teško je u potpunosti razlučiti i definirati njihovu toksičnost. Stoga se odnos koncentracije svakog elementa i njegovog učinka može opisati krivuljom na kojoj je moguće definirati područje tolerancije i/ili optimuma, s graničnim

gornjim i donjim koncentracijama metala, te područje toksičnosti i letalnosti (Slika 4). U slučaju esencijalnih elemenata, s obzirom da su neophodni živim organizmima, vidljivo je da postoji područje koje ukazuje na njihov nedostatak u organizmu, nešto širi raspon koncentracija koje predstavljaju njihov optimum, ali i područje toksičnosti i letalnosti uslijed pojačanog unosa (Slika 4).

One metale i metaloide koji se nalaze u prirodi, ali za koje do sada nije otkrivena nikakva biološka i metabolička uloga u živim organizmima nazivamo neesencijalnim, a to su primjerice As, Cd, Hg i Pb. Živi organizam podnosi vrlo uski koncentracijski raspon tih elemenata te oni mogu izazvati toksične učinke, pa i smrtnost, i već pri vrlo niskim koncentracijama (Slika 4) (Verma i Dwiwedi, 2013). Njihova toksičnost uvelike proizlazi iz sličnosti s esencijalnim metalima, zbog čega mogu zamijeniti esencijalne elemente te istim putovima ući u stanice i vezati se za važne biomolekule što dovodi do narušavanja metaboličkih funkcija, a time i toksičnosti (Wood i sur., 2012).



Slika 4. Odnos koncentracija esencijalnih i neesencijalnih metala i njihovih učinaka na organizme (preuzeto i prilagođeno iz Forstner i Wittmann, 1981)

2.3.3. Toksični učinci metala i mehanizmi detoksikacije

Kao i druge štetne tvari, metali u vodenim organizmima mogu imati široki spektar negativnih i toksičnih učinaka na svim biološkim razinama od stanice, organa, organizma, populacije pa sve do čitavog ekosustava (Slika 5).

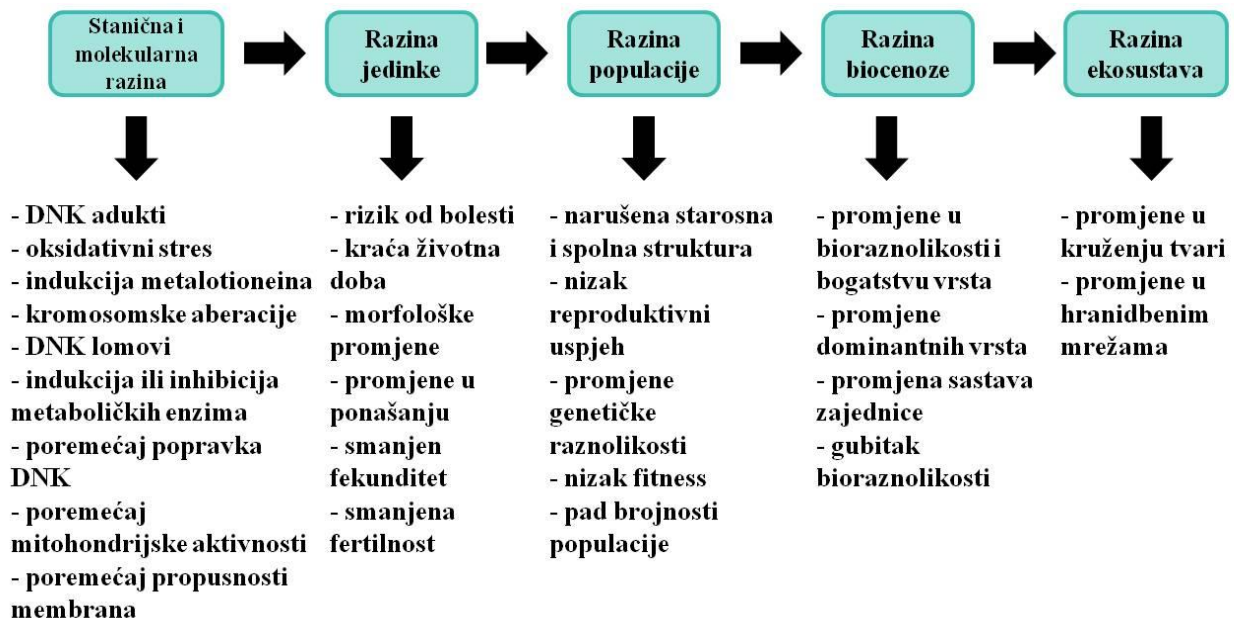
Promjene na razini populacija, biocenoza i ekosustava su manje specifične, poput promjena u sastavu, starosnoj strukturi, smanjenja genetičke raznolikosti te pada bogatstva vrsta i bioraznolikosti, kao i promjena u hranidbenim mrežama nekog sustava te se javljaju

nakon duge i kronične izloženosti i značajnog djelovanja zagađivala. Ipak, svaki toksični učinak počinje na molekularnoj i staničnoj razini, a posljedično može dovesti i do promjena na razini cijelog organizma (Mussali-Galante i sur., 2013). Učinci na razini organizma mogu biti morfološke (deformacija ljuštura, škrge, skeleta), bihevioralne (različite promjene u ponašanju) i fiziološke prirode (smanjen fekunditet i fertilitet, poremećaji i bolesti u radu tkiva i organa).

Toksični učinci na staničnoj razini su inhibiranje enzima, promjene funkcija proteina, oksidacijski stres, poremećaji mitohondrijske aktivnosti i propusnosti membrana, kao i različita oštećenja DNK (Phillips i Rainbow, 1993) (Slika 5). Naime, metali se mogu vezati na funkcionalne skupine biološki važnih molekula, tzv. metaloproteina, te ukoliko se radi o neesencijalnim elementima, dovesti do inhibicije ili promjene funkcija tih molekula (Viarengo, 1985). Metali imaju ulogu i u nastanku oksidacijskog stresa koji predstavlja metaboličko stanje organizma koje je praćeno povećanjem količine reaktivnih kisikovih spojeva. Reaktivni oblici kisika obuhvaćaju radikale kisika (superoksidni, hidroksilni, peroksidni) i reaktivne neradikalne derivate kisika (vodikov peroksid, hipokloritna kiselina). Iako su kisikovi radikali u ograničenim koncentracijama sastavni dio metabolizma, oni su visoko reaktivne molekule i u suvišku mogu oštetiti stanične strukture (staničnu membranu i organele), dovesti do inaktivacije enzima ili lipidne peroksidacije (Sevcikova i sur., 2011). Oksidacijski stres pogubno utječe na većinu staničnih struktura, uključujući membrane, lipide, proteine, lipoproteine i nukleinske kiseline te uzrokuje peroksidaciju lipida, s posljedičnim oštećenjima stanične membrane i lipoproteina. Proteini se djelovanjem kisikovih radikala podvrgavaju različitim konformacijskim modifikacijama koje mogu imati utjecaja na gubitak ili oštećenje njihove enzimске aktivnosti. Nadalje, mitohondriji koji čine stanični energetska pogon, mogu akumulirati veće količine metala što može dovesti do inhibicije njihove aktivnosti i oksidativne fosforilacije, čime se narušava energetska status stanice (Viarengo, 1985). Toksičan učinak metala dovodi i do poremećaja pasivnog i aktivnog prijenosa tvari uslijed poremećaja propusnosti membrane, bilo vezanjem određenih metala za fosfatne skupine u lipidnim slojevima membrane, inhibicijom enzima Na/K ovisne ATP-aze, ili posredno, smanjenjem količinom raspoloživog ATP-a (Viarengo, 1985).

Ipak, kako bi se spriječili i/ili smanjili toksični učinci metala, organizmi imaju različite homeostatske i detoksikacijske mehanizme koji održavaju koncentracije metala u uskom rasponu ili dovode do njihove eliminacije (Mason i Jenkins, 1995). Naime, metali se mogu akumulirati u obliku inertnih granula, vezati unutar lizosoma ili za specifične biomolekule koje imaju ulogu u njihovoj detoksikaciji poput metalotioneina (Viarengo, 1985). Nadalje, u

obrani od oksidacijskog stresa postoji čitav sustav antioksidansa koji nastoje održati ravnotežu u organizmu kako ne bi došlo do povećane proizvodnje kisikovih radikala. Antioksidansi se dijele na antioksidacijske enzime i antioksidacijske molekule. Najvažniji antioksidacijski enzimi su katalaza (CAT), superoksid dismutaza (SOD), glutation peroksidaza (GPx) te glutation S-transferaza (GST), dok su važne antioksidacijske molekule glutation (GSH), vitamin C te vitamin E (Martinez-Alvarez i sur., 2005). Nadalje, akumulirana količina metala, odnosno razlika između unosa i izlučivanja metala, pa tako i potencijalna toksična djelovanja metala, ovise o nizu abiotičkih (temperatura, salinitet, pH, intenzitet svjetlosti, količina otopljenih tvari) i biotičkih čimbenika (veličina, starost, spol, fiziologija i kondicijsko stanje organizma). Budući da se metali u organizmima akumuliraju u specifičnim organima i tkivima koji služe kao bioindikatorska tkiva, dodatan doprinos daje i afinitet nekog metala prema određenom tkivu, kao i struktura i funkcija samog tkiva.



Slika 5. Prikaz toksičnih učinaka zagađivala na različitim razinama biološke organizacije (preuzeto i prilagođeno iz Mussali-Galante i sur., 2013)

2.4. Bioindikatorski organizmi

Zbog dinamike vodenih sustava, mjerenje koncentracija metala u vodi ne odražava dugoročnu izloženost metalima, te se, kako bi se procijenilo stanje i kvaliteta okoliša, kao i utjecaj antropogenih aktivnosti na onečišćenje okoliša, najčešće koriste prikladni bioindikatorski organizmi koji daju pouzdaniju i zbog dugoročne izloženosti, vremenski precizniju procjenu utjecaja onečišćenja na okoliš (Kraemer i sur., 2006; Ovaskainen i sur.,

2019). Bioindikatori su organizmi koji svojom prisutnošću ili određenom reakcijom na čimbenike u okolišu mogu ukazivati na stanje okoliša, prisutnost određenih spojeva i promjene tijekom vremena. Mogu odražavati poremećaje u okolišu nastale i zbog prirodnih i antropogenih utjecaja, iako je fokus korištenja bioindikatora najčešće upravo na procjeni antropogenog utjecaja (Holt i Miller, 2010). Najčešće korišteni akvatički bioindikatorski organizmi su ribe, rakovi i školjkaši (Langston i Bebiano, 1998, Ivanković i sur., 2005; Geffard i sur., 2007; Filipović Marijić i sur. 2016; Krasnići i sur., 2019). Međutim, još uvijek postoji potreba za pouzdanim i osjetljivim bioindikatorima, koji bi brzo odražavali biorasploživu, a time i potencijalno toksičnu razinu metala, kao i ukazivali na kratkotrajne i dugotrajne promjene koncentracija metala u vodenom okolišu. Budući da je za najčešće korištena indikatorska tkiva poput jetre, škrge ili bubrega potrebno žrtvovati životinju, neletalne alternative poput ljusaka riba pokazuju potencijal kao dobri pokazatelji stanja okoliša koji bi smanjili invazivnost metoda i negativan utjecaj na bioraznolikost sustava i bogatstvo vrsta do kojeg može doći uslijed pojačanog uzorkovanja standardnih bioindikatorskih organizama te korištenja njihovih organa za analize.

Idealni bioindikatorski organizmi moraju zadovoljiti niz kriterija koje većinom ne može zadovoljiti samo jedna vrsta pa je dobro koristiti grupu organizama. Prije svega, bioindikatorski organizam treba biti široko rasprostranjen i zastupljen, treba imati ograničeno područje kretanja, nisku genetičku i ekološku varijabilnost, dovoljno dug životni vijek i veličinu za provođenje analiza, jasan trofički status te pokazivati osjetljivost na određenu grupu zagađivala. Odgovor dobrog bioindikatorskog organizma bi trebao upućivati i na odgovore drugih vrsta, pa čak i cijelog ekosustava. Praktičnost u smislu lakog uzorkovanja i determinacije vrsta također je važan kriterij. Nadalje, ponekad je bitna i socijalna komponenta kada se radi o ekonomski ili komercijalno važnim vrstama, onim vrstama koje se koriste u poljoprivredi ili u slučaju donošenja regulativa o zaštiti okoliša (Gerhardt, 2002; Holt i Miller, 2010).

S obzirom da upotreba bioindikatorskih organizama daje procjenu promjena u staništu, kumulativnih utjecaja i pristutnog zagađenja, njihova primjena ima prednost nad standardnim kemijskim testovima i mjerenjima fizikalnih parametara okoliša. Ipak, uz brojne prednosti bioindikatori imaju i nedostatke. Dok mnoge kemijske analize u okolišu predstavljaju stanje samo u trenutku uzorkovanja, prednost organizama je što dodaju i vremensku komponentu koja odgovara boravku organizma u određenom sustavu i na taj način daju integriranu informaciju o trenutnim i prošlim uvjetima u okolišu (Holt i Miller, 2010). Budući da organizmi imaju sposobnost akumulacije nekih zagađivala, primjerice metala i organskih

zagađivala, razina onečišćenja i utjecaja na organizme, posebno više trofičke razine, bi bila podcijenjena ukoliko bi se gledale samo kemijske i fizikalne analize. Također, odgovori bioindikatorskih organizama daju jasnija predviđanja kako bi cijeli ekosustavi reagirali na određene poremećaje i ukazuju na složene odgovore biocenoza i dinamične procese u ekosustavima, što je komponenta koju kemijska i fizikalna mjerenja nemaju. Međutim, na odgovore bioindikatora mogu utjecati i drugi abiotički (npr. temperatura, pH, otopljeni kisik) i biotički čimbenici (npr. bolest, starost, parazitizam, veličina organizma, kompeticija i predacija), što otežava razlikovanje prirodne i antropogene varijabilnosti (Steele i sur., 1984; Carignan i Villard, 2002; Holt i Miller, 2010). Zatim, ekosustavi su bogati organizmima različite razine i složenosti te uočeni odgovori na jednoj razini ne moraju ukazivati na isti obrazac kod organizama na drugoj razini. Primjerice, pokazatelji zabilježeni kod kralješnjaka ne moraju odgovarati onima kod beskralješnjaka. Vrste imaju i specifične ekološke niše unutar staništa pa upravljanje i eventualne mjere zaštite ekosustava prema nekom bioindikatoru neće nužno uspješno zaštititi i vrste koje imaju drugačije zahtjeve staništa (Holt i Miller, 2010). Iz navedenih razloga bitno je koristiti barem nekoliko organizama različitog stupnja organizacije kako bi se dobio što jasniji odgovor za kompleksne sustave i postigli učinkoviti načini zaštite primjenjivi na što veći udio organizama nekog ekosustava.

2.4.1. Ribe kao bioindikatorski organizmi

Ribe su među najčešće korištenim bioindikatorskim organizmima u slatkovodnim sustavima. Prednost njihove upotrebe je što imaju optimalnu veličinu za provođenje niza analiza, nalaze se na vrhu hranidbenih lanaca pa ukazuju na trofičko stanje u ekosustavu, imaju dug životni vijek te time ukazuju i na dugoročnu i trenutačnu izloženost, imaju sposobnost akumulacije zagađivala, a za mnoštvo vrsta već postoji i niz podataka (Chovanec i sur., 2003). S obzirom da su kralješnjaci, ribe imaju i fiziološke i funkcionalne sličnosti sa sisavcima, a čine i važan dio ljudske prehrane pa se koriste i u svrhu procjene opasnosti za zdravlje ljudi usporedbom izmjerenih razina pojedinih tvari, uključujući i metale, s pravilnicima i definiranim graničnim vrijednostima koje ne ugrožavaju zdravlje i okoliš. Problem ponekad može predstavljati mobilnost riba, osobito ukoliko se radi o migratornim vrstama, zbog čega je teško precizno definirati mjesto i vrijeme onečišćenja, kao i trajanje izloženosti (Chovanec i sur., 2003).

Opće je poznato da dugotrajna izloženost stresorima iz okoliša kao što su onečišćenje ili niska razina kisika uzrokuje štetne učinke na važne značajke riba poput metabolizma, rasta, otpornosti na bolesti, reproduktivnog potencijala te posljedično na zdravlje, stanje i

preživljavanje riba (Barton i sur., 2002; Benejam i sur., 2008). Zbog navedenih karakteristika ribe se između ostalog smatraju i jednim od najznačajnijih bioindikatora za procjenu onečišćenja metalima (Barak i Mason, 1990; Rashed, 2001; Chovanec i sur., 2003; Dragun i sur., 2018a).

Metali se akumuliraju u različitim organima riba, ovisno o vrsti i o kojem se metalu radi te se stoga često koriste pažljivo odabrani ciljni organi i tkiva. Najčešće se analize rade u jetri kao glavnom metaboličkom i detoksikacijskom organu (Giguere i sur., 2004; Dragun i sur., 2018a). Visoke koncentracije metala uočene su i u bubrezima koji su odgovorni za homeostazu tjelesnih tekućina, te ekskreciju mnogih metabolita i ksenobiotika kojima riba može biti izložena. Uz ove metabolički aktivne organe, često se koriste i škrge kao mjesto izravnog unosa toksikanata iz vode što je prikladno za praćenje trenutačne i kratkotrajne izloženosti metalima (Langston i Bebian, 1998; Chovanec i sur., 2003). Međutim, iako je u posljednjem desetljeću aktualna spoznaja da je unos metala putem hrane za ribe od jednakog ili čak većeg značaja, nego unos metala vodom (Clearwater i sur., 2000; Lapointe i Couture, 2009), probavilo riba je rijetko korišteno kao bioindikatorski organ u dosadašnjim okolišnim istraživanjima.

Ipak, izloženost riba metalima tijekom dugih vremenskih razdoblja može se najbolje opisati uporabom tvrdih, odnosno kalcificiranih struktura poput ljustica i otolita riba, koja predstavljaju neaktivna i metabolički inertna tkiva, što omogućava dobivanje trajnog zapisa o izloženosti metalima (Campana, 1999; Tzadik i sur., 2017).

2.4.1.1. Potočna pastrva (*Salmo trutta* Linnaeus, 1758) - rijeka Krka

Znanstvena klasifikacija:

Carstvo: Animalia

Koljeno: Chordata

Razred: Actinopterygii (zrakoperke)

Red: Salmoniformes (pastrvke)

Porodica: Salmonidae (pastrve)

Rod: *Salmo*

Vrsta: *Salmo trutta* Linnaeus, 1758

Potočna pastrva je autohtona vrsta riba u Hrvatskoj koja pripada porodici Salmonidae (pastrve) (Slika 6). Najbrojnija je i najrasprostranjenija autohtona vrsta pastrve u Europi, a unesena je u Sjevernu Ameriku, Australiju i srednju Afriku (Mrakovčić i sur., 2006). Široko

je geografski rasprostranjena zbog izražene sposobnosti adaptacije na novi okoliš, koloniziranja novih vodenih tokova, a poznata je i kao kvalitetna namirnica te je popularna vrsta za sportski ribolov (Klemetsen i sur., 2003). Reofilna je vrsta ribe koja uglavnom živi u gornjim i srednjim dijelovima rijeka te preferira hladnije i brže vodotoke temperature od 2 do 16 °C (Mrakovčić i sur., 2006).

Odrasle jedinke su predatori koji se hrane mnoštvom organizama uključujući manje ribe, žabe, školjkaše, rakušce, kao i kukce i njihove ličinke (Mrakovčić i sur., 2006), što ih stavlja na vrh hranidbenih mreža u slatkovodnim ekosustavima. Dosežu dužinu od 15 do maksimalno 70 cm te masu od 100 g do 6 kg, ovisno o starosti jedinke i okolišnim uvjetima. Postaju spolno zrele s 2-3 godine, a mrijest se odvija pri nižim temperaturama i uglavnom počinje u jesen (Klemetsen i sur., 2003; Mrakovčić i sur., 2006).

Prema Crvenoj knjizi slatkovodnih riba Hrvatske potočna pastrva se ubraja u osjetljive vrste, a glavni razlozi ugroženosti su onečišćenje i mehaničke promjene korita rijeka (pregrađivanje vodotoka, betoniranje, izgradnja hidroelektrana), čime se onemogućava migracija prema izvorišnim dijelovima što je potrebno za mrijest (Mrakovčić i sur., 2006). Negativan utjecaj ima i poribljavanje vodotoka koje može utjecati na genetičku raznolikost autohtone populacije, a dodatno utječe i na problematičnost taksonomskog statusa ove vrste. Naime, opisane su mnogobrojne forme i podvrste, poput jezerske i potočne forme čiji statusi u Hrvatskoj još nisu u potpunosti razjašnjeni i regulirani (Mrakovčić i sur., 2006).

Potočna pastrva se već pokazala kao dobar bioindikator (Linde i sur., 1998), a kako je zastupljena i u ljudskoj prehrani, zagađenje metalima u ove vrste je važan čimbenik vezan i uz ljudsko zdravlje (Culioli i sur., 2009).

Kao vrsta koja je u rijeci Krki zastupljena tijekom čitave godine, odabrana je kao bioindikatorski organizam u našem istraživanju za procjenu stanja kakvoće vode ove dinaridske krške rijeke i istraživanja utjecaja antropogenih aktivnosti, posebno industrijskih i komunalnih otpadnih voda, na živi svijet ovog ekosustava i obližnjeg NP Krka.



Slika 6. Prikaz potočne pastrve *Salmo trutta* Linnaeus, 1758 iz rijeke Krke

Autor fotografije: dr. sc. Vlatka Filipović Marijić

2.4.1.2. Babuška (*Carassius gibelio* Bloch, 1782) - rijeka Ilova

Znanstvena klasifikacija:

Carstvo: Animalia

Koljeno: Chordata

Razred: Actinopterygii (zrakoperke)

Red: Cypriniformes (šaranke)

Porodica: Cyprinidae (šarani)

Rod: *Carassius*

Vrsta: *Carassius gibelio* Bloch, 1782

Babuška je invazivna vrsta u Hrvatskoj koja pripada u porodicu Cyprinidae (šarani) (Slika 7). Ova vrsta je autohtona za područje Azije, točnije Sibira, ali danas je široko rasprostranjena po čitavoj Aziji i Europi (Britton i sur., 2011). Od staništa preferira pliće lagune, jezera i sporije vodotoke. Također, odgovaraju joj više temperature pa je stoga karakteristična za eutrofna područja s puno vegetacije.

Hrani se planktonom, biljem, detritusom i sitnijim bentosom. Babuške mogu narasti do 45 cm, a najčešća im je dužina 20-25 cm, a masa do 3 kg. Obično se mrijeste u proljeće i/ili ljeto između travnja i srpnja na temperaturi iznad 14 °C (Kottelat i Freyhof, 2007; Šaši, 2008), a s obzirom na sposobnost ginogeneze, jajašca ne moraju biti oplodena od mužjaka babuške što često rezultira populacijama u kojima su puno zastupljenije ženke (Šaši, 2008).

Ima visok invazivni potencijal koji ugrožava autohtone vrste kroz kompeticiju za hranu i stanište te inhibiciju reproduktivne aktivnosti drugih vrsta, čime babuška brzo može postati dominantna vrsta u nekom ekosustavu i čak dovesti i do nestanka nekih ribljih vrsta (Ergüden, 2015). Iako je slatkovodna vrsta, babuška tolerira i određene razine saliniteta pa ponekad naseljava i bočate vode. Ima široku ekološku toleranciju koja uključuje različite uvjete hipoksije i anoksije, zagađenja i varijacija u temperaturi (De Boeck i sur., 2004; Kottelat i Freyhof, 2007; Topić Popović i sur., 2016) te se pokazala kao dobar indikator u procjeni zagađenja okoliša (De Boeck i sur., 2004; Falfushynska i sur., 2011; Tsangaris i sur., 2011), uključujući i zagađenje metalima.

S obzirom na to da je dominantna vrsta u rijeci Ilovi, odabrana je kao bioindikatorski organizam u našem istraživanju za procjenu stanja kakvoće vode ove panonske rijeke i istraživanja utjecaja antropogenih aktivnosti (poljopriveda, ribnjačarstvo, industrijske i komunalne otpadne vode) na živi svijet ovog ekosustava i obližnjeg PP Lonjsko Polje.



Slika 7. Prikaz babuške *Carassius gibelio* Bloch, 1782 iz rijeke Ilove

Autor fotografije: dr. sc. Vlatka Filipović Marijić

2.4.1.3. Probavilo kao bioindikatorski organ

Iako je pokazano da je unos metala putem hrane za ribe od jednakog ili čak većeg značaja nego unos metala vodom, probavilo riba je još uvijek rijetko korišteno kao bioindikatorski organ u okolišnim istraživanjima. Ipak, unos hranom je u nekim slučajevima važniji put unosa i za esencijalne elemente poput Cu, Zn i Fe (Clearwater, 2000; Bury i sur., 2003), ali i neesencijalne poput Tl (Lapointe i Couture, 2009).

Unos metala putem hrane je od velike važnosti jer uključuje metale koji se talože u sedimentu, što zapravo daje podatak o kroničnoj izloženosti organizama metalima, za razliku od unosa samo putem vode kroz škrge koji ukazuje isključivo na trenutačno stanje. Međutim, uz laboratorijska istraživanja koja se provode u kraćem trajanju i pri višim koncentracijama metala nego što su u okolišu, postoji svega nekoliko radova o akumulaciji metala u probavilu autohtonih slatkovodnih vrsta riba (Dallinger i Kautzky, 1985; Ünlü i sur., 1996; Sun i Jeng, 1998; Sures i sur., 1999; Andres i sur., 2000; Giguère i sur., 2004; Staniskiène i sur., 2006; Filipović Marijić i Raspor, 2010, 2012, 2014; Jarić i sur., 2011; Nachev i Sures, 2016; Yeltekin i Sağlam, 2019) te se navode samo ukupne koncentracije metala koje ne ukazuju na biološki i metabolički dostupnu, a time i potencijalno toksičnu frakciju metala, već uključuju i detoksicirane oblike metala (Wallace i sur., 2003; Urien i sur., 2018). Uz nekoliko istraživanja na klenovima iz rijeke Save u Hrvatskoj (Filipović Marijić i Raspor, 2010, 2012), do naših istraživanja potočne pastrve i babuške nije bilo literaturnih podataka o citosolskim koncentracijama metala u probavilu niti jedne vrste riba, što ukazuje na nedostatak detaljnijih informacija o toksičnim učincima metala na organizme do kojih dolazi putem prehrane.

Važno je procijeniti bioraspoloživost metala, odnosno količinu metala koju organizam može koristiti nakon probavljanja i apsorpcije što ovisi o vrsti prehrane i samim fiziološkim potrebama pojedinog organizma (Fairweather Tait, 1983). Općenito se probavni sustav u riba sastoji od usne šupljine, ždrijela, jednjaka, želuca, srednjeg i stražnjeg crijeva, a većina

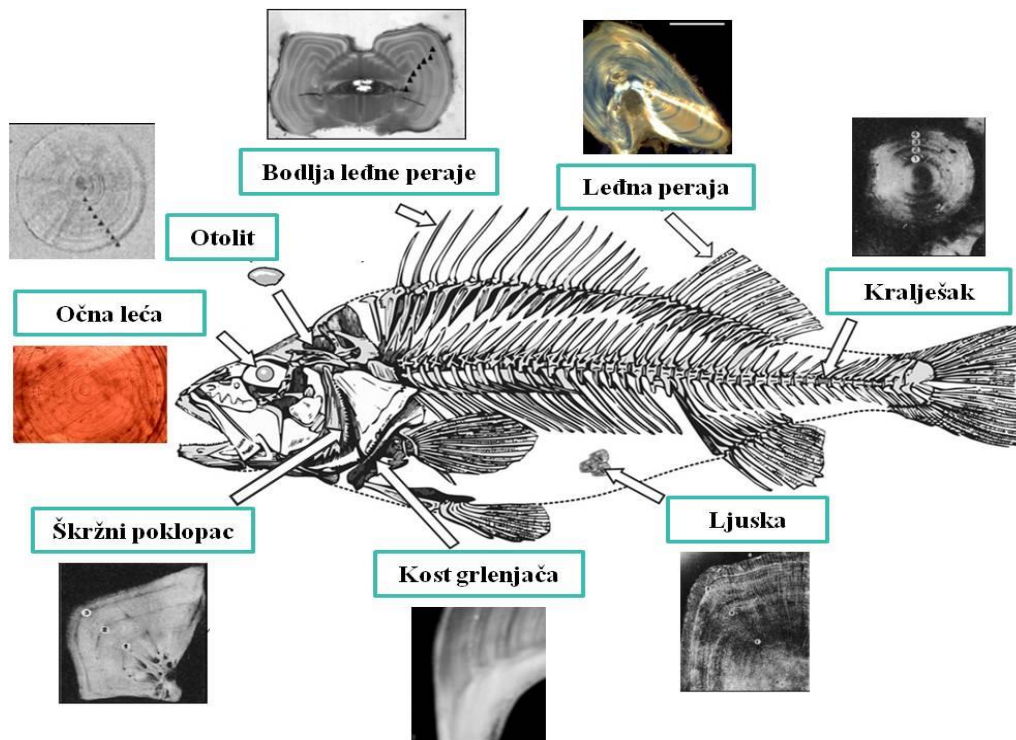
apsorpcije tvari odvija se u crijevu (Ojo i Wood, 2008). Osnovna građa stijenke probavila riba je sljedeća:

- a) Sluznica (*tunica mucosa*) koju čine pokrovni epitel, sloj lamine proprije i lamine muskularis mukoze. Stanice epitela tvore mikrovile, a glavni tipovi stanica su enterociti (odgovorni za razgradnju i apsorpciju) te vrčaste stanice (luče mukus). Laminu propriju čini rahlo vezivno tkivo, žile, žlijezde i limfno tkivo, a muskularis mukoze je tanki mišićni sloj.
- b) Podsluznica (*tunica submucosa*) koju čini gušće vezivno tkivo, krvne i limfne žile te živčano tkivo.
- c) Mišićni sloj (*tunica muscularis*) koji čine glatke mišićne stanice koje obavijaju sluznicu i podsluznicu i često se sastoji od dva sloja mišića, unutarnjeg kružnog i vanjskog uzdužnog.
- d) Seroza koju čini jednoslojni pločasti epitel te tanki sloj vezivnog tkiva s masnim stanicama, krvnim i limfnim žilama.

Glavnu funkciju epitela probavne cijevi predstavlja njegova uloga selektivno propusne barijere koja omogućava prijenos i probavu hrane, apsorpciju proizvoda probave, proizvodnju hormona, te proizvodnju sluzi. Metali se pri tome u probavnom sustavu vežu na mukozni sloj, odakle se apsorbiraju u organizam ili izlučuju putem mukusa, ovisno o koncentraciji i potrebama organizama.

2.4.1.4. Kalcificirane („tvrde“) strukture (otoliti i ljuske) kao indikatorska tkiva

Tvrde strukture riba su strukture bogate kalcijem koje mogu pružiti informacije o životu ribe zbog vidljivih zona rasta. Uključuju strukture poput otolita, ljuski, peraja, kralješaka ili očne leće te su uglavnom sastavljene od tri glavna tipa matriksa - kalcijevog karbonata, hidroksiapatita, i organskog matriksa (Slika 8). Primjerice, očna leća je sastavljena uglavnom od organskog matriksa, ali ima isti potencijal za razjašnjavanje događaja u životu riba (Dove i Kingsford, 1998; Clarke i sur., 2007) kao i druge tvrde strukture bogate kalcijem.

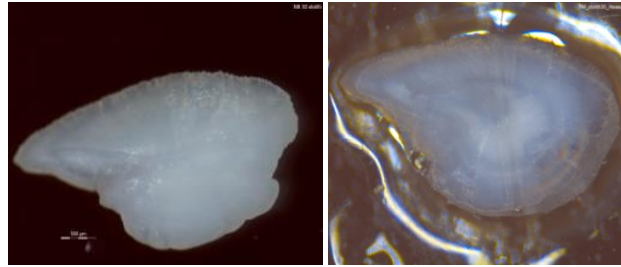


Slika 8. Kalcificirane/tvrde strukture riba (preuzeto i prilagođeno iz Tzadik i sur., 2017 i Vilizzi, 2018)

Unatoč tome što na akumulaciju metala u kalcificiranim tkivima (otoliti, ljuske, kosti) gotovo zanemarivo utječu procesi detoksikacije ili metaboličke transformacije, različita kalcificirana tkiva su u dosadašnjim istraživanjima uglavnom korištena za utvrđivanje sastava i veličine populacija riba (Mulligan i sur., 1983; Campana i sur., 2000; Milton i sur., 2008), istraživanje migracija riba (Hamer i sur., 2006; Sturrock i sur., 2012; Tabouret i sur., 2012; Prohaska i sur., 2016), a samo u manjem obujmu kao bioindikator onečišćenja okoliša (Saquet i sur., 2002; Darafsh i sur., 2008; Ranaldi i Gagnon, 2008, Sultana i sur., 2017). Nadalje, postoji svega nekoliko istraživanja koja uspoređuju koncentracije metala u više struktura odjednom, neovisno o tome radi li se o procjeni onečišćenja ili istraživanju ribljih migracija i sastava populacija (Gillanders, 2001; Clarke i sur., 2007; Wolff i sur., 2013; Kalantzi i sur., 2019).

Otoliti (Slika 9) su kalcificirane strukture u unutarnjem uhu riba koštunjača sastavljene od slojeva aragonita u proteinskom matriksu koji se taloži tijekom čitavog života riba (Campana i Nielson, 1982), što rezultira koncentričnom prstenastom strukturom koja se sastoji od širih prozirnih dijelova kalcijeva karbonata i uskih neprozirnih zona organskog matriksa (Brothers i sur., 1976). Ove metabolički inertne strukture pokazuju prstenove rasta u svojoj mikrostrukturi koji odražavaju starost ribe i privremeni rast ribe u odnosu na uvjete okoliša (Campana, 1999; Radhakrishnan i sur., 2009). Stoga analiza njihove mikrokemije daje

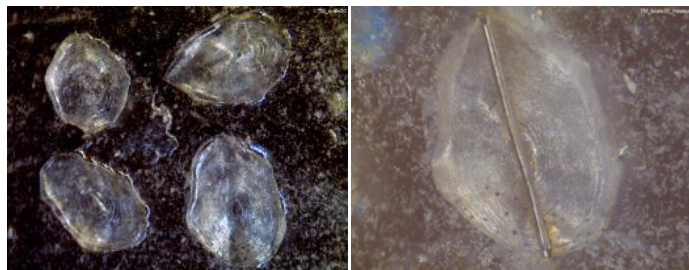
vremensku razlučivost izloženosti različitim metalima, budući da i anorganski i organski dijelovi otolita imaju sposobnost ugradnje metala u svoje strukture (Saquet i sur., 2002).



Slika 9. Prikaz otolita potočne pastrve iz rijeke Krke prije i nakon postupka poliranja te laserske ablacije

Autor fotografija: Tatjana Mijošek

Ljuske (Slika 10) riba koštunjača sastoje se od tankog, tvrdog, vanjskog, dobro mineraliziranog sloja koji je uglavnom izgrađen od kalcijem siromašnog (nestehiometrijskog) hidroksiapatita $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ koji prekriva deblji, slabo mineralizirani sloj. Smanjuju otpor ribama pri kretanju kroz vodu, služe kao skladište minerala i hranjivih tvari te pružaju zaštitu tijelu, osobito za bočnu prugu koja je odgovorna za prepoznavanje vibracija i kretanje u vodi (Able i Lamonaca, 2006). Zbog koncentričnog rasta često se koriste za određivanje dobi riba, kao i za identifikaciju vrsta. Njihova prstenasta struktura omogućava i procjenu unosa metala tijekom vremena i može dati podatke o dugoročnoj izloženosti riba metalima, za razliku od koncentracija metala u mekim tkivima. Ukoliko dođe do oštećenja ili gubitka ljusaka, ribe ih mogu regenerirati. Međutim, nove ljuske ne pokazuju karakteristične koncentrične uzorke u središtu te se stoga ne mogu koristiti za analizu čitave povijesti života ribe, uključujući i migracije, izloženost metalima te okolišne uvjete općenito jer ne predstavljaju sve faze u njihovom životu (Ohira i sur., 2007). Budući da prekrivaju površinu tijela riba, prednost je što upotreba ljusaka omogućava neletalnu alternativu u procjeni izloženosti riba metalima i ostalim zagađivačima (Wells i sur., 2003, Muhlfield i sur., 2005).



Slika 10. Prikaz ljusaka potočne pastrve iz rijeke Krke prije i nakon postupka laserske ablacije

Autor fotografija: Tatjana Mijošek

2.4.2. Rakušci kao bioindikatorski organizmi

Rakovi porodice Gammaridae, a osobito roda *Gammarus* naseljavaju brojna slatkovodna staništa te su često dominantni organizmi u zajednicama makrozoobentosa svojom brojnošću i masom (MacNeil i sur., 1997). Često su korišteni kao bioindikator onečišćenja okoliša zbog svoje široke rasprostranjenosti, velike gustoće populacija i brojnosti, spolnog dimorfizma, jednostavnog uzorkovanja i determinacije, sposobnosti kolonizacije ekosustava, relativno dugog životnog vijeka te osjetljivosti na različite toksikante (Ritterhof i sur., 1996; Geffard i sur., 2007). Nadalje, imaju važnu ulogu u slatkovodnim ekosustavima jer su važan posrednik između primarne i sekundarne produkcije u hranidbenim mrežama, kao i vršni predatori, tako da smanjena ili povećana brojnost može radikalno utjecati na strukture čitavih zajednica (Kunz i sur., 2010; Syrovátka i sur., 2020). Razgradnjom nežive organske tvari pomažu oslobađanju i kruženju hranjivih tvari vezanih u detritusu, međutim recentna istraživanja ukazuju i na vršnu regulatornu ulogu rakušaca u slatkovodnim ekosustavima (Syrovátka i sur., 2020). Naime, čak i nativne vrste rakušaca mogu pokazivati omnivornu, pa čak i predatorsku prehranu te utjecati na brojnost i sastav čitavih vodenih zajednica, a osobito pojedinih skupina beskralješnjaka (Syrovátka i sur., 2020). Nadalje, rakušci su rezerva hrane za mnoge beskralješnjake, ribe, vodozemce i ptice te imaju ključnu ulogu u usitnjavanju listinca producirajući sitne čestice organskog materijala koji može biti korišten od drugih funkcionalnih grupa manjih organizama (Wallace i Webster, 1996) te povećavaju biodostupnost nutrijenata razbijajući organsku tvar u finije čestice (Boeker i Geist, 2015) što je ključno za opskrbu energijom u gornjim dijelovima vodotoka olakšavajući produktivnost u vodenim ekosustavima gdje je smanjena primarna produkcija (Collins i sur. 2016).

2.4.2.1. *Gammarus balcanicus* Schäferna, 1922 i *Echinogammarus acarinatus* Karaman, 1931 - rijeka Krka

Znanstvena klasifikacija:

Carstvo: Animalia

Koljeno: Arthropoda

Razred: Malacostraca

Red: Amphipoda

Porodica: Gammaridae

Rod: *Gammarus* i *Echinogammarus*

Vrste: *Gammarus balcanicus* Schäferna, 1922 i *Echinogammarus acarinatus* Karaman, 1931

Rakušci (Amphipoda) u rijeci Krki su prema istraživanju Gottstein i sur. (2007) zastupljeni s 13 vrsta iz 7 rodova i 5 porodica. Predstavnici porodice Gammaridae prisutni su s vrstama iz rodova *Echinogammarus* (*E. acarinatus*, *E. pungens*, *E. stammeri*, *E. veneris*), *Fontogammarus* (*F. dalmatinus krkensis*) i *Gammarus* (*G. balcanicus*, *G. aequicauda*). Pri tome je najbrojnija vrsta, zabilježena na 13 lokaliteta toka rijeke Krke, vrsta *G. balcanicus*, koja je i inače široko rasprostranjena vrsta na području Europe (Karaman i Pinkster, 1987; Pinkster, 1993; Gottstein i sur., 2007; Hou i Sket, 2016).

U našem istraživanju u gornjem dijelu toka Krke, kod izvora kao referentnoj postaji, zabilježene su i uzorkovane dvije vrste rakušaca (*G. balcanicus* i *E. acarinatus*, Slika 11), dok su na onečišćenoj postaji nizvodno od grada Knina bile prisutne samo jedinke vrste *G. balcanicus*. Vrsta *E. acarinatus* je endemska vrsta ograničena na rijeke Jadranskog sliva na području Hrvatske, Bosne i Hercegovine te Crne Gore (Žganec i sur., 2016).



Slika 11. Prikaz rakušaca *Gammarus balcanicus* Schäferna, 1922 i *Echinogammarus acarinatus* Karaman, 1931

Autor fotografija: prof. dr. sc. Sanja Gottstein

2.4.2.2. *Gammarus fossarum* Koch, 1936 i *Gammarus roeselii* Gervais, 1835 - rijeka Ilova

Znanstvena klasifikacija:

Carstvo: Animalia

Koljeno: Arthropoda

Razred: Malacostraca

Red: Amphipoda

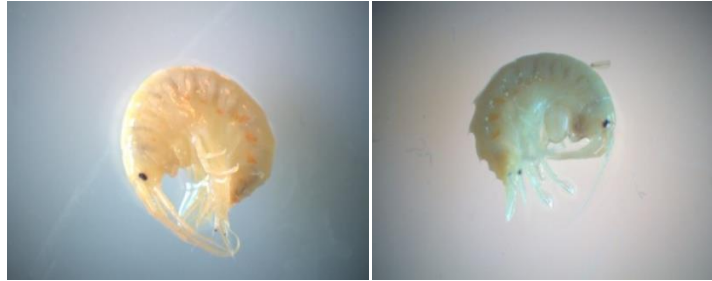
Porodica: Gammaridae

Rod: *Gammarus*

Vrste: *Gammarus fossarum* Koch, 1936 i *Gammarus roeselii* Gervais, 1835

U rijeci Ilovi bile su zastupljene dvije vrste rakušaca: *Gammarus fossarum* s većom zastupljenošću te *Gammarus roeselii* (Slika 12) u manjem broju, i to samo na najuzvodnijoj

postaji rijeke Ilove kod sela Maslenjača, na kojoj su uzorkovani samo voda i sediment, dok na postajama kod sela Ilova i sela Trebež, gdje su uzorkovane i ribe, su zabilježeni samo pojedinačni nalazi invazivne vrste rakušaca *Dikerogammarus haemobaphes* (Eichwald, 1841).



Slika 12. Prikaz rakušaca *Gammarus fossarum* Koch, 1936 i *Gammarus roeselii* Gervais, 1835

Autor fotografija: Tomislav Kralj

2.4.3. Kukaši

2.4.3.1. Tipologija, rasprostranjenost i sistematika kukaša

Mnogobrojne vrste slatkovodnih riba su domadari različitih vrsta obligatnih ili fakultativnih nametničkih vrsta koje im mogu nanijeti određenu štetu, ali najčešće bez smrtnog ishoda. Utjecaj nametnika može predstavljati koegzistenciju koja ne dovodi do narušavanja homeostaze domadara, ali može uzorkovati i opće i laganije simptome koji ukazuju na povećani broj nametnika, kao i poremećaje određenih fizioloških procesa domadara koji povremeno mogu dovesti čak i do smrti (Macrogliese, 2004; Sures, 2006; Timi i Poulin, 2020). Nametničke organizme moguće je podijeliti na ektoparazitske i endoparazitske vrste. Ektoparaziti se nalaze na vanjskoj površini domadara, dok se endoparaziti nalaze u unutrašnjim dijelovima uključujući mišiće, membrane i unutrašnje organe. Kod riba, ektoparaziti se najčešće nalaze na koži, ljuskama škrgama ili perajama, dok su endoparaziti najčešći u probavnom sustavu.

Česti crijevni nametnici u probavnom sustavu slatkovodnih riba pripadaju koljenu kukaša (Acanthocephala). Ime im dolazi od grčkih riječi acanthias (bodljikav) i cephalo (glava) (Crompton i Nikol, 1985). Životni ciklus im je složen, a uključuje međudomadare, krajnje domadare i potencijalno fakultativne domadare (Kennedy, 2006).

Imaju izrazito složenu sistematiku te njihov položaj i odnosi prema drugim skupinama i dalje nisu u potpunosti razjašnjeni. Svrstavaju se u natkoljeno Gnathifera zajedno s kolnjacima (Rotifera) te su filogenetske analize pokazale da je razred Bdelloidea sestrinska

grupa s kukašima (Steinauer i sur., 2005; Gazi i sur., 2012; García-Varela i Pérez-Ponce de León, 2015). Dijele se na 4 razreda, te 26 porodica s 122 roda s opisanih 1298 vrsta (Amin, 2013). Tri glavna razreda su Archiacanthocephala, Palaeacanthocephala i Eoacanthocephala, dok je četvrti razred Polyacanthocephala zastupljen s vrlo malim brojem vrsta koje koriste južnoameričke kajmane kao krajnje domadare (Kennedy, 2006). Pripadnici različitih razreda morfološki su različiti, imaju različite međudomadare i konačne domadare te ekologiju i staništa (Kennedy, 2006; García-Varela i Pérez-Ponce de León, 2015). Eoacanthocephala su u cijelosti vodeni nametnici koji koriste člankonošce, pripadnike skupina Ostracoda i Copepoda, kao međudomadare te ribe, vodozemce i gmazove, posebno kornjače, kao krajnje domadare. Nasuprot tome, predstavnici razreda Archioacanthocephala su isključivo kopneni nametnički organizmi koji koriste kopnene kukce kao međudomadare, a ptice i sisavce kao konačne domadare. Ipak, najveći broj poznatih vrsta kukaša pripada razredu Palaeacanthocephala, čiji su najčešći krajnji domadari ribe (38,8 %), ptice (36,9 %) i sisavci (20,5 %). Iako su se uspješno prilagodili i kopnenom životu zaražavajući sve razrede kralješnjaka, većina ih ipak zaražava vodene životinje (62,7%), najčešće slatkovodne (Kennedy, 2006). Međutim, i zaraženost riba varira ovisno o ekologiji ribljih vrsta i njihovim prehrambenim navikama. Česti domadari kukaša su slatkovodne omnivorne i predatorske vrste, dok herbivorne vrste nisu domadari s obzirom da se te ribe ne hrane međudomadarima (rakovima) potrebnim za razvoj kukaša.

2.4.3.2. Vanjska i unutrašnja građa kukaša

Kukaši imaju crvoliko i bilateralno simetrično tijelo te su najčešće bijele, krem ili žućkaste do narančaste boje, a dužina im varira od svega nekoliko mm do 65 cm. Tijelo nije segmentirano, ali se može podijeliti na područje trupa te rila (proboscisa) s kukicama koje se mogu uvući i u unutrašnjost tijela u strukturu receptakula. Rilo najčešće ima radijalnu simetriju te se broj, oblik i raspored kukica na njemu razlikuje između vrsta i rodova pa se to svojstvo koristi i u određivanju vrsta kukaša (Kennedy, 2006). Pomoću rila kukaši probijaju stijenku probavila domadara te se trajno pričvršćuju (Fijan, 2006). Uz kukice, neke vrste kukaša imaju i dodatne hitinizirane strukture, nazvane trnovima, po ostatku tijela čija je uloga također vezana uz pričvršćivanje u domadaru (Kennedy, 2006).

Stijenku tijela kukaša čini vanjski tegumentum te slojevi prstenastih i uzdužnih mišića, a na površini se nalazi glikokaliks koji ima ulogu u resorpciji hrane i obrani od probavnih enzima domadara (Habdija i sur., 2011). Kroz te dijelove se proteže i sustav lakuna te čitava stijenka ima ulogu u metabolizmu, prehrani i zaštiti kukaša. U prednjem dijelu tijela se nalazi

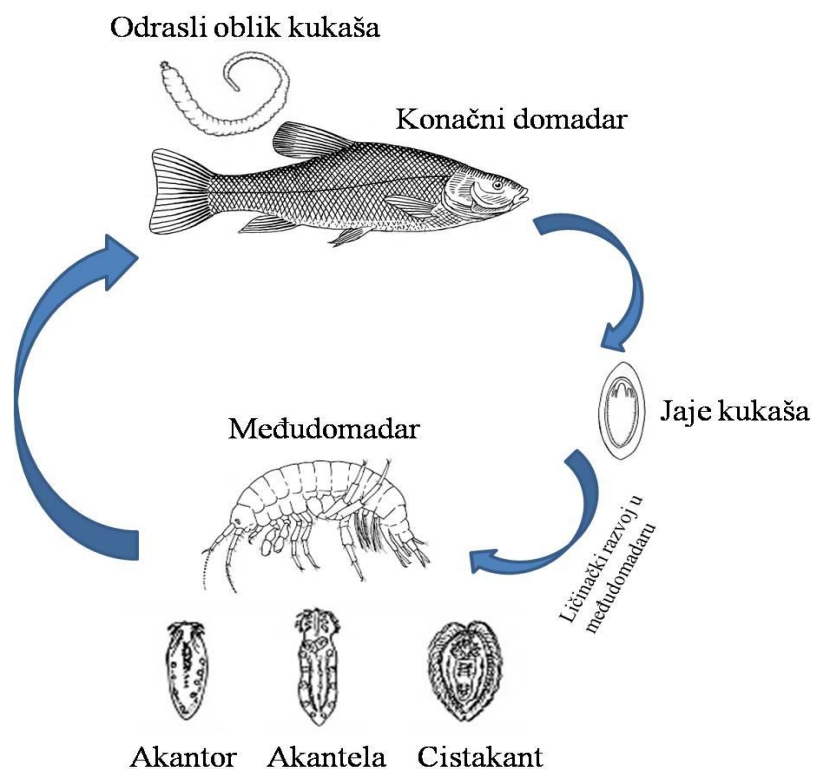
receptakul, vrećasta struktura u koju se uvlači rilo uz koju je smješten i jedan par lemniska za koje se ne zna imaju li još neku dodatnu uloga, ali povećavaju slobodnu površinu stijenke tijela u unutrašnjosti kukaša te kao hidaruličke vrećice olakšavaju ispužanje rila (Crompton i Nickol, 1985; Habdija i sur., 2011).

Unutrašnje strukture su malobrojne jer kukaši nemaju usta, probavilo, i sustav za izlučivanje te hranjive tvari vjerojatno apsorbiraju kroz stijenkiju tijela iako sam mehanizam nije u potpunosti razjašnjen (Crompton i Nickol, 1985). Kroz površinu tijela se vjerojatno odvija i izlučivanje, premda neke vrste ovih nametnika posjeduju i protonefridije (Crompton i Nickol, 1985). Međutim, imaju razvijen živčani te osobito reproduktivni sustav. Živčani sustav čine veliki središnji ganglij ispod vrećice rila s pripadajućim živcima, dok u stražnjem dijelu tijela postoje genitalni gangliji koji su kod mužjaka podijeljeni na dva simetrična ganglija na bazi kopolaturnih organa, a kod ženki ih čini nakupina stanica oko gonopore. Kukaši su razdvojenog spola, a razmnožavanje je isključivo spolno uz unutarnju oplodnju (Kennedy, 2006). Muški reproduktivni sustav čine najčešće dva testisa, ispod kojih se nalazi nekoliko parova cementnih žlijezdi kroz koje luče ljepljivu izlučevinu na gonoporu nakon kopolacije. Uz to, dijelovi reproduktivnog sustava mužjaka su i ekskretorni kanal, kopolaturni organ te kopolaturni tobolac (Crompton i Nickol, 1985; Kennedy, 2006). Reproductivni sustav ženki je jednostavniji i čine ga jajnici, koji u obliku ovalnih granuloznih tvorbi plutaju oko ligamenta, te se dalje izdvajaju vagina, maternica, zvonoliko proširenje maternice i jajovodi. Oplodena jajašca plutaju u trbušnoj šupljini ženki dok nisu dovoljno zrela da dođu u probavni sustav domadara i putem fecesa u okoliš gdje započinju složen ličinački životni ciklus od prvog stadija nazvanog akantor (Slika 14).

2.4.3.3. Životni ciklus kukaša

Zreli, zaštićeni akantori, odnosno jaja, ispuštaju se u probavni trakt domadara kojeg napuštaju putem fecesa (Slika 14). Zvonoliko proširenje maternice kod ženki kukaša funkcionira na principu uređaja za razdvajanje jaja koji dopuštaju da se u okoliš otpuštaju isključivo zreli akantori zarazni za međudomadara. Jaja predstavljaju mirujući i otporni stadij koji može preživjeti nepovoljne uvjete, uključujući i velike temperaturne fluktuacije (-16 °C do +26 °C, Kennedy, 2006). Nadalje, jaja različitih vrsta kukaša imaju određene specifične prilagodbe kako bi njihov prijenos bio što uspješniji. Kod nekih vrsta, jaja ispuštaju filamente čime se lakše pričvrste za vodeno bilje, kod nekih se vrsta grupiraju u klastere, ili pak uzimaju veće količine vode čime se šire i lakše plutaju u zoni svojih međudomadara (Kennedy, 2006). Životni ciklus se nastavlja kad akantore pojede odgovarajući međudomadara, što je uvijek neka

vrsta člankonožaca – Arthropoda (Slika 14). U slučaju kukaša koji zaražavaju ribe, međudomadari su najčešće rakovi (Kennedy, 2006). U tijelu međudomadara akantor se prvo razvija u ličinku akantelu, koja prelazi u hemocel gdje se razvija u konačni i infektivni ličinački stadij, cistakant (Slika 14). To je mirujući stadij koji može živjeti jednako dugo kao i međudomadar (najčešće do godinu dana) te omogućava preživljavanje nepovoljnih uvjeta. Ciklus se zatvara kad konačni domadar (kralješnjak) pojede međudomadara i cistakant dospijeva u probavni sustav domadara i razvija se u odrasli, spolno zreli oblik koji ponovno može stvoriti jajašca (Slika 15, Kennedy, 2006). Životni vijek odraslog oblika kukaša uglavnom ne prelazi nekoliko mjeseci (Kennedy, 2006). Međutim, u nekim slučajevima, ribe, gmazovi ili vodozemci se mogu uključiti u životni ciklus kao paratenični ili fakultativni domadari koji nisu prikladni kao konačni domadari, ali sudjeluju u prijenosu cistakanta do konačnog domadara (García-Varela i Pérez-Ponce de León, 2015). Cistakant u parateničnom domadaru preživljava iako neće rasti i dalje se razvijati sve dok takvog domadara ne pojede prikladni krajnji domadar u kojemu cistakant dalje nastavlja svoj razvoj do odraslog oblika kukaša (Kennedy, 2006; García-Varela i Pérez-Ponce de León, 2015).



Slika 14. Životni ciklus kukaša (preuzeto i prilagođeno iz García-Varela i Pérez-Ponce de León, 2015)

2.4.3.4. Kukaši kao bioindikatorski organizmi

U proteklih dvadesetak godina raste interes za otkrivanjem veze između onečišćenja i parazitizma u vodenim ekosustavima te mogućom primjenom nametnika kao indikatora kakvoće vode, što je dovelo do stvaranja novog znanstvenog područja nazvanog “okolišna parazitologija” (Sures, 2001; Sures i sur., 2017). Kao i većina nametnika, i kukaši pokazuju mnogobrojne osobine dobrih bioindikatora zagađenja okoliša: imaju poznat životni ciklus, reproduciraju se unutar domadara, uglavnom nemaju značajnog utjecaja na patologiju i ponašanje domadara, široko su rasprostranjeni te se lako uzorkuju (Kennedy, 2006). Ipak, kukaši imaju i neke karakteristike koje ne pogoduju indikatorskim organizmima: zbog mobilnosti njihovih domadara postoji velika varijabilnost u akumulaciji zagađivala između jedinki kukaša, što otežava procjenu razlika između lokaliteta, te imaju relativno kratak životni vijek (Siddal i Sures, 1998).

Unatoč određenim nedostacima, pokazuju potencijal kao bioindikator zagađenja metalima, eutrofikacije ili pak zakiseljavanja okoliša (Kennedy, 2006). Naime, zakiseljavanje voda, odnosno pad pH vrijednosti uzrokuju manju brojnost parazita tako da promjene u strukturi i raznolikosti zajednica kukaša i ostalih nametnika mogu poslužiti kao bioindikator zagađenja (MacKenzie, 1999). Organsko zagađenje također može utjecati na razinu invazije kukašima iako su promjene razine nametnika uglavnom uvjetovane utjecajem na brojnost člankonožaca kao međudomadara te su stoga člankonošci zapravo bolji indikatori organskog zagađenja.

Dosadašnja istraživanja nekoliko znanstvenih grupa u svijetu potvrdila su vrlo učinkovitu sposobnost akumulacije metala u nekoliko vrsta kukaša (*Pomphorynchus laevis*, *Acanthocephalus lucii*, *Acanthocephalus anguille*), koja je viša u odnosu na druge vodene organizme, uključujući i školjkaša vrste *Dreissena polymorpha* kao često korištenog bioindikatorskog organizma (Sures i sur., 1997, 2017; Sures, 2001, 2004; Filipović Marijić i sur., 2013, 2014; Nachev i Sures, 2016). Kukaši apsorbiraju hranjive tvari preko stijenke probavila domadara, čime ujedno unose i esencijalne metale kao neophodne mikronutijente. Uz esencijalne se, zbog kemijske sličnosti, istim putevima unose i neesencijalni metali, za koje se pokazalo da se posebno učinkovito akumuliraju u kukašima. Posljedično, kukaši potencijalno pružaju i zaštitnu ulogu svojim domadarima akumulirajući dio unesenih metala (Filipović Marijić i sur., 2013; Sures i sur., 2017). Omjer koncentracija metala u kukašima i pojedinim tkivima riba nazvan je biokoncentracijski faktor (BCF) te predstavlja omjer kratkotrajne i dugotrajne izloženosti metalima s obzirom da je životni vijek kukaša relativno

kratak (50-140 dana) u usporedbi s puno dužim životnim vijekom riba (10-15 godina) (Kennedy, 1985; Kottelat i Freyhof, 2007). Stoga viši omjer ukazuje na porast nedavne izloženosti metalima s obzirom na brz unos metala u nametnike, dok niži omjer upućuje na dugotrajniju izloženost (Siddal i Sures, 1998). Uz to, koncentracije metala u kukašima predstavljaju i biološki raspoloživu frakciju koja je unesena iz probavila ribe u nametnike. Osim podataka o koncentracijama metala te BCF u kukašima, do sada nije razjašnjen mehanizam učinkovitog unosa i vezanja metala, kao niti mehanizam zaštite od toksičnih djelovanja metala. Uz to, u dostupnoj znanstvenoj literaturi nema podataka o akumulaciji metala u vrsti *Dentitruncus truttae*, uzorkovanoj u probavilu potočnih pastrva iz rijeke Krke.

2.4.3.5. *Dentitruncus truttae* Sinzar, 1955

Znanstvena klasifikacija:

Carstvo: Animalia

Koljeno: Acanthocephala

Razred: Palaeacanthocephala

Red: Echinorhynchida

Porodica: Illiosentidae

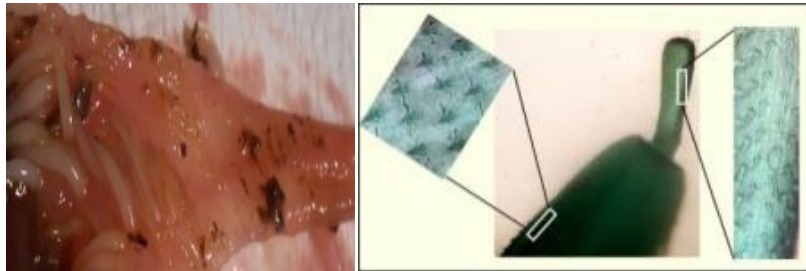
Rod: *Dentitruncus*

Vrsta: *Dentitruncus truttae* Sinzar, 1955

Vrsta *D. truttae* (Slika 15) jedina je vrsta svojeg roda unutar porodice kukaša Illiosentidae te ima ograničen areal rasprostranjenosti. Zabilježena je u nekim područjima Bosne i Hercegovine (Šinžar, 1956; Čanković i sur., 1968), Italije (Moravec, 2004; Dezfuli i sur., 2008) te Hrvatske (Topić-Popović i sur., 1999; Barišić i sur., 2018). Najčešći zabilježeni domadar ove vrste kukaša je potočna pastrva iako su nađeni i u nekim drugim ribljim vrstama poput mekousne i kalifornijske pastrve te jegulje (Čanković i sur., 1968; Dezfuli i sur., 2009, 2012). Visoka invadiranost potočnih, ali i kalifornijskih pastrva (*Oncorhynchus mykiss*) ovom vrstom kukaša u rijeci Krki potvrđena je tijekom više godina (2005.-2008., 2015.-2016.) i iznosila je 70-100% (Vardić Smrzlić i sur., 2013; Barišić i sur., 2018) te je pretpostavljeni životni ciklus ove vrste u našem podneblju *D. truttae* → *E. acarinatus*/*G. balcanicus* → *S. truttae*/*O. mykiss* (Vardić Smrzlić i sur., 2013).

Ultrastrukturalno istraživanje ove vrste ukazalo je na postojanje cilindričnog proboscisa s 18 longitudinalnih redova kukica. U svakom redu nalazi se 18 kukica, ponekad 19-20 (Dezfuli i sur., 2008). Na tijelu postoji i veći broj trnova čija brojnost se smanjuje prema

posteriornom dijelu tijela. Analiza elektronskim mikroskopom ukazala je i na postojanje prugica na kukicama *D. truttae*, čija funkcija nije poznata, a neuobičajene su za druge vrste kukaša (Dezfuli i sur., 2008).



Slika 15. Prikaz kukaša *Dentitruncus truttae* Sinzar, 1955

Autori fotografija: dr. sc. Vlatka Filipović Marijić i dr. sc. Irena Vardić Smrzlić

Ova vrsta kukaša u probavilu domadara prodire vrlo duboko pri čemu može dovesti do progresivnih i regresivnih promjena poput upalnih procesa, oštećenja crijevnih resica, smanjenog broja mukoznih stanica ili povećanog broja eozinofila (Dezfuli i sur., 2011; Barišić i sur., 2018).

2.5. Biomonitoring i biomarkeri

S obzirom na to da su slatkovodni ekosustavi među najugroženijim, a često i najosjetljivijim ekosustavima, potrebno je sveobuhvatnim i interdisciplinarnim pristupom i metodama procijeniti stvarno stanje tih sustava te pratiti dinamiku unosa te utjecaj zagađivala i njegove posljedice, odnosno provoditi redoviti monitoring. Monitoring obuhvaća proučavanje utjecaja okolišnih čimbenika u prostoru i vremenu, s ciljem prikupljanja podataka o prisutnosti onečišćujućih tvari, njihovih izvora, kao i određivanja njihovih koncentracija na mjernim točkama kako bi se prepoznale eventualne opasnosti za promatrani ekosustav. Može biti kemijski (CM, eng. *Chemical Monitoring*), bioakumulacijski (BAM, eng. *Bioaccumulation Monitoring*), biološki (BEM, eng. *Biological Effects Monitoring*), zdravstveni (HM, eng. *Health Monitoring*) i monitoring ekosustava (EM, eng. *Environmental Monitoring*).

2.5.1. Biomonitoring

S obzirom na to da uobičajeno provodeći kemijski monitoring koji obuhvaća mjerenje razine metala u vodi/ili sedimentu, ne daje pouzdanu informaciju o mogućem učinku na same organizme, uobičajeno je mjeriti i bioakumulaciju u prikladno odabranim akvatičkim bioindikatorskim organizmima, kao dio biomonitoringa koji omogućava procjenu mogućih

učinaka metala na akvatičku biotu. Također, mjerenje koncentracija metala u vodi odražava samo stanje u trenutku uzorkovanja, dok je sediment ipak bolji pokazatelj dugoročne izloženosti, ali ti metali nisu uvijek u potpunosti bioraspoloživi organizmima (Phillips i Rainbow, 1993) niti dobivamo informacije o potencijalno toksičnim frakcijama metala i njihovim toksičnim učincima na biotu.

Biološki monitoring ili biomonitoring stoga koristi različite metode praćenja stanja ekosustava određivanjem bioloških promjena (biomarkera) u indikatorskim organizmima, odnosno njihovim organima i tkivima, što omogućava otkrivanje štetnih učinaka. Cilj biomonitoringa je što ranije odrediti promjene na staničnoj razini organizama uslijed izloženosti metalima, prije nego što (sub)letalno djelovanje zahvati više sinekološke razine. Biomonitoring omogućava procjenu integriranog učinka složenih skupina zagađivala, omogućava i otkrivanje učinaka niskih koncentracija akumuliranih metala zbog brzih molekularnih i staničnih odgovora organizama te ne zahtijeva kontinuirano uzorkovanje (Zhou i sur., 2008). Zhou i sur. (2008) navode različite tehnike biomonitoringa: analizu bioakumulacije i biokemijskih promjena (biokemijski biomarkeri), morfološka praćenja (npr. impanseks), praćenja ponašanja (npr. biotestovi), analize na razini populacije (npr. gustoća, veličinske kategorije) i zajednice (npr. bogatstvo vrsta, sastav zajednice) te modeliranje (npr. razvoj modela akumuliranja i otpuštanja metala ili predviđanja njihovog mogućeg mehanizma metabolizma i toksičnosti), a u ovom su istraživanju primijenjene tehnike procjene bioakumulacije te biokemijski biomarkeri. Međutim, uz prednost koju organizmi pružaju u odnosu na isključivo kemijske analize, i oni mogu pokazivati varijabilne rezultate na to da na njihove odgovore utječu mnogi abiotički i biotički čimbenici, fiziologija organizma i fizikalno-kemijski čimbenici staništa. Stoga se preporuča koristiti kombinaciju kemijskog i multibiomarkerskog pristupa koja realnije ukazuje na stvarne uvjete okoliša te na prisustvo i učinke određenih grupa zagađivala (Monseratt i sur., 2007), kao i sezonsko uzorkovanje, kako bi se razlučili abiotički i biotički utjecaji od antropogenih utjecaja na biološke odgovore organizama.

2.5.2. Biomarkeri

Biomarkeri predstavljaju promjene staničnih struktura ili funkcija koje upućuju na međudjelovanje biološkog sustava i nekog potencijalno šetnog fizikalnog, kemijskog ili biološkog čimbenika. Kada šetne tvari dospiju do mjesta djelovanja izazivaju mjerljivi i specifični učinak na molekularnoj i staničnoj razini, poput oštećenja te pobudne sinteze ili inhibicije biološki važnih molekula, te stoga imaju velik toksikološki značaj i smatraju se

ranim pokazateljima izloženosti onečišćenju (Erk i sur., 2002). Osim na staničnoj razini, zagađenje može uzrokovati posljedice i na višim sinekološkim razinama, poput promjena na razini populacije ili ekosustava. Međutim, poremećaji na razini populacije su manje specifični i daju odgovor tek nakon dugotrajne izloženosti, kada je toksičnim učinkom zahvaćena već čitava populacija, dok su biomarkeri na razini stanice rani signali za uzbunu na značajne promjene u organizmu, budući da svakom stresu na razini populacije mora prethoditi onaj na nižoj (staničnoj i molekularnoj) razini (Lam, 2009).

Biomarkeri se dijele u tri osnovne kategorije: biomarkeri izloženosti, biomarkeri učinka i biomarkeri osjetljivosti (van der Oost i sur., 2003). Biomarkeri izloženosti ukazuju na izloženost organizma određenim kemikalijama, ali ne daju informaciju o toksičnim učincima supstanci (npr. indukcija metalotioneina). Biomarkeri učinka ukazuju na učinak kemikalije na organizam i mjere biokemijske ili fiziološke promjene, poput oštećenja DNK, inhibicije enzima odgovornih za homeostazu, dok su biomarkeri osjetljivosti pokazatelji naslijeđene ili stečene sposobnosti organizama da odgovori na izloženost nekoj kemijskoj tvari (van der Oost i sur., 2003).

Iako vrlo korisni, biomarkeri u ekotoksikološkim istraživanjima nisu idealni, jer bi njihov odgovor trebao odražavati stupanj onečišćenja te varirati isključivo ovisno o njemu, što je u praksi vrlo rijetko. Naime, često postoji sezonska i starosna varijabilnost, varijacije s obzirom na dostupnost hrane ili reproduktivni status, kao i na razne okolišne uvjete, što treba uzeti u obzir prilikom interpretacije rezultata. Kako bi se nadvladali navedeni problemi, nužno je koristiti set biomarkera, odnosno multibiomarkerski pristup, što omogućava pouzdanije razlikovanje utjecaja okolišnih čimbenika od antropogenog utjecaja (Hamer i sur., 2008). Iz tog razloga je potrebno odrediti i bazalne koncentracije biomarkera u što više bioindikatorskih organizama i tkiva, kako bi se promjene uzrokovane onečišćenjem mogle razlikovati od prirodne varijabilnosti biomarkera, što omogućuje donošenje valjanih zaključaka te usporedbu u ekološki sličnim sustavima diljem svijeta.

2.5.2.1. Metalotioninini (MT) - biomarkeri izloženosti metalima

Metalotioneini (MT) su niskomolekulski, termostabilni proteini s visokim udjelom cisteinskih ostataka na čije tiolne skupine (-SH) vežu metale. Prisutni su kod prokariotskih i eukariotskih organizama te su identificirani u svim skupinama kralješnjaka, kao i u 50 različitih vrsta beskralješnjaka iz pet koljena (Hamza-Chaffai i sur., 2000). Unatoč postojanju različitih izoformi, struktura im je ostala konzervirana tijekom evolucije, okarakterizirana specifičnim aminokiselinskim sastavom s oko 30 % cisteina, nedostatkom aromatskih

aminokiselina i histidina, niskom molekulskom masom od 6000-7000 Da te toplinskom stabilnošću (Shaw i sur., 1992). Postojanje izoformi ovih proteina može ukazivati na to da različiti oblici imaju različite uloge, što može biti vrlo važno za funkcioniranje stanice, kao i regulaciju metala. U molekuli MT postoji sedam veznih mjesta za dvovalentne metale (Zn^{2+} , Cd^{2+}) koji su tetraedarski koordinirani u dvije metalotioneinske domene: karboksi-terminalna α -domena sa stehiometrijom M_4S_{11} (*klaster* s 4 atoma metala) te amino-terminalna β -domena sa stehiometrijom M_3S_9 (*klaster* s 3 atoma metala). Ako se radi o jednovalentnim ionima, poput Cu^+ i Ag^+ , struktura može biti digonalna ili trigonalna (Blindauer i sur., 2010). U stanicama se MT najčešće nalaze u citosolu, ali i u lizosomima te u jezgri stanica (Langston i Bebbiano, 1998).

Kao njihova primarna funkcija često se ističu održavanje homeostaze esencijalnih (Zn i Cu) i detoksikacija neesencijalnih metala (Cd, Hg i Ag) (Vašak, 2005; Amiard i sur., 2006), ali i zaštita od oksidacijskog stresa te transport metala, iako sve funkcije i mehanizmi zapravo ni nisu potpuno razjašnjeni jer se radi o multifunkcionalnim proteinima (Isani i Carpenè, 2014). U vodenih organizama koncentrirani su u jetri, bubrezima, škragama i probavnom sustavu, odnosno organima vezanim uz unos i/ili detoksikaciju tvari (Roesijadi, 1992).

S obzirom na to da u prisustvu povišenih koncentracija metala dolazi do pobudne sinteze MT, redovito se koriste kao biomarkeri izloženosti metalima u procjeni stanja okoliša. Takvu indukciju prvi je opisao Piscator (1964) u jetri zeca izloženog Cd te je otkriveno da su za vezanje metala odgovorne -SH skupine. Zaštitno djelovanje metalotioneina (MT) sastoji se u pojačanoj sintezi apoproteina (apoMT) koja nastupa vezanjem unesenih metala na “*metal-responsive transcription factor*” (MTF), koji time mijenja konformaciju i veže se na “*metal regulatory elements*” (MREs) metalotioneinskog gena (MT gen), čim se povećava intenzitet transkripcije MT gena, što uzrokuje povećanje koncentracije metalotioneina (Roesijadi, 1992; Filipović Marijić i Raspor, 2005).

Međutim, iako se MT koriste kao indikatori izloženosti metalima, njihova koncentracija i pobudni odgovor ovise o mnogim abiotičkim i biotičkim čimbenicima, kao što su vrsta i/ili starost organizma, spol, stanište i prehrambene navike, sezona, reproduktivna aktivnost, fiziološko stanje i rast (Viarengo i sur., 1999). Stoga je u okolišnim istraživanjima vrlo bitno razlučiti biotičke i abiotičke uzroke indukcije MT od antropogenih utjecaja. Istraživanja o unosu metala hranom i posljedičnom odgovoru MT na izloženost metala u probavilu su rijetka (Handy i Taylor, 1996).

2.5.2.2. Biomarkeri oksidacijskog stresa i antioksidacijskog kapaciteta

Oksidacijski stres je metaboličko stanje u kojem dolazi do poremećaja u ravnoteži oksidacijsko-redukcijskih procesa u organizmu pri čemu dolazi do prekomjernog stvaranja kisikovih reaktivnih spojeva (ROS, eng. *reactive oxygen species*), odnosno narušavanja mehanizama antioksidacijske obrane (Betteridge, 2000). Reaktivni oblici kisika obuhvaćaju slobodne radikale i neradikalne molekule koje mogu uzrokovati oštećenja stanica. Slobodni radikali su atomi, molekule ili ioni s nesparenim elektronom zbog čega često stupaju u kemijske reakcije (Halliwell i Gutteridge, 1984; Lushchak, 2011), dok neradikalne molekule nemaju nespareni elektron, ali mogu oksidirati biomolekule te se brzo i nepredvidivo spajaju s bilo kojom prostorno bliskom molekulom proteina, lipida, ugljikohidrata ili nukleinske kiseline (Fogarasi i sur., 2016). Iako mogu biti i izuzetno štetni, slobodni radikali kisika su neophodni za fiziološke procese u organizmima (sinteza nekih hormona, antibakterijski kapacitet makrofaga i neutrofila, antitumorsko djelovanje, apoptoza, signalizacija u staničnim procesima, regulacija transkripcije) te se njihova proizvodnja stalno odvija u stanicama živih organizama (Halliwell i Gutteridge, 2007; Rada i Leto, 2008).

Najvažniji reaktivni kisikovi spojevi su: superoksidni anion ($O_2^{\bullet-}$), perhidroksilni radikal (HOO^{\bullet}), hidroksilni radikal (OH^{\bullet}), vodikov peroksid (H_2O_2), hidroksidni ion (OH^-), hipokloritna kiselina ($HClO$) i drugi. Pri tome se hidroksilni radikal (OH^{\bullet}) smatra najreaktivnijim oblikom, jer ga karakterizira niska specifičnost prema supstratu i kratko vrijeme poluživota čime lako oduzima elektrone susjednim molekulama te je važan pokretač lipidne peroksidacije (Lončar, 2015). Svi ti spojevi mogu nastati kao posljedica endogenih (unutarnjih) izvora kao što su proces staničnog metabolizma, razgradnja makromolekula, upalni procesi, fagocitoza; ili pak egzogenih (vanjskih) izvora poput radijacije, onečišćenja metalima ili organskim otapalima (Kohen i Nyska, 2002; Rada i Leto, 2008).

Kako bi nadvladali štetne posljedice ROS-a, organizmi imaju dobro razvijen antioksidacijski sustav koji obuhvaća enzimске i neenzimске komponente. Njihova zadaća je ukloniti slobodne radikale i zaustaviti lančanu reakciju stvaranja novih radikala čime prestaje njihovo štetno djelovanje. Djelovanje antioksidansa može se opisati kroz nekoliko mehanizama: uklanjanje kisika ili smanjivanje njegovih lokalnih koncentracija, uklanjanje metalnih iona, uklanjanje ciljnih ROS-a poput superoksida ili vodikovog peroksida, uklanjanje slobodnih radikala ili singletnog kisika (Gutteridge, 1995; Štefan i sur., 2007). Najvažniji antioksidacijski enzimi su: katalaza (CAT), superoksid dismutaza (SOD), glutation peroksidaza (GPx), glutation reduktaza (GR), glukoza-6-fosfat dehidrogenaza (G6PD) te

glutation S-transferaza (GST) (Halliwell i Gutteridge, 2000; van der Oost i sur., 2003). Nisko-molekularni antioksidansi poput glutationa (GSH), β -karotena (vitamin A), askorbinske kiseline (vitamin C) ili pak α -tokoferola (vitamin E) čine neenzimatsku komponentu antioksidacijskog sustava organizama i također imaju značajnu ulogu u procesu uklanjanja radikala (van der Oost i sur., 2003). Međutim, unatoč ovom načinu zaštite i obrane, mnoga zagađivala (uključujući i metale) mogu dovesti do poremećaja u ravnoteži nastanka i uklanjanja oksidansa, što u organizmima uzrokuje oksidacijski stres (Valko i sur., 2005; Sevcikova i sur., 2011).

Zna se da su metali jedan od čimbenika koji utječu na razinu oksidacijskog stresa pri čemu željezo (Fe), bakar (Cu), krom (Cr), vanadij (V) i kobalt (Co) stvaraju radikale preko reakcija redoks ciklusa, dok živa (Hg), kadmij (Cd) ili nikal (Ni) djeluju neizravno preko vezanja za glutation i sulfhidrilne skupine proteina (Valko i sur., 2005; Sevcikova i sur., 2011). Treći način djelovanja metala je i kroz Fentonovu reakciju u kojoj mogu sudjelovati Fe, Cu, Cr, V, Co i njihovi kompleksi te kroz koju nastaje i hidroksilni anion (Valko i sur., 2005; Luschak, 2011).

Glavne posljedice oksidacijskog stresa su: oštećenje DNK, lipidna peroksidacija, oksidacija aminokiselina u proteinima, inaktivacija enzima, narušavanje strukture biomembrana, poremećaj mitohondrijskog transporta elektrona te u konačnici i smrt stanice (Pinto i sur., 2003; Martínez-Alvarez i sur., 2005). Tijekom lipidne peroksidacije dolazi i do formiranja lipooksidacijskih produkata koji se često koriste kao biomarkeri oksidacijskog stresa u *in vivo* uvjetima (Petlevski i sur., 2006).

Budući da se pokazalo da aktivnost antioksidacijskih enzima može biti vrijedan čimbenik u procjeni stanja okoliša, smatra se nužnim korištenje široke lepeze biomarkera vezanih uz oksidacijski stres koji daju potpuniju sliku učinaka koje određeni ksenobiotici imaju na oksidacijski stres u organizmima. Ti biomarkeri bi trebali uključivati i samu oksidacijsku štetu, ali i promjene u antioksidacijskom kapacitetu organizama (Carney Almroth i sur., 2008).

2.5.2.2.1. Malondialdehid (MDA) – biomarker oksidacijskog stresa

Lipidna peroksidacija je proces kojim slobodni radikali uzimaju elektrone s polinezasićenih masnih kiselina u staničnim membranama pri čemu dolazi do oštećenja lipida i nastanka lipidnih peroksila te krajnjih produkata, reaktivnih aldehida poput malondialdehida (MDA) (Petlevski i sur., 2006). Najčešće je uzrokuje hidroksilni radikal ($\text{OH}\cdot$), ali i pojedini drugi radikali poput superoksida O_2^- također mogu pokrenuti proces oksidacije nezasićenih

masnih kiselina (Štefan i sur., 2007). Sam proces lipidne peroksidacije sastoji se od inicijacije, propagacije i terminacije. Tijekom inicijacije dolazi do oksidacije lipida u kojoj visoko reaktivni oksidans oduzima atom vodika višestruko nezasićenoj masnoj kiselini pri čemu nastaje alkilni, odnosno lipidni radikal. Ukoliko ne dođe do učinkovitog djelovanja antioksidansa slijedi faza propagacije i nastanak lančane reakcije lipidne peroksidacije u kojoj sudjeluju i ioni željeza. Lipidni hidroperoksidi lako reagiraju s Fe^{2+} i Fe^{3+} ionima, pri čemu nastaju lipidni peroksilni radikali i alkoksi radikali. Nastali lipidni peroksil radikal je vrlo reaktivan i dolazi do grananja lančane reakcije procesa lipidne peroksidacije. Zadnji korak događa se ako neki antioksidans donira vodik lipidnom peroksil radikalumu i zaustavi proces otimanja vodika susjednom lipidu, čime nastaju krajnji produkti lipidne peroksidacije poput malondialdehida (MDA), ili 4-hidroksi-2-nonenala (HNE) (Štefan i sur., 2007; Alaya i sur., 2014).

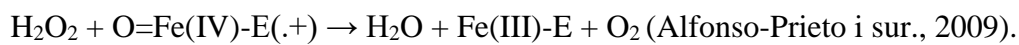
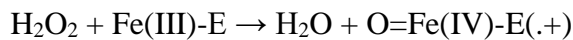
Lipidna peroksidacija ima značajan utjecaj na biološke sustave jer dovodi do gubitka fluidnosti membrana koje postaju krute, opadanja vrijednosti membranskog potencijala, povećanja propusnosti, pucanja stanica i otpuštanja njihovog sadržaja (Štefan i sur., 2007).

Najštetniji, najčešći i jedan od najvažnijih produkata lipidne peroksidacije je upravo MDA, koji se dugi niz godina koristi kao prihvaćeni biomarker oksidacijskog stresa (Del Rio i sur., 2005; Valko i sur., 2005). Dokazano je kako je MDA vrlo dobar pokazatelj onečišćenja okoliša te se koristi u procjenama stanja vodenog okoliša kao biomarker oksidacijskog stresa uzrokovanog onečišćenjem (Farombi i sur., 2007). Organski je spoj kemijske formule $CH_2(CHO)_2$ te ima izrazite citotoksične učinke. U fiziološkim uvjetima se najčešće nalazi u obliku enolatnog iona te reagira s proteinima, s osobitim afinitetom prema lizinskom aminokiselinskom ostatku. Gvaninska baza DNK je drugo ciljno mjesto MDA što može dovesti do mutacija. U organizmu MDA se metabolizira do malonatne kiseline koja je kompetitivni inhibitor mitohondrijske sukcinat dehidrogenaze (Štefan i sur., 2007).

U procjeni stanja vodenih okoliša pod utjecajem zagađenja metalima razine MDA su uglavnom mjerene u škrigama, mišiću i jetri (Banerjee i sur., 1999; Fatima i sur., 2000; Durmaz i sur., 2006; Dragun i sur., 2017), dok za probavno tkivo postoji manje podataka te se radi o izlaganju određenim metalima (Berntssen i sur., 2000; Carriquiriborde i sur., 2004), dok su istraživanja u okolišnim uvjetima izloženosti onečišćenju rijetka (Dragun i sur., 2017).

2.5.2.2.2. Katalaza (CAT) i ukupni glutation (GSH) – biomarkeri antioksidacijskog kapaciteta

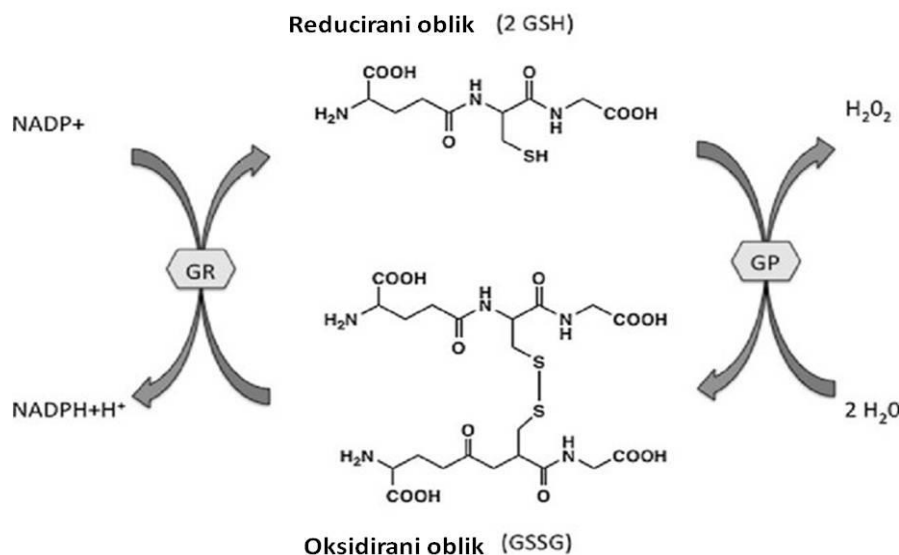
Katalaza je antioksidacijski enzim koji katalizira dvostupanjsku konverziju dviju molekula vodikovog peroksida (H_2O_2) u dvije molekule vode i jednu molekulu kisika, štiteći stanicu od vodikovog peroksida (Young i Woodside, 2001). U prvom se koraku jedna molekula H_2O_2 reducira do vode, uz oksidaciju željeza u hem skupini katalaze; a u drugom se koraku, druga molekula H_2O_2 oksidira dajući molekulu vode i molekulu kisika, uz istovremenu redukciju željeza unutar hem skupine katalaze. Taj molekularni mehanizam razgradnje vodikovog peroksida uz pomoć katalaze odvija se u prema slijedećim reakcijama:



Prisutna je u svim živim organizmima koji su izloženi kisiku. Iako je najčešća u peroksisomima (Radovanović i sur., 2010), može se naći i u mitohondrijima, kloroplastu, citosolu i izvan stanice kao slobodan enzim ili vezana za membranu (Đorđević, 2004; Sharma i sur., 2014). Molekula katalaze je tetramer sastavljen od četiri polipeptida, odnosno četiri podjedinice od kojih svaka sadrži preko 500 aminokiselina i prostetičku grupu hem u katalitičkom centru. Katalaza je enzim vrlo velike aktivnosti, odnosno jedna molekula katalaze može u jednoj minuti reducirati i više milijuna molekula H_2O_2 do vode i kisika. Međutim, uz veliku katalitičku sposobnost, CAT zapravo ima nizak afinitet prema supstratu, tako da se njezina aktivnost očituje tek pri višim koncentracijama H_2O_2 . Uvjeti blagog oksidacijskog stresa djeluju pobudno na ekspresiju i aktivnost CAT kao način obrane od štetnih učinaka radikala, ali jaki oksidacijski uvjeti mogu dovesti do inhibicije enzima, što se bilježi u uvjetima jakog onečišćenja okoliša i narušavanja homeostaze oksidacijsko-redukcijskih procesa. Često se koristi kao biomarker antioksidacijskog kapaciteta u istraživanju utjecaja pesticida i metala na kopnene i vodene okoliše (Saint-Denis, 1998; Ribera i sur., 2001; Atli i sur., 2006; Hernández-Moreno i sur., 2014).

Glutation je jedna od najznačajnijih neenzimatskih molekula u antioksidacijskoj obrani organizama. Radi se o tripeptidu aminokiselinskog sastava Glu-Cys-Gly s reduciranom tiolnom skupinom. Glutation ima nekoliko važnih uloga u obrani od oksidacijskog stresa: kofaktor je za detoksikaciju enzima kao što su glutation peroksidaza (GP_x) i glutation transferaza (GST), uklanja hidroksilne radikale i singletni kisik izravno, dok razinu vodikovog peroksida regulira putem katalitičkog djelovanja glutation peroksidaze te može regenerirati oksidirani vitamin C i vitamin E (Forman i sur., 2009). U slučaju obrane od oksidacijskog stresa uzrokovanog metalima, glutation djeluje kao prva linija obrane stanice, dok još nije

došlo ni do indukcije metalotioneina (Canesi i sur., 1999). Osim toga, ima i brojne fiziološke uloge u procesima poput prijenosa signala, transporta aminokiselina, konjugacije metabolita i detoksikacije različitih ksenobiotika (Masella i sur., 2005). U organizmu glutation postoji u reduciranom stanju (GSH) i oksidiranom stanju (GSSG) (Slika 16) čiji se omjeri održavaju pomoću procesa reverzibilne oksidacije i redukcije. U zdravim stanicama i tkivu većina ukupnog glutaciona je u reduciranom obliku (GSH). U stanicama se održava u reduciranom obliku uz pomoć enzima glutation reduktaze (GR) koristeći NADPH kao izvor elektrona (Rahman i MacNee, 1999). S druge strane, u reakciji redukcije vodikovog peroksida i lipidnih hidroperoksida u vodu i odgovarajući alkohol, kataliziranoj glutation peroksidazom (GP_X), dolazi do oksidacije tiolne skupine cisteina u GSH te tada reducirani glutation (GSH) prelazi u oksidirani oblik (GSSG) koji je zapravo štetan za stanicu. Budući da oksidacijski stres u stanicama može uzrokovati smanjenje koncentracije GSH i povećanje razine oksidiranog GSSG oblika (Luperchio i sur., 1996), promjene u koncentraciji GSH služe kao jedan od bioloških biljega izloženosti zagađivalima koji uzrokuju oksidacijski stres, odnosno kao biomarker antioksidacijskog kapaciteta stanica. S obzirom na to da i metali značajno doprinose nastanku i povećanju razina oksidacijskog stresa, i GSH se, kao i enzimi vezani uz ciklus GSH, često koristi u istraživanju utjecaja metala na okoliš i biotu, uključujući i slatkovodne sustave i ribe kao bioindikatore.



Slika 16. Metabolizam glutationa u organizmu (preuzeto i prilagođeno iz Xiong i sur., 2011)

Različiti odgovori opisanih antioksidansa zabilježeni su kod riba izloženih metalima, ovisno o dozi, elementu, vrsti ili putu izlaganja (Liu i sur., 2005; Atli i sur., 2006; Tsangaris i sur., 2011; Greani i sur. al., 2017) te literaturni podaci izvještavaju o višim, nepromijenjenim

ili nižim aktivnostima i/ili koncentracijama antioksidansa kao odgovor na izloženost onečišćujućim tvarima u laboratorijskim i terenskim istraživanjima (van der Oost i sur., 2003). Stoga je u istraživanjima najbolje kombinirati odgovore nekoliko antioksidansa, zajedno s markerom oksidacijskog stresa, kako bi se provela pouzdanija procjena stanja okoliša i stupnja oksidacijskog stresa i oštećenja.

2.5.2.3. Acetilkolinesteraza (AChE) - biomarker izloženosti organskim zagađivalima i metalima

Enzim acetilkolinesteraza (AChE) je važan molekularni biomarker u ekotoksikologiji te esencijalni enzim za ispravno prenošenje živčanih impulsa koji katalizira razgradnju acetilkolina, najvažnijeg neurotransmitera u mnogih životinja, na acetat i kolin u sinapsama, odnosno mjestima prijenosa živčanog impulsa s jednog neurona na drugi. Pripada porodici enzima kolinesteraza (ChE), specijaliziranih hidrolaza koje sudjeluju u razgradnji kolinskih estera. AChE je serinska hidrolaza s iznimno visokom i specifičnom katalitičkom aktivnošću. Iako se prvenstveno nalazi u živčano-mišićnim sinapsama živčanog tkiva, AChE se u organizmu nalazi u nekoliko različitih molekularnih oblika u raznim tkivima i stanicama: mišićima i živcima, središnjim i perifernim tkivima, motornim i senzornim vlaknima te na membranama eritrocita u krvi (Daniels, 2007; Lionetto i sur., 2011; Čolović i sur., 2013). Primarna uloga joj je završetak sinaptičkog prijenosa impulsa što sprječava kontinuirano podražavanje receptora na živčanim završetcima. Dakle, inhibicija aktivnosti AChE dovodi do neprestanog prijenosa podražaja (hiperstimulacije) što može dovesti do paralize mišića.

Premda se inhibicija aktivnosti AChE prvenstveno koristi kao biomarker izloženosti karbamatnim i organofosforinim spojevima, pokazalo se da i mnogi drugi spojevi u okolišu, poput ugljikovodika, deterdženata ili metala uzrokuju promjene u aktivnosti ovog enzima (Lionetto i sur., 2003, 2011; Jebali i sur., 2006; de Lima i sur., 2012). U mnogobrojnim istraživanjima potvrđen je i utjecaj metala na inhibiciju aktivnosti AChE (Frasco i sur., 2005; Lionetto i sur., 2011; de Lima i sur., 2012), ali mehanizam inhibicije razlikuje se od organskih spojeva te još nije u potpunosti razjašnjen i nije sigurno radi li se izravnoj ili neizravnoj inhibiciji enzima, odnosno o fiziološkim promjenama u organizmu koje posljedično dovode i do smanjene aktivnosti enzima, ili o izravnom utjecaju metala na spomenuti enzim (de Lima i sur., 2012).

Kod riba je aktivnost AChE uglavnom mjerena u mišiću, jetri, srcu i mozgu (Romani i sur., 2003; Yadav i sur., 2009; Hernández-Moreno i sur., 2014), dok su drugi organi poput škrge ili probavila rijetko korišteni. Što se tiče probavila riba, dostupno je samo istraživanje

Szabó i sur. (1991) u kojem je uspoređena aktivnost AChE u mozgu, mišićima, srcu i probavilu 11 vrsta riba, pri čemu je probavilo pokazalo najnižu aktivnost od svih organa u okolišu koji nije izložen onečišćenju. U znanstvenoj literaturi vidljivo je da je aktivnost ovog enzima češće mjerena u vodenim beskralješnjacima, osobito školjkašima, ali i rakovima, nego u kralješnjacima. Naime, organska zagađivala se češće određuju u ovim organizmima koji su osjetljiviji na njih nego kralješnjaci te koji su često ključne vrste u hranidbenim mrežama (Lionetto i sur., 2011).

Budući da nije toliko specifičan biomarker, korištenje AChE pojedinačno ne daje sigurne i potpune podatke te se određivanje AChE uključuje u set biomarkera koji se koriste kao sredstva procjene stanja okoliša i organizama. Naime, razumijevanje međusobnog odnosa biomarkera pri izloženosti zagađivalima i načina na koji se odgovori razlikuju među vrstama daje ključne podatke za pravilno tumačenje rezultata. Stoga se AChE koristi integrirano, osobito u kombinaciji s promjenama u antioksidacijskim enzimima i razinama peroksidacije lipida, s ciljem praćenja okoliša i otkrivanja moguće izloženosti ili učinka izazvanog zagađivalima na žive organizme.

2.5.2.4. Ukupni citosolski proteini (TP) - biomarkeri općeg stresa

Kao nespecifičan biomarker općeg stresa organizma često se koristi i koncentracija ukupnih citosolskih proteina (TP). Citosol je unutarstanična tekućina koja se najvećim dijelom sastoji od vode, ali i niza makromolekula poput nukleinskih kiselina, ugljikohidrata, proteina i lipida te je stoga i mjesto aktivnog metabolizma, prijenosa signala i metabolita. Proteini imaju važnu ulogu kao esencijalne makromolekule za građu stanica i tkiva, prijenos i pohranu tvari, prijenos informacije između stanica, kontrolu rasta i diferencijaciju stanica, obranu od infekcija te osiguravanje fizioloških funkcija poput disanja. Nadalje, kao enzimi kataliziraju gotovo sve kemijske reakcije u biološkim sustavima pa tako imaju ulogu u gotovo svim aktivnostima u stanici (Robinson, 2015).

Smanjena ili pojačana sinteza proteina stoga odražava stanje ravnoteže u organizmima te ukazuje na poremećaje općeg stanja organizama koji mogu nastati pod utjecajem vanjskih čimbenika. Određivanje ukupnog sadržaja citosolskih proteina može ukazati na moguću izloženost nekim zagađivalima, uključujući metale, ali kao i kod većine biomarkera, utjecaj imaju i abiotički i biotički čimbenici te koncentracija proteina ovisi i o vrsti organizma, tkivu, fiziološkom stanju organizma, reproduktivnom ciklusu, temperaturi, a često odražava i prehrambene navike vrsta i dostupnost hrane (Peragón i sur., 1994; Filipović Marijić i Raspor, 2010). Stoga se TP koristi kao dodatna i popratna informacija u procjeni stanja okoliša, kao

rani pokazatelj određenih poremećaja, ali u kombinaciji s drugim, specifičnijim biomarkerima.

2.6. Analitičke metode

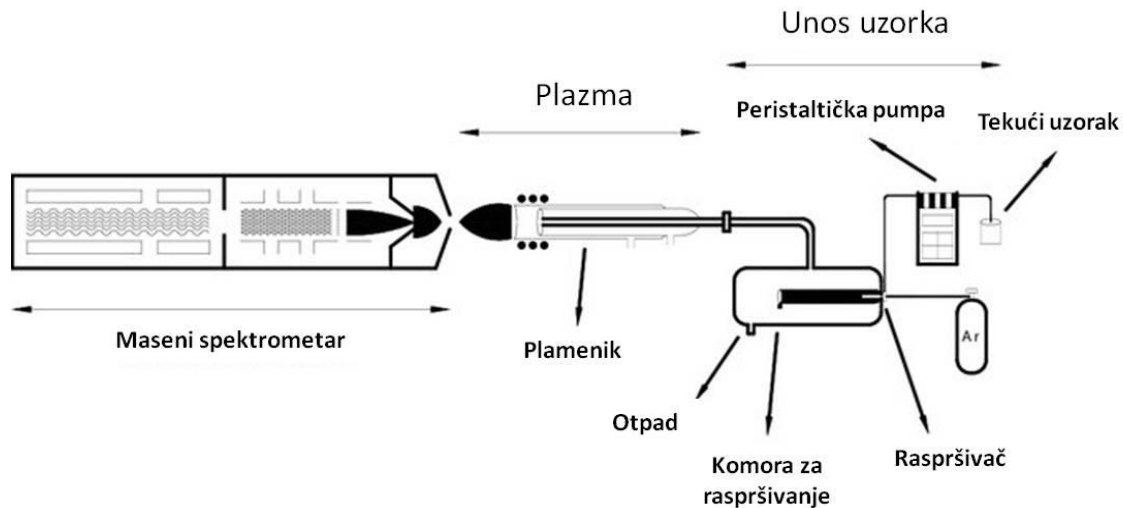
2.6.1. Analitičke metode za određivanje koncentracija metala u uzorcima iz okoliša

Danas je u upotrebi niz tehnika kojima se mogu određivati koncentracije metala u uzorcima iz okoliša, poput atomske apsorpcijske spektrometrije (AAS), atomske emisijske spektrometrije s induktivno spregnutom plazmom (ICP-AES), spektrometrije masa s induktivno spregnutom plazmom (ICP-MS), atomske fluorescencijske spektrometrije (AFS), rendgenske fluorescentne analize (XRF) te elektrokemijskih metoda (Lobinski i Marczenko, 1997; Szpunar, 2004; Michalke and Nischwitz, 2010; Dragun i sur., 2018b). Dodatno se mogu koristiti i analize neutronske aktivacije (NAA) ili djelomično inducirane rendgenske emisije (PIXE) te sustav laserske ablacije povezan s ICP-MS koji omogućava mjerenje u čvrstim biološkim uzorcima (Prohaska i sur., 2016; Walkner i sur., 2017).

2.6.1.1. Masena spektrometrija s induktivno spregnutom plazmom (ICP-MS)

ICP-MS je tehnika u kojoj se induktivno spregnuta plazma koristi kao ionizacijski izvor, a detekcija se vrši spektrometrijom masa. Koristi se kao pouzdana metoda za mjerenje metala u tekućim uzorcima, dok se kruti uzorci analiziraju neizravno nakon razgradnje i prevođenja u tekućinu. Određivanje kemijskih elemenata može se podijeliti na nekoliko faza: atomizacija, pretvaranje atoma u ione, razdvajanje iona na osnovu omjera njihovih masa i naboja te detekcija i kvantifikacija iona prisutnih u uzorku (Montaser, 1998). Glavni dijelovi su: sustav za uvođenje uzorka, plazma za kao ionizacijski izvor, analizator masa te detektor (Slika 17).

Tekući uzorak se unosi u sustav pomoću pumpe (na primjer, peristaltičke) te otopina ulazi u raspršivač gdje se pomoću argona pretvara u aerosol. Dalje se u komori za raspršivanje velike kapi odvajaju iz aerosola i izlaze iz komore, a aerosol ulazi u plazmu u plameniku. Uzorci u plameniku prolaze kroz faze desolvacije, isparavanja, atomizacije i ionizacije. Molekule aerosola putuju plazmom te se formiraju pozitivno nabijeni ioni koji se kroz dva metalna konusa prenose u sučelje masenog spektrometra, koji se sastoji od sustava leća, analizatora i detektora (Slika 17). Pozitivno nabijeni ioni se odvajaju od ostalih čestica te se na temelju omjera masa/naboj razdvajaju i potom detektiraju upotrebom multiplikatora elektrona (Montaser, 1998).



Slika 17. Osnovni dijelovi i princip rada spektrometrije masa s induktivno spregnutom plazmom (preuzeto i prilagođeno iz Kashani i Mostaghimi, 2010)

U odnosu na druge metode, ICP-MS omogućava multielementnu i brzu analizu, čime se omogućava analiza više uzoraka u kraćem vremenu, ima niske granice detekcije, sposobnost mjerenja izotopa te su dobiveni spektri relativno jednostavni za tumačenje i analizu (Garcia i sur., 2006). Pomoću ICP-MS-a moguće je provoditi kvalitativne, polukvantitativne i kvantitativne analize.

Posebno osjetljiv i pouzdan uređaj je spektrometar masa visoke rezolucije s induktivno spregnutom plazmom (HR ICP-MS) koji kombinacijom fizičkog ograničavanja snopa iona prolaskom kroz usku pukotinu različitih dimenzija, te specifične konstrukcije spektrometra masa s dvostrukim fokusiranjem u električnom i magnetskom polju omogućava znatno preciznije fokusiranje izotopa i korištenje tri različite rezolucije (niske, srednje i visoke). Izborom odgovarajuće rezolucije za pojedini element postiže se njihovo maksimalno razdvajanje od mogućih interferencija, koje su time svedene na minimum (Pröfrock i Prange, 2012).

2.6.1.2. Spektrometrija masa s induktivno spregnutom plazmom i laserskom ablacijom (LA ICP-MS)

Nadogradnju tehnike ICP-MS čini i laserska ablacija (LA ICP-MS) koja omogućava analizu koncentracije metala izravno u čvrstim uzorcima bez prethodne obrade i razgradnje uzoraka. S obzirom na to, smanjuje se mogućnost onečišćenja, za mjerenje su potrebne izuzetno male količine uzorka ($< \mu\text{g}$) te se postiže dobra rezolucija (Russo i sur., 2002). Uzorak za analizu se priprema slično mikroskopskim preparatima, kao tanki presjek

materijala pričvršćen za podlogu, ali bez pokrova, tako da površina bude izložena ablaciji pulsirajućeg lasera koji stvara aerosol i koji se dalje prenosi u ICP sustav te se analiza dalje odvija kao u slučaju tekućih uzoraka (Russo i sur., 2002).

Ubrzo nakon prve upotrebe 1985. godine, za analizu uzoraka stijena (Gray, 1985), metoda LA ICP-MS je dobila široku primjenu u različitim okolišnim i geološkim istraživanjima, forenzici te arheološkim istraživanjima (Russo i sur., 2002; Friedrich i Halden, 2008; Holá i sur., 2009; Liu i sur., 2013; Pozebon i sur., 2014, 2017; Almirall i Trejos, 2016; Tanner i sur., 2016).

U istraživanjima slatkovodnih ili morskih sustava, korištenjem riba kao indikatorskih organizama, LA ICP-MS omogućava preciznu i detaljnu analizu elemenata u tragovima, istovremeno može detektirati i mjeriti mnoge elemente, a mjerenja duž laserskih linija indikatorskih kalcificiranih tkiva omogućavaju praćenje razine onečišćenja tijekom čitavog života riba, što daje informacije o trajanju i intenzitetu izloženosti (Zitek i sur., 2010).

2.6.2. Analitičke metode za razdvajanje biomolekula na koje se vežu metali

S obzirom na to da se metali u organizmima javljaju vezani na čitav niz različitih biomolekula, uz tradicionalno određivanje njihove koncentracije sve važniji postaje i sveobuhvatni pristup proučavanja njihove specijacije, lokalizacije i uloge unutar organizama i ekosustava (Thiele i Gitlin, 2008). S tim ciljem se relativno nedavno razvila i nova grana nazvana metalomika koja je, prema Szpunar (2004), definirana kao "sveobuhvatna analiza svih oblika metala i metaloida u stanicama ili tkivima," a obuhvaća specijaciju metala u najširem smislu, uključujući kompleksiranje elemenata, kao i utjecaj kompleksiranja na okoliš te i na ljudsko zdravlje. Jedan od ciljeva je identificirati i razjasniti fiziološku funkciju biomolekula koje vežu metale u biološkim sustavima kombinacijom različitih znanstvenih područja poput geokemije, biologije, farmakologije te fiziologije (Mounicou i sur., 2009). Cjelokupna istraživanja u području metalomike provode se pomoću više metodoloških pristupa te se analize sastoje od mnoštva koraka kako bi se došlo do konačnog cilja.

Osnovu za analizu biomolekula koje vežu metale čine različite kromatografske i elektroforetske tehnike (Szpunar i Lobinski, 1999; Mounicou i sur., 2009). Kromatografija je fizikalno-kemijska metoda odjeljivanja u kojoj se sastojci razdvajaju između dviju faza od kojih je jedna pokretna (mobilna), odnosno kreće se u određenom smjeru, dok je druga nepokretna (stacionarna). Jedna od osnovnih i najčešće korištenih tehnika je tekućinska

kromatografija koja se primjenjuje u modernim biološkim znanostima, te u analitičkoj ili preparativnoj kemiji (Cindrić i sur., 2009).

2.6.2.1. Tekućinska kromatografija visoke djelotvornosti s isključenjem po veličini (SEC-HPLC)

Tekućinska kromatografija se koristi za razdvajanje otopljenih tvari pri čemu tvari iz otopina stupaju u interakciju sa stacionarnom (nepokretnom) i tekućom mobilnom (pokretnom) fazom zbog razlika u adsorpciji, ionskoj izmjeni, razlici u veličini čestica ili stereokemijskih interakcija. Stoga se tvari ovisno o tehnici i svojstvima različito dugo zadržavaju na stacionarnoj fazi (Cindrić i sur., 2009).

Kod kromatografije s isključenjem po veličini razdvajanje komponenti se provodi uglavnom na osnovi veličine čestica te djelomično prema njihovom obliku (Szpunar, 2004). U slučaju ove metode stacionarna faza sadrži pore različitih veličina što utječe na mehanizam razdvajanja molekula koje prolaze kroz kolonu. Naime, najmanje molekule koje mogu ući u pore svih veličina punila zaostaju duže na koloni, dok veće samo prolaze između čestica punila, zaobilazeći pore i na taj način ranije izlaze iz kolone. S obzirom na razliku u veličini pora, samo najveće molekule će vrlo lako i vrlo brzo proći kroz kolonu, dok će druge ovisno o svojoj veličini ulaziti u pore s većom ili manjom učestalosti i razdvajati se kroz kolonu od najvećih, srednjih pa do najmanjih molekula (Burgess, 2018).

Razdvajanje se u ovoj metodi odvija pod blagim fiziološkim uvjetima, što omogućava da proteini i druge važne biomolekule ostanu strukturno i funkcionalno očuvani, a što je važno u analizi metaloproteina (de la Calle Guntiñas i sur., 2002). Nadalje, moguće je povezati ovu metodu s ICP-MS analizom za određivanje koncentracije metala te se na taj način povezivanjem kromatografa i spektrometra masa mogu dobiti profili raspodjele metala vezanih za pojedine biomolekule te odgovarajući rasponi molekulskih masa biomolekula koje vežu metale.

Identifikacija i izučavanje biomolekula koje vežu metale u vodenim organizmima još uvijek se rijetko provodi i kao biomarker se koristi tek nekoliko poznatih metaloproteina (Hauser-Davis i sur., 2012), prije svega metalotioneini. Ipak, kombinacija SEC-HPLC-a i ICP-MS-a u okolišnim istraživanjima kao prvi korak razdvajanja biomolekula već je primijenjena za analizu raspodjele pojedinih elemenata u školjkašima, i to dagnji (*Mytilus galloprovincialis*; Strižak i sur., 2014) te smeđoj dagnji (*Perna perna*; Lavradas i sur., 2016), kao i u tkivima različitih vrsta riba, poput limande (*Limanda limanda*; Lacorn i sur., 2001), jegulje (*Anguilla anguilla*; Van Campenhout i sur., 2008) ili šarana (*Cyprinus carpio*;

Goenaga Infante i sur., 2003). Analize niza metala u ribama u svrhu određivanja njihove citosolske raspodjele pomoću kombinacije SEC-HPLC i ICP-MS metoda provedene su za škrge i jetru europskog klana (*Squalius cephalus*) iz rijeke Sutle u Hrvatskoj (Krasnići i sur., 2013, 2014) te vardarskog klana (*Squalius vardarensis*) iz triju makedonskih rijeka - Bregalnice, Zletovske i Krive (Krasnići i sur., 2018, 2019), a slična su istraživanja napravljena i u jetri mladih američkih žutih grgeča (*Perca flavescens*; Caron i sur., 2018) te jetri i gonadama bijelih sisača (*Catostomus commersonii*; Urien i sur., 2018). Nadalje, citosolska raspodjela Cd, Co, Cu, Fe, Mn, Mo, Se, Tl i Zn određena je i za jetru potočnih pastrva (*Salmo trutta*) iz rijeke Krke (Dragun i sur., 2018b), dok je raspodjela Cd, Cu, Fe, Mo, Se i Zn među citosolskim molekulama određena za jetru i škrge babuški (*Carassius gibelio*) iz rijeke Ilove (Dragun i sur., 2020). Međutim, u dosad dostupnoj znanstvenoj literaturi nema podataka o raspodjeli metala među citosolskim biomolekulama probavila niti jedne vrste riba, čime je i dalje zanemaren značajan unos metala putem hrane u ovih organizama i moguće specifične razlike u raspodjeli metala među različitim organima ovisno o njihovoj fiziološkoj funkciji i građi.

2.6.3. Spektrofotometrijske metode za određivanje koncentracija i/ili aktivnosti biomarkera

Spektrofotometrijske metode omogućavaju određivanje koncentracija, količina ili aktivnosti analita u uzorku mjerenjem količine svjetlosti koju je uzorak apsorbirao. Spektrofotometar se u osnovi sastoji od nekoliko dijelova - izvora zračenja, monokromatora i detektora. Princip rada se zasniva na tome da svjetlost iz izvora zračenja putuje kroz monokromator koji propušta svjetlost određene valne duljine, koja zatim prolazi kroz mjereni uzorak. Valna duljina koju monokromator propušta može se mijenjati, ovisno o specifičnoj metodi te tipu uzorka i produkta reakcije koji se žele mjeriti. Naposljetku se mjeri propušteni intenzitet svjetlosti kroz uzorak pomoću nekog svjetlosnog senzora, odnosno detektora (Reule, 1976).

Ukoliko se radi o koncentraciji nekog uzorka, mjerenjem intenziteta svjetlosti koju je analit u uzorku apsorbirao te usporedbom sa specifičnim kalibracijskim pravcem, dobivenim mjerenjem apsorpcije svjetlosti pri poznatim koncentracijama analita, može se odrediti koncentracija analita u uzorku. Ukoliko se radi o aktivnosti enzima, mjeri se promjena količine supstrata ili produkta u određenom vremenskom razdoblju. Pri tome se aktivnost enzima može mjeriti izravno, ako su supstrat ili produkt jednostavno mjerljivi, odnosno neizravno, ukoliko ih nije moguće izravno izmjeriti. Kontinuirani test se koristi kao izravna

mjera, pri čemu se prati promjena koncentracije supstrata ili produkta u kratkim intervalima tijekom određenog vremenskog razdoblja. Aktivnost enzima se određuje i testovima fiksnog vremena, što podrazumijeva da se enzimska reakcija odvija neko vrijeme te se zatim prekida bilo inhibicijom ili denaturacijom enzima, nakon čega se odredi količina nastalog produkta ili utrošenog supstrata. Takav oblik testa se najčešće koristi kad promjene nije moguće pratiti izravno te se u tom slučaju količina potrošenog supstrata ili nastalog produkta najčešće određuje dodatkom nekog reagensa koji dovodi do obojenja otopine (Bisswanger, 2014).

Ovakve spektrofotometrijske metode imaju široku primjenu u fizici, kemiji, medicini, kemijskoj tehnologiji, molekularnoj biologiji, a koriste se i u mnogim biokemijskim eksperimentima koji uključuju izolaciju DNK, RNK i proteina, kinetiku enzima i različite biokemijske reakcije (Trumbo i sur., 2013).

2.7. Dosadašnja istraživanja u rijeci Krki i Ilovi

Što se tiče istraživanja kakvoće vode i ekosustava rijeke Krke, dosadašnja istraživanja su uključivala mjerenje koncentracija metala u sedimentu (Cukrov i Barišić, 2006, Cukrov i sur., 2007, 2008a, 2013) te vodi (Cukrov i sur., 2008b, 2012, Cindrić i sur., 2015), ugljikohidrata (Tepić i sur., 2007), kao i ukupnog i otopljenog ugljika (Vojvodić i sur., 2007, Marcinek i sur., 2020), uglavnom u području NP Krka te području riječnog estuarija.

U području uzvodno od NP Krka, koje je pod potencijalnom opasnošću zbog ispusta neprikladno pročišćenih komunalnih voda grada Knina i industrijskih otpadnih voda tvornice vijaka samo 2 km uzvodno od granice parka, rađena su istraživanja kakvoće vode (Štambuk-Giljanović i Smolčić, 1982), citotoksičnosti i genotoksičnosti površinskih voda i sedimenata (Mihaljević i sur., 2011; Ternjej i sur., 2013), utjecaja otpadnih voda na morfologiju i oštećenja DNK u rakušaca u rijeci Kosovčici (Ternjej i sur., 2014), te mikrobiološka istraživanja (Kolda i sur., 2019). Nadalje, nekoliko lokacija rijeke Krke predmet su i stalnog monitoringa Hrvatskih voda (Izvješće o stanju površinskih voda u 2019. godini). Međutim, nedostatna su istraživanja koja istovremeno povezuju kemijsku kakvoću okolišnih uzoraka vode i sedimenata te utjecaja na organizme ovog osjetljivog krškog ekosustava. Stoga su kakvoća vode te biološki odgovori različitih bioindikatorskih organizama (ribe, rakušci, kukaši) u tom dijelu toka rijeke Krke bili prvo istraživani u okviru projekta koji je financirala Zaklada ADRIS, „Procjena kakvoće vodotoka rijeke Krke i potencijalne opasnosti za Nacionalni park Krka primjenom novih bioindikatora i biomarkera” (voditeljica dr. sc. Vlatka Filipović Marijić), te zatim i projekta koji je financirala Hrvatska zaklada za znanost, „Akumulacija, unutarstanično mapiranje i učinci metala u tragovima u akvatičkih

organizama” (AQUAMAPMET, voditeljica dr. sc. Marijana Erk), a u okviru kojega je izrađena i ova doktorska disertacija.

Prethodna istraživanja rijeke Ilove obuhvaćaju promjene morfoloških značajki porječja rijeke Ilove, prema Okvirnoj direktivi EU o vodama, pri čemu je otkriveno da 38,5 % ukupnog sliva, odnosno 21 vodno tijelo u porječju Ilove, ne zadovoljava ciljeve Okvirne direktive EU o vodama jer su dobili ocjenu umjereno promijenjeno, loše ili vrlo loše morfološko stanje (Plantak i sur., 2016). Uz ova hidrološka i hidrogeografska istraživanja, u području toka rijeke Ilove ranijih je godina istraživana ihtiofauna (Delić, 1989; Jelić i sur., 2009) te kvalitativni i kvantitativni sastav makrozoobentosa (Delić, 1991), no područje rijeke Ilove, osobito u nizvodnom dijelu, je uglavnom zanemareno u ekotoksikološkim i kemijskim istraživanjima unatoč blizini zaštićenog područja PP Lonjsko polje i potencijalnim opasnostima do kojih dolazi zbog ispusta otpadnih voda tvornice gnojiva, mnogobrojnih ribnjaka i razvijenih poljoprivrednih aktivnosti na tom području. Dva takva istraživanja na ovom području (Durgo i sur., 2009; Radić i sur., 2013) bila su fokusirana na (geno)toksični potencijal površinske vode rijeke Ilove onečišćene otpadnim vodama tvornice gnojiva, pri čemu je došlo do značajnog oksidacijskog stresa u odabranim modelnim organizmima prilikom izlaganja, kao i pojačane akumulacije određenih metala, poput Cd, Cr, Ni, Pb i Zn, ali sveobuhvatna analiza izloženosti metalima kao posljedica svih navedenih antropogenih aktivnosti (poljoprivreda, ribnjačarstvo, industrijske i komunalne otpadne vode) nije provedena sve do HRZZ AQUAMAPMET projekta, osobito ne u okolišnim i terenskim uzorcima vode, sedimenata i bioindikatorskih organizama.

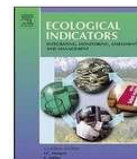
3. ZNANSTVENI RADOVI

Ecological Indicators 105 (2019) 188–198



Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Comparison of electrochemically determined metallothionein concentrations in wild freshwater salmon fish and gammarids and their relation to total and cytosolic metal levels

Tatjana Mijošek^{a,*}, Vlatka Filipović Marijić^a, Zrinka Dragun^a, Dušica Ivanković^a, Nesrete Krasnići^a, Marijana Erk^a, Sanja Gottstein^b, Jasna Lajtner^b, Mirela Sertić Perić^b, Renata Matoničkin Kepčija^b

^a Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička cesta 54, 10000 Zagreb, Croatia

^b University of Zagreb, Faculty of Science, Department of Biology, Division of Zoology, Rooseveltov trg 6, 10000 Zagreb, Croatia



ARTICLE INFO

Keywords:

Biomarkers
Metallothioneins
Fish intestine
Amphipods
Karst aquatic environment

ABSTRACT

Application of metallothionein (MT) as an early warning sign of metal exposure in aquatic organisms is common in biomonitoring, but there is a huge variability in MT concentrations among different studies. Present research aims to assess MT responses in freshwater fish brown trout (*Salmo trutta* Linnaeus, 1758) and gammarids (*Gammarus balcanicus* Schäferna, 1922 and *Echinogammarus acarinatus* Karaman, 1931) as indicators of metal exposure within the freshwater karst environment (Krka River, Croatia). Sampling was performed upstream (reference site) and downstream (anthropogenically impacted site) of the wastewater discharges in autumn and spring seasons. Brown trout intestine was applied as a bioindicator tissue due to its role in dietborne metal uptake while gammarids were chosen as fish food and potential metal uptake source. Moreover, there is a lack of data on intestinal MT levels, so our results on MT and metal/metalloid concentrations, measured as total and metabolically available cytosolic levels, represent the first data of this kind for the selected indicator species. The results indicated that the ecotoxicological impact of technological and municipal wastewaters on the biota of the karst Krka River was moderate, although higher metal levels at the affected site were evident in both, fish and gammarids. The modified Brdička reaction applied in this study was confirmed as reliable electrochemical technique for MT quantification in both vertebrates and invertebrates, and it indicated higher MT levels in gammarids (1.9–4.1 mg g⁻¹ w.w.) than in fish intestine (0.5–2.8 mg g⁻¹ w.w.). Due to the lack of the data on MT concentrations in *S. trutta* and gammarid species *G. balcanicus* and *E. acarinatus*, presented results can serve as a preliminary data to establish MT background levels in intestine of wild freshwater fish and gammarids. Obtained MT levels showed species-, tissue- and method-specific differences, so comparison between MT levels should always involve the same species, tissue and measurement method.

1. Introduction

First measurable changes related to the exposure of contaminants and their impacts on the aquatic organisms are biochemical responses used as cellular and histological biomarkers (Hinton and Lauren, 1990). One of the major biomarkers pointing to metal exposure of aquatic organisms is the increase in metallothionein (MT) levels as a consequence of the induction of MT synthesis associated with increased capacity for metal binding and MT involvement in protection against

metal toxicity (Roesijadi, 1992). MTs are low molecular mass cysteine- and metal-rich proteins containing sulphur-based metal clusters that have significant roles in maintaining the homeostasis of essential trace metals (Zn and Cu), sequestration of toxic metals (Cd, Ag and Hg), and protection against oxidative damage (Vašák, 2005; Amiard et al., 2006). Although often used as the best known biochemical responses to metal exposure in the environment, MTs are also inducible by other biotic and abiotic factors, e.g., starvation, freezing (Amiard et al., 2006), reproductive state, age and sex, temperature, seasonal

* Corresponding author.

E-mail addresses: tmjosek@irb.hr (T. Mijošek), vfilip@irb.hr (V. Filipović Marijić), zdragun@irb.hr (Z. Dragun), djuric@irb.hr (D. Ivanković), nkrasnic@irb.hr (N. Krasnići), erk@irb.hr (M. Erk), sanja.gottstein@biol.pmf.hr (S. Gottstein), jasna.lajtner@biol.pmf.hr (J. Lajtner), msertic@biol.pmf.hr (M. Sertić Perić), rmatonic@biol.pmf.hr (R. Matoničkin Kepčija).

<https://doi.org/10.1016/j.ecolind.2019.05.069>

Received 20 February 2019; Received in revised form 20 May 2019; Accepted 25 May 2019
1470-160X/ © 2019 Elsevier Ltd. All rights reserved.

environmental changes (Viarengo et al., 1999; Isani et al., 2000), which contribute to variations of the MT-cellular concentrations.

As organisms at the top of aquatic food chains, fish are commonly used as bioindicator species for the assessment of metal accumulation. Metal uptake in fish occurs through gills and skin (i.e., sites of waterborne uptake), and intestine (i.e., site of dietborne uptake). Most studies dealing with metal contaminant exposure involved gills (Dragun et al., 2009), liver (Podrug and Raspor, 2009) and kidney (Sevcikova et al., 2013) as indicator organs in fish. The investigations on the uptake and effects of dietary metals in fish and the respective MT responses to metal exposure in fish intestine are still rare (Handy and Taylor, 1996). It was reported that metals are accumulated in the epithelial cell layer of the intestinal tissue and can be eliminated from the organism by desquamation of mucus layer (Sorensen, 1991). MT induction is evident in intestinal absorptive cells, enterocytes, and serves as a biological mechanism which reduces transfer of metals from the luminal to the serosal side. Most of the previous studies considered laboratory experiments in which applied metal concentrations were often higher than their environmental levels, and metals sourcing from the diet were usually ignored, due to complexity of wild fish nutrition (Schlekat et al., 2005; Giguère et al., 2006).

In the present study MT and metal levels were estimated in the gastrointestinal tissue of the salmonid fish brown trout (*Salmo trutta* Linnaeus, 1758), selected as a widely spread freshwater species in rivers in Europe. The potential of fish intestinal tissue to be applied as bioindicator organ of metal contamination in the aquatic environment was evaluated. Additionally, the present study included the assessment of the MT and metal levels in amphipod crustaceans, *Gammarus balcanicus* Schäferna, 1922 and *Echinogammarus acarinatus* Karaman, 1931. Crustaceans of the genus *Gammarus* are often used as bioindicators of environmental pollution due to their wide distribution, high abundance, clear sexual dimorphism, easy sampling and identification, and due to their sensitivity to different kinds of toxicants (Geffard et al., 2007). Gammarids often play a central role in freshwater ecosystems because they represent an important link between detritus and fish in the aquatic food webs and a reduction in their number can have deleterious effects on the structures of biological communities (MacNiel et al., 1997; Kunz et al., 2010). Moreover, in a pilot-study of benthos-drift relationship in the Krka River it was suggested that gammarids, which were found most numerous in drift, could be considered as the most suitable bioindicators of a contaminant (i.e., metal) accumulation and mobilization within karst aquifers (Sertić Perić et al., 2018).

Metal exposure assessment of the organisms (i.e., fish, gammarids) was conducted in anthropogenically impacted karst Krka River. The study involved evaluation of MT and metal levels, including total metal concentrations in fish intestine and cytosolic metal concentrations in fish intestine and whole gammarids, as a fraction which presents metabolically available and therefore potentially toxic metals (Caron et al., 2018; Mijošek et al., 2019). Aquatic systems, especially the sensitive karst ecosystems, are nowadays threatened by a variety of contaminants, often originating from different anthropogenic sources. Among them, metals/metalloids represent one of the most troublesome pollutants in the aquatic environment due to their high toxicity, long persistence and tendency of bioaccumulation and biomagnification in the food chain (Eisler, 1993). Heavy metals can originate from direct atmospheric deposition, geologic weathering as natural sources or through the discharge of different waste products; agricultural, municipal or industrial, as one of the main anthropogenic sources (Demirak et al., 2006).

Our main goals were to evaluate the impact of known pollution sources (technological and municipal wastewaters) on the biota inhabiting the Krka River using electrochemically measured MTs as biomarkers of metal exposure, and metal/metalloid concentrations in the whole intestinal tissue of brown trout and additionally in cellular cytosol of fish intestine and whole gammarids. Cytosolic metal fraction involves respective metal levels which are available of binding to

biomolecules in the cell cytosol of the organisms and therefore may constitute metal-sensitive (enzymes) or metal detoxified fraction (metallothioneins) (Filipović Marijić et al., 2010; Caron et al., 2018). Moreover, seasonal and spatial differences in metal and MT levels in fish and gammarids from the two sites (reference site – upstream of the wastewater discharge, and pollution impacted site – downstream of the wastewater discharge) were compared, as well as MT levels measured by electrochemical method among different wild freshwater bioindicator organisms.

2. Experimental section

2.1. Study area

The samplings of both *S. trutta* and gammarids were performed at the two sampling sites of the Krka River and involved two sampling campaigns, autumn (October 2015) and spring (May 2016). Coordinates for the reference site were 44°04.11' N 16°23.24' E and for the contaminated site 44°03.37' N 16°19.04' E.

Krka River watercourse is situated in the Dinaric area of the Republic of Croatia. Due to its unique tufa-barriers, a large part of the watercourse was proclaimed national park in 1985. Based on the previously published data on the physico-chemical water parameters and total dissolved metal levels in the river water (Cukrov et al., 2008; Filipović Marijić et al., 2018), river source was chosen as the reference site, whereas a location downstream of the town of Knin, located 2 km upstream of the northern border of the Krka National Park, was selected as the contaminated site. Krka River is threatened by two known sources of contamination: technological wastewaters of the screw factory and municipal wastewaters of the town of Knin. The wastewaters are released without a proper treatment into the river watercourse just 2 km upstream of the border of the Krka National Park. Krka River water analyses, conducted at the same time as fish and gammarids upstream and downstream of the wastewater discharge, have shown that metal/metalloid concentrations were increased at the affected site (Dragun et al., 2018; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Dissolved metals in water with the highest concentrations at the anthropogenically impacted site were Fe, Li, Mn, Mo, Sr, Rb and Ca and among them Fe and Mn are metals specific for technological wastewaters and often used in the manufacture of iron and steel alloys, and manganese products (Dragun et al., 2018; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). All measured physico-chemical water parameters indicated slightly deteriorated environmental conditions at the site downstream of wastewater discharge, of which temperature, conductivity, total dissolved solids and total water hardness showed significant between-site differences (Sertić Perić et al., 2018). The map and detailed description of the area have already been published (Filipović Marijić et al., 2018).

2.2. Sampling procedure

Sampling was conducted in two campaigns; in the autumn 2015, a total of 36 individuals of brown trout were sampled (16 from the reference site and 20 from the contaminated site), while in the spring 2016, a total of 32 fish were sampled (16 per each site).

Electrofishing was applied as a fish sampling tool, according to the Croatian standard HRN EN 14011. After capture, fish were placed in an opaque plastic tank with aerated river water in order to be kept alive until further processing in the laboratory. Fish were euthanized using freshly prepared anaesthetic tricaine methane sulphonate (MS 222, Sigma Aldrich, USA) and sacrificed. Body mass and the total length of the fish were recorded. The posterior part of the intestinal tissue was dissected, weighed and stored at -80°C until further analyses.

Gammarids were collected by benthos hand net (625 cm² and mesh size 250 μm) in aquatic macrophytes and on the stony substrate at the same sampling sites as fish. At least 200 individuals of *G. balcanicus*

were collected at each location in both seasons, while *E. acarinatus* was recorded only at one location, the Krka River source, and at least 180 individuals were sampled. Gammarids were stored at -80°C until further analyses in the laboratory. Prior to homogenization process, individuals were dried on the filter paper, subjected to manual exclusion of detritus that might contaminate the samples, and pooled together due to their small masses. At the reference site, 14 pooled samples of *G. balcanicus* in autumn and 10 samples in spring were obtained, whereas at the contaminated site, 16 samples were obtained in autumn, and 17 in spring. From the reference site, 5 pooled samples of the *E. acarinatus* were obtained in autumn and 6 in spring. A pooled sample of the *G. balcanicus* from the reference site consisted of 11–15 individuals, whereas 6–12 individuals were pooled at the contaminated site. For the appropriate sample of *E. acarinatus* collected at the reference site, 20–30 individuals were pooled.

2.3. Homogenization of posterior intestinal tissue and whole gammarids

Samples of the fish intestine were cut in small pieces and diluted 6 times with cooled homogenization buffer. The homogenizing buffer contained 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at 4°C) in which 1 mM DTT (dithiothreitol, Sigma, USA) was added as a reducing agent and 0.5 mM PMSF (phenylmethylsulfonyl fluoride, Sigma, USA) and 0.006 mM leupeptin (Sigma, USA) as protease inhibitors (Filipović Marijić and Raspor, 2007). Intestinal tissue was homogenized by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice cooled tube at 6000 rpm. Whole gammarids were homogenized in a same way; only pooled samples were 10 times diluted with the homogenization buffer to get enough material for the measurements (Filipović Marijić et al., 2016). Considering fish intestinal tissue, one part of the obtained homogenates was separated and subjected to the digestion procedure, in order to determine the total metal content (insoluble and soluble tissue fraction) in this tissue. The other part of the homogenates was centrifuged to obtain cytosolic cellular fraction, which was also digested for subsequent metal measurements. Cytosolic metals represent only soluble tissue fraction (Wallace and Luoma, 2003). In whole gammarids, due to the existence of chitin exoskeleton metals were measured only in cytosolic fractions.

2.4. Preparation of cytosolic and heat-treated cytosolic fractions

Fish and gammarid homogenates were centrifuged in the Avanti J-E centrifuge (Beckman Coulter, USA) at $50,000 \times g$ for 2 h at 4°C . Resulting supernatants (S50), representing the water soluble tissue fractions (cytosol), were used for metal analyses, while MT measurements were performed in the heat treated S50 fraction (HT S50). Heat-treatment was applied because this procedure denatures high molecular mass cytosolic proteins, which would otherwise interfere with the electrochemical MT determination, while MT as a thermostable protein remains in the solution after heat-treatment (Erk et al., 2002). The cytosolic S50 fraction was firstly 10 times diluted with 0.9% NaCl (Suprapur, Merck) to prevent co-precipitation of MTs with denatured proteins and then heat-treated at 85°C for 10 min in the Dri Block (Techne, GB). Afterwards, heat-treated samples were placed on ice at 4°C for 30 min and centrifuged at $10,000 \times g$ in Biofuge Fresco centrifuge (Kendro, USA). The resulting supernatant (HT S50), containing heat-stable proteins was stored at -80°C until further analyses, while the pellet was discarded.

2.5. Electrochemical determination of MT concentrations

MT concentrations were measured by differential pulse voltammetry (DPV) following the modified Brdička procedure (Raspor et al., 2001). Voltammetric measurements were performed on 797 VA Computrace (Metrohm, Switzerland) with a three-electrode system (hanging mercury drop electrode, HMDE, as a working electrode, an Ag/AgCl/

saturated KCl reference electrode and a platinum counter electrode). Measurements were done in duplicate (A and B subsample) in 10 mL of an electrolyte solution consisting of 5 mL of 2 M $\text{NH}_4\text{Cl}/\text{NH}_4\text{OH}$ and 5 mL of 1.2×10^{-3} M $\text{Co}(\text{NH}_3)_6\text{Cl}_3$, pH = 9.5 which was thermostated to 20°C and purged with the pure nitrogen. The applied measurement parameters for DPV were the following: potential scan from -0.9 V to -1.65 V; scan rate 0.013 Vs^{-1} ; voltage pulse amplitude 0.02502 V; duration of the pulse application 0.057 s and a step time 0.2 s (Mijošek et al., 2018). MT concentrations were derived from the straight calibration line, constructed with the commercially available standard rabbit liver MT-2 (Enzo, USA) dissolved in 0.25 M NaCl. Final results were expressed as mg MT g^{-1} of wet tissue (w.w.). In order to enable comparison with other available studies reporting MT levels on protein content, our data on MT levels were also standardized by the protein content ($\mu\text{g mg}^{-1}\text{prot}$). Protein concentrations were measured according to Lowry et al. (1951). Calibration was accomplished using a bovine serum albumin (BSA) (Serva, Germany) as a reference standard (0.25 – 2 mg ml^{-1} BSA).

2.6. Digestion of homogenates and cytosolic cellular fractions

Prior to the metal measurement, homogenates of fish intestine and cytosolic fractions of fish intestine and whole gammarids were digested in duplicates by adding the oxidation mixture of concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany). In all cases, concentrated acid and hydrogen peroxide were added in the volume ratio of 3:1. Digestion was performed in the laboratory dry oven at 85°C for 3.5 h. Cooled samples were afterwards diluted with Milli-Q water, 1:20 for Na, K, and Mg analyses, and 1:5 for the remaining elements (Dragun et al., 2018). The validation of acid digestion efficiency was performed by the digestion of dogfish muscle certified reference material for trace metals (DORM-2, National Research Council of Canada, NRC, Canada). Recoveries means of the trace elements studied from the certified reference material ranged from 95 to 105% as follows: As (103%), Cd (105%), Co (99%), Cu (100%), Fe (101%), Mn (101%); Se (102%), Tl (100%) and Zn (95%).

2.7. Determination of total and cytosolic metal concentrations

Elements were analyzed using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2; Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA). During the metal measurements, three resolution modes were used. Measurements of ^{82}Se , ^{98}Mo , ^{111}Cd , ^{133}Cs , and ^{205}Tl were all operated in low resolution mode; of ^{23}Na , ^{24}Mg , ^{42}Ca , ^{55}Mn , ^{56}Fe , ^{59}Co , ^{63}Cu and ^{66}Zn in medium resolution mode; and high resolution mode was used for ^{39}K and ^{75}As determination. External calibration for macro elements was made using multielement stock standard solution containing $\text{Ca } 2.0 \text{ g L}^{-1}$, $\text{Mg } 0.4 \text{ g L}^{-1}$, $\text{Na } 1.0 \text{ g L}^{-1}$, and $\text{K } 2.0 \text{ g L}^{-1}$ (Fluka, Germany). Calibration solution for the trace elements was prepared by dilution of multielement stock standard solution (Analitika, Czech Republic) supplemented with Cs (Fluka, Germany). Indium ($1 \mu\text{g L}^{-1}$, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to all solutions as an internal standard (Fiket et al., 2007). Quality control samples were used to test the accuracy and the precision of measurements; QC Minerals, Catalog number 8052, UNEP GEMS, Burlington, Canada for the macro elements and QC trace metals, catalog no. 8072, UNEP GEMS, Burlington, Canada for the trace elements. A generally good agreement was observed between our data and certified values, with the following recoveries based on five measurements in the control sample (%): As (101.4 ± 10.3), Ca (95.7 ± 1.3), Cd (95.6 ± 0.6), Co (97.0 ± 1.6), Cu (95.7 ± 2.2), Fe (95.4 ± 5.1), K (90.7 ± 5.1), Mg (93.3 ± 2.5), Mn (96.5 ± 1.8), Na (97.3 ± 3.9), Se (99.1 ± 3.6), Tl (96.3 ± 0.8) and Zn (96.9 ± 2.3). Limits of detection (LOD) were calculated based

on three standard deviations of ten consecutively determined trace element concentrations in blank sample (100 mM Tris-HCl/Base, 1 mM DTT) which was digested the same way as samples. LODs for trace elements measured within this study were the following (ng g^{-1}): As, 6.72; Cd, 0.430; Co, 0.266; Cs, 0.102; Cu, 13.5; Fe, 141; Mn, 0.810; Mo, 0.680; Se, 2.93; Tl, 0.001 and Zn, 635, while for macro elements ($\mu\text{g g}^{-1}$): Ca, 1.07; K, 0.112; Mg, 0.024; and Na, 0.320.

2.8. Statistical methods

Basic calculations were performed in Microsoft Office Excel 2007, while SigmaPlot 11.0 (Systat Software, USA) was used for all statistical analyses. Since assumptions of normality and homogeneity of variance were not always met, the significance of differences between seasons or locations was tested by application of Mann-Whitney *U* test. Differences were regarded as significant when $p < 0.05$. Correlation between different parameters was calculated using Spearman correlation analysis. Levels of significance of applied statistical tests were indicated in the text. Fulton condition indices (FCI) were expressed according to Rätz and Lloret (2003), i.e. $K = W/L^3$, where *W* is the body mass (g) and *L* is the total length of fish (cm).

3. Results and discussion

3.1. Biological responses in brown trout

Comparison of biometric parameters of sampled fish from two sampling sites indicated comparable total length but higher body mass of fish from the wastewater impacted site in both seasons, although not significantly. Only FCI values were significantly higher at the contaminated site in both investigated seasons ($U = 6.00$; $p = 0.001$ in autumn and $U = 9.00$; $p = 0.002$ in spring). This could be due to the higher fish masses, which are likely a consequence of better nutrient availability (Lambert and Dutil, 1997) originating from municipal and industrial wastewaters discharged into the Krka River water downstream of the town of Knin. Average total length and body mass both pointed to significantly higher fish biometric parameters in the autumn than spring season at both locations ($U = 14.5$; $p = 0.008$ and $U = 20$; $p = 0.026$ for fish length in reference and contaminated site, respectively; $U = 16$; $p = 0.011$ and $U = 21$; $p = 0.031$ for fish mass in reference and contaminated site, respectively). Other than that, FCI values were elevated in spring, although not significantly which could be a result of the seasonal mobilization of energy reserves needed for reproductive development (Maddock and Burton, 1999). However, in different studies the opposite trend of lower FCI values in polluted sites is also often observed (Couture and Rajotte, 2003; Jenkins, 2004; Shobikhuliatul, 2013; Zhelev et al., 2016, 2018). Our results might suggest that the wastewater impact at the contaminated site was not high enough to induce defense mechanism of fish in a way which would require a lot of energy and consequently result in decreased FCI values.

3.1.1. MT concentrations in the heat-treated cytosol of intestine of brown trout

Average fish intestinal MT levels were higher at the contaminated site (downstream of the town of Knin) compared to the river source in both seasons, but the site- or season-specific differences were not proven significant (Fig. 1). Average MT concentrations in the intestine of fish from the reference site in autumn and spring campaign were 0.85 and 0.96 mg g^{-1} w.w., and from polluted site 1.5 and 1.45 mg g^{-1} w.w., respectively (Fig. 1). To our knowledge, MT levels reported in this study represent the first data set for the intestinal tissue of brown trout measured by electrochemical method DPV. Different research groups in the world use variety of spectrometric, immunochemical and electrochemical methods for MT determination but obtained MTs levels are highly variable depending on the measurement method (Isani et al., 2000; Dabrio et al., 2002; Zorita et al., 2005). Therefore, it would not

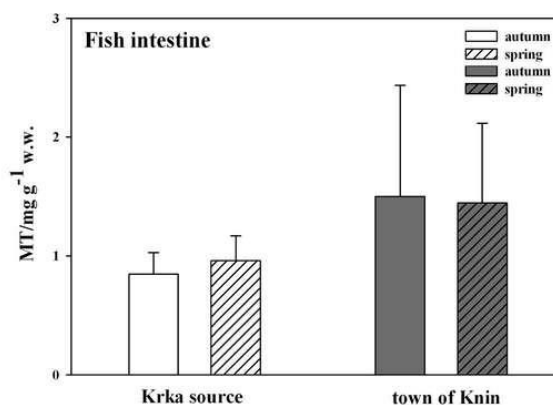


Fig. 1. MT levels (mg g^{-1} w.w., mean values and standard deviations) in intestinal tissue of *S. trutta* from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: town of Knin) in two samplings (autumn 2015 and spring 2016).

be relevant or correct to compare our records with the MT levels obtained by different methods. Data on MT levels determined by electrochemical method were reported for different tissues of wild freshwater fish species, i.e., European chub (Dragun et al., 2009; Filipović Marijić and Raspor, 2010; Dragun et al., 2013), rainbow trout (Roch et al., 1982), common carp, perch, pike, bream, roach and rudd (Sevcikova et al., 2013). Filipović Marijić and Raspor (2010) reported average MT concentrations in the intestine of European chub from the Sava River to be around 3 mg g^{-1} w.w., which is therefore 2–3 times higher than values observed for the brown trout from the Krka River in our study, but also higher compared to MT levels in gills (around 2 mg g^{-1} w.w) and liver (around 1.5 mg g^{-1} w.w) of the same species (Dragun et al., 2009; Podrug and Raspor, 2009; Dragun et al., 2015). As gastrointestinal tissue and gills are organs which are known to be involved in the uptake, detoxification and excretion processes (Van Cleef et al., 2000), higher MT concentrations observed in these tissues could probably be linked to the important function of MTs in metal homeostasis and detoxification. In humans, higher MT concentrations can even indicate more serious disorders in the body, such as carcinoma (Krizkova et al., 2009b). However, there is no real connection of higher MT concentrations with carcinoma in fish. Barišić et al. (2018) made the investigation on architectural and histopathological biomarkers in the intestine of the same brown trout specimens as used in this research and concluded that serious histopathological lesions, such as neoplasia, were not evident in fish from the Krka River.

3.1.2. Total and cytosolic metal concentrations in intestine of brown trout

Metal levels measured in digested homogenate, presenting total metal concentrations in insoluble and soluble tissue fractions, were, as expected, higher compared to their levels measured in cytosolic intestinal fraction, i.e. soluble fraction (Table 1). For Ca, Cu, Fe, Mn and Zn < 50% of the total metal levels were present in the insoluble cellular fractions (Fig. 2a), pointing that these metals are partially present in tissue fraction which is not considered as metabolically available (such as metal rich granules) and partially in cytosolic fraction, which represents potentially toxic part of metals which can bind to physiologically important molecules (Wallace and Luoma, 2003; Vijver et al., 2004; Caron et al., 2018). The proportion of other measured metals, As, Cd, Cs, Mo, Se, K, Mg, and Na, was over 67% in cytosolic fraction (Fig. 2b), indicating that these metals are mostly found in soluble fraction where they can be bound to cytosolic biomolecules, for example metallothioneins (detoxified metal fraction) or enzymes (metal-sensitive fraction) (Wallace and Luoma, 2003; Caron et al., 2018). Presented relation of total and cytosolic metal/metalloid concentrations

Table 1

Total and cytosolic metal and metalloid concentrations ($\mu\text{g kg}^{-1}$ or mg kg^{-1} (macroelements)) in the intestinal tissue of *S. trutta* from the Krka River at two sampling sites in two sampling campaigns. For each element first row represents the total levels and the second cytosolic levels. Results are showed as mean values \pm standard deviations.

	Krka River source				Town of Knin			
	October 2015		May 2016		October 2015		May 2016	
	For all elements: first row – total levels; second row – cytosolic levels							
As	20.27 \pm 12.14 [*]	12.45 \pm 7.19 ^{*, A}	32.53 \pm 9.24 [*]	20.63 \pm 7.40 [*]	30.28 \pm 14.73	33.94 \pm 34.94 ^B	42.83 \pm 17.35	37.06 \pm 15.03
Cd	88.87 \pm 123.78	64.76 \pm 91.80 ^A	135.47 \pm 125.79 ^A	85.39 \pm 89.94 ^A	27.68 \pm 25.30 [*]	30.80 \pm 51.54 ^{*, B}	3.81 \pm 2.90 ^{*, B}	3.12 \pm 2.58 ^{*, B}
Co	39.12 \pm 17.42	15.38 \pm 11.57 ^A	24.33 \pm 58.86 ^A	13.73 \pm 5.93 ^A	61.34 \pm 37.73	46.33 \pm 56.26 ^B	58.86 \pm 27.45 ^B	57.62 \pm 45.03 ^B
Cs	10.03 \pm 2.18 ^A	9.28 \pm 2.31 ^{*, A}	7.82 \pm 1.83	7.01 \pm 1.93 ^{*, A}	5.79 \pm 4.50 ^B	4.25 \pm 2.86 ^B	5.97 \pm 1.84	4.82 \pm 1.70 ^B
Cu	777.88 \pm 242.17	253.10 \pm 126.32 ^A	942.37 \pm 221.62	356.22 \pm 115.68	966.58 \pm 413.62	597.41 \pm 560.92 ^B	897.67 \pm 312.04	345.58 \pm 158.98
Fe	19116.76 \pm 11560.96	8185.41 \pm 3138.92	11009.87 \pm 2281.04	5939.63 \pm 3153.94	17529.05 \pm 4137.32	6614.67 \pm 3321.66	14749.16 \pm 5247.41	7037.54 \pm 1597.50
Mn	921.23 \pm 478.61	399.26 \pm 308.03	783.62 \pm 139.55	282.13 \pm 60.16	881.87 \pm 209.90	266.48 \pm 112.80	953.15 \pm 435.44	316.30 \pm 115.86
Mo	50.90 \pm 39.22	31.96 \pm 10.89	48.28 \pm 13.06 ^A	30.67 \pm 8.55	42.48 \pm 8.90 [*]	41.10 \pm 20.60 [*]	31.00 \pm 5.31 ^{*, B}	23.81 \pm 7.72 [*]
Se	807.83 \pm 323.81 ^A	721.83 \pm 329.89 ^A	845.26 \pm 172.90 ^A	677.11 \pm 69.57 ^A	1201.36 \pm 385.90 ^B	1120.86 \pm 511.20 ^B	1173.95 \pm 292.24 ^B	1056.38 \pm 296.96 ^B
Tl	45.97 \pm 31.73 ^A	29.62 \pm 15.38 ^A	44.62 \pm 12.96 ^A	30.78 \pm 10.62 ^A	19.24 \pm 7.90 ^B	8.68 \pm 4.04 ^B	19.89 \pm 8.15 ^B	11.76 \pm 5.53 ^B
Zn	98677.54 \pm 39032.26	42579.36 \pm 12009.36	107033.66 \pm 49100.97	45995.21 \pm 12593.36	138929.69 \pm 86549.18	46981.93 \pm 20645.43	124701.27 \pm 23088.43	54950.47 \pm 6834.28
Ca	221.28 \pm 160.72	91.37 \pm 93.31	136.74 \pm 49.24 ^A	53.95 \pm 18.77 ^A	292.98 \pm 298.14	112.42 \pm 117.82	245.27 \pm 93.49 ^B	94.67 \pm 41.56 ^B
K	2935.06 \pm 357.63	2842.10 \pm 326.10	2887.32 \pm 364.96	2749.35 \pm 171.76	2938.30 \pm 430.18	2681.73 \pm 402.69	2911.28 \pm 364.25	2811.05 \pm 287.30
Mg	154.53 \pm 21.07	103.16 \pm 18.64	163.59 \pm 21.51	103.52 \pm 11.10	164.01 \pm 23.72	100.45 \pm 15.67	148.39 \pm 24.96	100.55 \pm 23.17
Na	1107.76 \pm 132.20 [*]	1071.33 \pm 111.14 [*]	932.87 \pm 177.15 [*]	904.32 \pm 125.92 [*]	1117.55 \pm 115.57 [*]	981.17 \pm 173.13	974.32 \pm 162.13 [*]	976.78 \pm 127.09

Significant difference at $p < 0.05$ level between two seasons at each sampling site is marked with asterisk (*) and significantly different values at two sampling sites within the same sampling campaign are assigned with different superscript letters (A and B).

in *S. trutta* intestinal tissue is in accordance to the proportions of total metals in hepatic cytosol of the same fish (Dragun et al., 2018). Exceptions were only Co, Cu, Mn and Zn, with around 20% higher proportion of total levels in liver cytosol than intestinal cytosol. Total concentrations of trace elements in *S. trutta* intestinal tissue followed the descending order $\text{Zn} > \text{Fe} > \text{Se} > \text{Mn} \geq \text{Cu} > \text{Cd} > \text{Co} \geq \text{Mo} > \text{Tl} \geq \text{As} > \text{Cs}$, which is quite similar to total metal trends observed in hepatic tissues of the same fish (Dragun et al., 2018). Due to the lack of data on cytosolic metal levels in fish intestine, comparison

with other literature was only possible for total metal levels and also confirmed the common trend of the highest Fe, Zn, Mn and Cu levels in the intestine of rainbow trout from rivers Augraben and the Leiferer Graben in Italy (Dallinger and Kautzky, 1985), perch from the lake Mondsee in Austria (Sures et al., 1999), different freshwaters fish species in waters of Lithuania (Staniskiene et al., 2006), starlet from the Danube River in Serbia (Jarić et al., 2011), barbel from the Danube River in Bulgaria (Nachev and Sures, 2016) and in *Salmo trutta macrostigma* and rainbow trout from Çatak River in Turkey (Yeltekin and

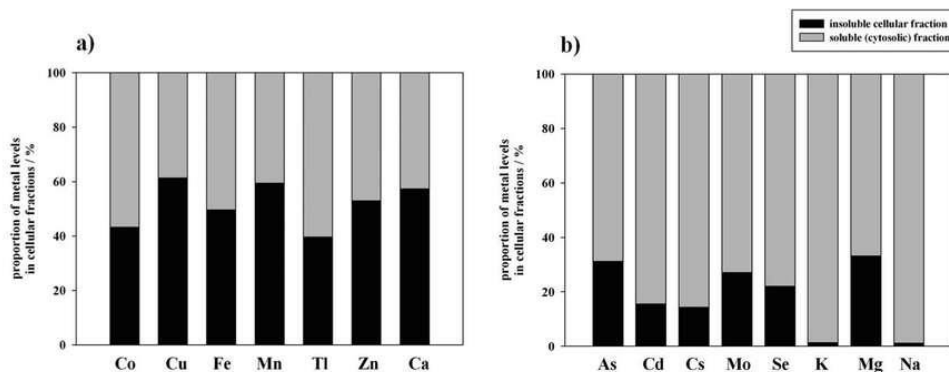


Fig. 2. Proportions (%) of metal/metalloid levels present in cellular cytosolic fraction (soluble) and insoluble fraction of the intestine of *S. trutta* caught in the Krka River: a) metals present in cytosolic fraction up to 60% and b) metals present in cytosolic fraction above 60%.

Sağlamer, 2019). In fish from Croatian rivers, average total Cu, Fe and Mn levels in the intestinal tissue of European chub from the lowland Sava River were either comparable or lower than their values in intestine of brown trout from the karst Krka River, depending on the season and location, while total Cd and Zn levels were mostly higher in the intestine of European chub from the Sava River (Filipović Marijić and Raspor, 2012; Dragun et al., 2015).

Despite differences in total and cytosolic metal concentrations, their relation between two locations indicated similar pattern, with higher total and cytosolic concentrations of As, Ca, Co, Se and Zn in brown trout from the contaminated compared to the reference site (Table 1). Such pattern of elevated intestinal metal/metalloid levels at the contaminated site might reflect higher metal/metalloid exposure level in the river water at the location near the town of Knin, influenced by technological and municipal wastewaters, as already reported by Filipović Marijić et al. (2018) and Sertić Perić et al. (2018). Other measured metals, Fe, K, Mg, Mn and Na did not show a clear trend between two locations (Table 1), while Cd, Cs, Mo and Tl concentrations were higher in the intestinal homogenate and cytosolic fraction of fish from the Krka River source in at least one season (Table 1). Presented results are in accordance with the trend reported for hepatic metal levels of *S. trutta* from the same locations (Dragun et al., 2018) but the exact cause of significantly higher Cd, Cs, Mo and Tl concentrations in fish from the reference site requires further investigation, with special emphasis on river sediment and food as metal sources, considering dietary intake as the important uptake route in fish (Clearwater et al., 2000). Cd, Cs and Tl were also significantly higher in gammarids from the same site, which might serve as possible fish prey and consequently as a possible metal source for fish.

Regarding seasonal differences, majority of studied metal/metalloids in intestinal homogenate and cytosolic fraction had higher levels in autumn than spring season. Significant differences were observed in both fractions only for As and Na at the reference site, and for Mo and Cd at the contaminated site, with the elevated metal levels in autumn, except for As (Table 1). Unique seasonal differences at both locations, but without significant differences, were evident as higher Co, Cs, Fe, Mo and Na levels in autumn in intestinal homogenate and as higher Mo, Se and Na levels in autumn in intestinal cytosol (Table 1). Mostly lower intestinal metal levels in spring could be due to the lower dissolved metal levels in the river water accompanied by the more effective self-purification process of the Krka River in that period (Cukrov et al., 2008; Filipović Marijić et al., 2018).

Since one of the main MT roles in the organisms is the regulation of essential metals (Cu and Zn), and detoxification of heavy metals (Cd, Hg, Ag) (Amiard et al., 2006), which increased levels may induce MTs synthesis, we evaluated possible contribution of intestinal metals to the observed MT levels. Spearman correlation analysis confirmed a significantly positive relation of MT with cytosolic Cd ($r = 0.762$; $p = 0.02$) and Cu levels ($r = 0.786$; $p = 0.0149$) in fish from the reference site in spring, while in autumn with cytosolic Cu in fish from contaminated site ($r = 0.782$; $p = 0.005$). Total metal levels did not show significant correlation with MT, probably because cytosolic metals are those which might be directly bound to biomolecules and have impact on their concentrations, activities or structures (Caron et al., 2018). However, metal content obviously cannot completely explain variability and complexity in MT levels, which may be affected by other parameters such as season, temperature, pH values, size, fish gender or nutritional status (Hyland et al., 1998; Dragun et al., 2009; Filipović Marijić and Raspor, 2010). Intestinal MT and metal levels did not show significant correlation with brown trout biometry, what is in agreement with the existing literature data where intestine has already been reported as an organ with no additional metal accumulation with fish age and growth (Giguère et al., 2004; Filipović Marijić and Raspor, 2007). Of physico-chemical factors, temperature, conductivity, total dissolved solids and total water hardness showed significant differences between the sites and pointed to deteriorated ecological status near the town of

Knin (Sertić Perić et al., 2018), possibly influencing MT levels as well. Since in polluted environment organisms are exposed to a mixture of different metals and contaminants, it is generally impossible to connect the elevated MT synthesis only to specific elements, especially knowing that a combination of various biotic and abiotic factors greatly affects MT induction.

3.2. Biological responses in gammarids

In both seasons, individuals of *G. balcanicus* were bigger at the contaminated site with the average weight of 27 and 23 mg in autumn and spring, respectively. At the Krka source, average weights were about 15 mg in both seasons. *E. acarinatus* individuals were sampled only at Krka River source and they were much smaller than *G. balcanicus*, which is the inherent property of this species. The average weights of *E. acarinatus* were of 6 mg in autumn, and 8 mg in spring. Gammarid mass differences were most likely caused by habitat or microhabitat conditions. In the source part of the rivers, higher water velocity takes away nutrients and consequently can affect size of the organisms. Žganec et al. (2016) also observed dominance of smaller species of gammarids in both microhabitat types, stones and mosses, at the upper course of the Krka River, which represents food limited location due to the lack of packs of detritus/leaves – likely as a result of a very strong water current and absence of detritus in upstream sections of the river. Usually, higher abundance and bigger gammarid individuals are found in the downstream parts where more fine particulate organic matter can be found.

3.2.1. MT concentrations in the heat-treated cytosol of *Gammarus balcanicus*

Opposite to the intestinal MT levels in brown trout, MT concentrations in *G. balcanicus* differed significantly between locations and seasons (Fig. 3a). Spatial differences were observed in spring with significantly higher MT levels in gammarids from the wastewater impacted location ($U = 23.00$; $p = 0.002$), while in autumn MT concentrations were comparable between the reference and contaminated site. Significant seasonal differences were present at both locations, pointing to increased MT levels in autumn ($U = 6.00$; $p < 0.001$ in the reference site and $U = 32.00$; $p < 0.001$ at the contaminated site) (Fig. 3a). Average MT concentration in gammarids in autumn was around $3.30 \text{ mg g}^{-1} \text{ w.w}$ in both locations while average MT levels in spring were lower, $2.43 \text{ mg g}^{-1} \text{ w.w}$ in individuals from the reference site and $2.87 \text{ mg g}^{-1} \text{ w.w}$ in individuals from the Krka near Knin (Fig. 3a). These values were comparable or a bit higher than the MT levels obtained in the research on *G. fossarum* from the Sutla River, where reported average MT values were around $2.50 \text{ mg g}^{-1} \text{ w.w}$ (Filipović Marijić et al., 2016).

3.2.2. Cytosolic metal concentrations in *Gammarus balcanicus*

Reported differences in MT levels might be, to some extent, linked to cytosolic metal/metalloid concentrations, which were higher in *G. balcanicus* from the contaminated site, and this trend was proven significant for Co, Fe, Mn, Mo, K and Na in both seasons and for As, Cu and Zn in one season (Table 2). Therefore, a significant difference in MT levels between the two sites in spring could be linked to the much higher concentrations of Cu and Zn as important MT inducers at the location downstream of the town of Knin (Table 2). Zn was also significantly correlated to MT levels in *G. balcanicus* in autumn at the contaminated site ($r = 0.621$; $p = 0.0101$). On the other hand, Cd, Cs and Tl levels were significantly elevated in *G. balcanicus* from the reference site in both seasons, the same as recorded in fish intestine (Table 1), and Ca and Se only in autumn (Table 2). Again, as dissolved metal concentrations in water do not follow such pattern, the exact cause of these higher concentrations at the reference site needs to be further investigated. Ternjeje et al. (2014) reported total metal levels in *G. balcanicus* from the Kosovčica River, which is Krka tributary, and

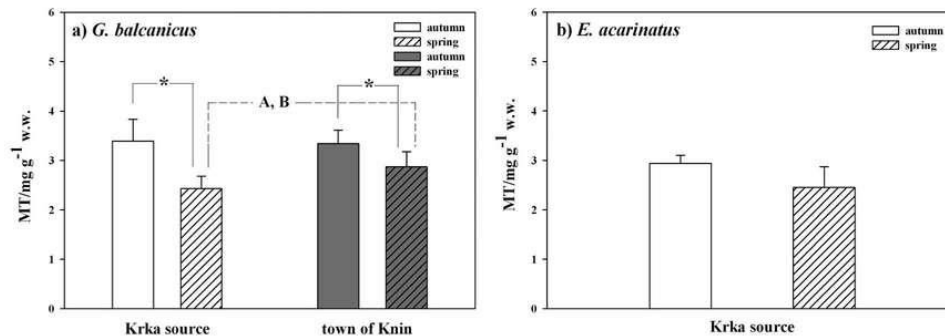


Fig. 3. MT levels (mg g^{-1} w.w., mean values and standard deviations) in a) *G. balcanicus* and b) *E. acarinatus* from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: town of Knin) in two samplings (autumn 2015 and spring 2016). Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ level between two seasons at each sampling site are marked with solid line and asterisk (*) and between two sampling sites within the same season are assigned with dashed line and different superscript letters (A and B).

also pointed to higher Cd levels in gammarids from the river spring compared to the pollution impacted river watercourse.

Significant seasonal differences in metal accumulation in *G. balcanicus* were observed for As, Cd, Cu, Fe, Mn, Mo, Ca, Mg and Na at the reference site with mostly higher values in autumn. Only As and Na levels were higher in spring, similar to As concentrations in fish intestine (Table 1). At the contaminated site, statistically significantly higher Cd, Cs and Mo levels were evident in autumn and Co, Se, Tl, Zn and Na levels in spring (Table 2). In addition, cytosolic As and Ca levels were higher in spring than autumn, but without significant differences, what is in accordance to the seasonal trend of As found in intestine of brown trout (Table 1).

Correlation of Cd, Cu or Zn with MT was mostly not significant, except Zn ($r = 0.621$; $p = 0.0101$) in autumn at Knin location, but as in other organisms, MT induction in gammarids might be impacted by other factors such as season, temperature, size, gender or reproductive status (Rainbow and Moore, 1986; Correia et al., 2004; Geffard et al., 2007). However, data on the impact of these parameters are not always consistent. For example, Geffard et al. (2007) concluded that MTs levels in *G. pulex* were significantly negatively related to the organism weight, while on the other hand, Filipović Marijić et al. (2016) have not

observed any significant differences in MT concentration in relation to the *G. fossarum* size. No significant differences in MT levels were observed between the different age-groups of *G. locusta* either (Correia et al., 2004).

There is not much detailed data on the life cycle of *G. balcanicus* in the world, but in the area of Bieszczady Mountains in Poland, the breeding period of *G. balcanicus* lasts from the beginning of April to the end of October (Zieliński, 1995). However, depending on the water temperature and geographical region, in localities with constant water temperatures, this species may have acyclic breeding without a winter pause (Dedju, 1980). In the case of the Krka River, the exact life cycle of the species is not known yet, but observed seasonal differences in MT levels might be associated with the different reproductive stages. Levels of MT were significantly higher in autumn at both locations, likely during the reproduction period for this species. Generally, as most of the gammarids have similar life-cycles, MT synthesis is directly related to seasons, with higher values in autumn and winter and lower in spring, as for example observed in *G. pulex* (Geffard et al., 2007). Many studies on different invertebrate species like *Corbicula fluminea* (Baudrimont et al., 1997), *Mytilus galloprovincialis* (Raspor et al., 2004; Ivanković et al., 2005) or *Mytilus edulis* (Geffard et al., 2005) have also

Table 2

Cytosolic metal and metalloid concentrations ($\mu\text{g kg}^{-1}$ or mg kg^{-1} (macroelements)) in *G. balcanicus* from the Krka River at two sampling sites in two sampling campaigns and *E. acarinatus* from the Krka River source in two campaigns. Results are showed as mean values \pm standard deviations.

	Krka River source		Town of Knin		Krka River source	
	October 2015	May 2016	October 2015	May 2016	October 2015	May 2016
	<i>Gammarus balcanicus</i>				<i>Echinogammarus acarinatus</i>	
As	79.11 \pm 19.89 ^A	152.70 \pm 24.5 ^A	135.17 \pm 11.60 ^B	147.18 \pm 23.88	157.46 \pm 23.51*	220.35 \pm 13.36*
Cd	204.30 \pm 38.05 ^A	100.18 \pm 12.11 ^A	15.81 \pm 3.71 ^A	12.42 \pm 2.62 ^B	180.79 \pm 23.42*	121.98 \pm 14.67*
Co	17.87 \pm 2.0 ^A	19.09 \pm 1.94 ^A	34.41 \pm 3.16 ^B	70.07 \pm 11.11 ^B	24.57 \pm 8.22	21.96 \pm 0.59
Cs	4.01 \pm 0.4 ^A	3.84 \pm 0.25 ^A	3.03 \pm 0.46 ^B	2.62 \pm 0.22 ^B	4.43 \pm 0.28*	4.10 \pm 0.16*
Cu	5365.41 \pm 1207.69 ^A	4184.47 \pm 563.15 ^A	5525.71 \pm 529.37	5684.54 \pm 651.55 ^B	4464.71 \pm 1106.95	4345.17 \pm 299.37
Fe	1417.96 \pm 552.66 ^A	977.09 \pm 126.11 ^A	2124.95 \pm 353.67 ^B	1972.47 \pm 359.78 ^B	1638.18 \pm 497.07	1284.98 \pm 509.41
Mn	335.80 \pm 37.43 ^A	281.40 \pm 22.21 ^A	648.00 \pm 88.88 ^B	622.53 \pm 78.17 ^B	433.74 \pm 30.44*	325.71 \pm 28.83*
Mo	39.25 \pm 6.85 ^A	27.60 \pm 3.06 ^A	68.06 \pm 8.70 ^B	57.02 \pm 7.94 ^B	35.80 \pm 5.58*	26.71 \pm 1.54*
Se	321.81 \pm 45.96 ^A	299.68 \pm 44.38	290.65 \pm 31.94 ^B	323.91 \pm 47.21 ^B	322.77 \pm 18.79	330.11 \pm 18.23
Tl	19.41 \pm 2.49 ^A	18.55 \pm 1.02 ^A	5.57 \pm 0.77 ^B	7.38 \pm 1.20 ^B	27.66 \pm 1.51*	22.83 \pm 2.18*
Zn	6806.64 \pm 719.10	6675.64 \pm 595.40 ^A	6794.99 \pm 393.87 ^A	8061.22 \pm 838.43 ^B	7252.82 \pm 886.40	6883.24 \pm 596.36
Ca	4698.53 \pm 322.75 ^A	4041.23 \pm 293.38 ^A	4140.15 \pm 252.09 ^B	4310.13 \pm 415.00	5084.05 \pm 454.71*	4414.88 \pm 335.72*
K	1627.99 \pm 206.60 ^A	1530.02 \pm 70.29 ^A	1762.77 \pm 145.89 ^B	1746.65 \pm 95.18 ^B	1726.35 \pm 120.58*	1579.29 \pm 53.14*
Mg	269.72 \pm 27.09 ^A	220.81 \pm 19.49 ^A	228.92 \pm 18.45 ^B	239.34 \pm 19.50 ^B	280.43 \pm 24.66*	224.40 \pm 18.67*
Na	1178.64 \pm 70.27 ^A	1352.78 \pm 87.68 ^A	1060.26 \pm 73.87 ^B	1291.18 \pm 50.19 ^B	1244.49 \pm 100.92*	1491.57 \pm 75.54*

Significant difference at $p < 0.05$ level between two seasons at each sampling site is marked with asterisk (*) and significantly different values at two sampling sites within the same sampling campaign are assigned with different superscript letters (A and B).

already shown that variations in MT levels are often related to the physiological conditions of organisms, especially to their reproductive stage.

3.2.3. MT concentrations in the heat-treated cytosol of *Echinogammarus acarinatus*

Krka River source has already been reported as a habitat of another two gammarid species – *Echinogammarus acarinatus* Karaman, 1931 and *Fontogammarus dalmatinus krkensis* S. Karaman, 1931, both being endemic species in Dinaric karst rivers (Gottstein et al., 2007; Žganec et al., 2016). These species do not inhabit the area of the chosen contaminated site, so in our research results on *E. acarinatus* are presented only for the reference location in October 2015 and May 2016, whereas *F. dalmatinus krkensis* was not recorded in macrophytes of the Krka spring, but reaches the highest densities in the moss microhabitats of the spring head (Žganec et al., 2016). Absence of *E. acarinatus* and *F. dalmatinus krkensis* in the anthropogenically impacted area of the Krka River was already reported and explained as a consequence of their sensitivity on pollution impact, so their habitat in the Krka River comprises only clean parts of the watercourse (Gottstein et al., 2007).

MT levels in *E. acarinatus* were higher in autumn, the same as in *G. balcanicus*, but the seasonal differences were not significant. Average MT values in *E. acarinatus* were 2.94 and 2.53 mg g⁻¹ w.w in autumn and spring, respectively (Fig. 3b). These values were also similar and comparable to the MT concentrations observed in *G. balcanicus*, so the average MT levels were not significantly different between the two gammarid species at the Krka source in any season (Fig. 3).

3.2.4. Cytosolic metal concentrations in *Echinogammarus acarinatus*

Since *E. acarinatus* species only inhabit unpolluted area of the Krka River, cytosolic metals were presented regarding their seasonal differences in gammarids from the river source. Among investigated metals only As and Na were significantly increased in the spring campaign, while Cd, Cs, Mn, Mo, Tl, Ca, K and Mg were significantly increased in autumn (Table 2). Such pattern was found in *G. balcanicus*, in which As and Na were the only elements elevated in spring (Table 2), as also showed for As in fish intestine (Table 1). Significant differences between the two gammarid species were observed for As, Co, Cs, Mn and Tl in both seasons, while for Cd, Ca and Na only in the spring season. All of these elements had higher concentrations in *E. acarinatus* (Table 2). As *E. acarinatus* individuals were much smaller, the differences in metal accumulation might be caused by the gammarid size differences. For example, Rainbow and Moore (1986) showed that the smallest amphipods accumulated the highest concentrations of Cu, Zn, Fe and Pb. Moreover, even closely related species like these two gammarid species may be feeding on different food sources which results in different dietary inputs of metals (Rainbow and Moore, 1986). If we consider Cd, Cu and Zn, as the main MT inducers, their levels were not significantly correlated with MT levels in *E. acarinatus*, as already stated for other gammarid species, *G. balcanicus* (Table 2).

3.3. Comparison of cytosolic metal concentrations in intestine of freshwater fish and whole gammarids

Most of the cytosolic metal levels were higher in gammarids than in brown trout intestine, therefore confirming that most of the metals are not expected to biomagnify in aquatic food webs (Mathews and Fisher, 2008). The highest difference existed for Ca, Cu and As, which average levels were around 50, 15 and 8 times higher in gammarids than in fish cytosolic fraction, respectively. Twice as higher cytosolic Cd and Mg levels were recorded in gammarids than in fish intestine, while few metals showed the opposite pattern, i.e. K, Se and Cs levels were 2–3 times lower and Fe and Zn about 5 times lower in gammarids (Tables 1 and 2). Such results are in accordance to trophic transfer factors obtained for metals in marine food chain, which indicated that possible biomagnification is specific for Cs, Se and Zn (Mathews and Fisher,

2008). Descending order of cytosolic metal levels in intestine of brown trout from the Krka River was the following: K > Na > Mg > Ca > Zn > Fe > Se (average metal levels higher than 1000 µg kg⁻¹) and Cu ≥ Mn > Cd ≥ Co > Mo > As > Tl > Cs (average metal levels lower than 1000 µg kg⁻¹) (Table 1). Comparison of cytosolic metal levels between two gammarid species, *E. acarinatus* and *G. balcanicus* indicated higher metal levels in *E. acarinatus*, but the concentration range in both species was comparable. Thus, descending order of cytosolic metal levels in both gammarid species from the Krka River was the following: Ca > K > Na > Mg > Zn > Cu > Fe (average metal levels higher than 1000 µg kg⁻¹) and Mn > Se > As ≥ Cd > Mo > Co ≥ Tl > Cs (average metal levels lower than 1000 µg kg⁻¹) (Table 2).

To our knowledge, comparison of intestinal cytosolic metal/metalloid concentrations in brown trout with other fish species was possible only for cytosolic metal levels in the intestinal tissue of European chub from the Sava River, which showed the same descending order of investigated metal levels and mostly comparable concentrations (Zn > Fe > Cu > Mn > Cd) (Filipović Marijić and Raspor, 2012). Cytosolic metal levels in gammarids can be compared with levels in *G. fossarum* from the Sutla River where Cs, Cu, Mn and Zn levels were approximately 2 times higher than in our research, while the levels of Ca and Tl were about 2 and 6 times higher in gammarids from the Krka River, respectively (Filipović Marijić et al., 2016). Such differences in cytosolic metal levels between different gammarid species are probably influenced by variability in metal exposure and environmental conditions of their habitat.

3.4. Comparison of MT concentrations measured by electrochemical methods in freshwater fish and gammarids from different studies

Modified Brdička reaction is recognized as a commonly and widely used electrochemical method for MT determination in biological samples (Fabrik et al., 2008; Dragun et al., 2009; Krizkova et al., 2009a; Filipović Marijić et al., 2016). In our research, newly modified Brdička method (Mijošek et al., 2018) was confirmed as a fast and reliable technique for quantification of MTs in both intestinal fish tissue and the whole individuals of gammarids species. One of the main advantages of the applied method is that it requires a small amount of the sample to conduct the assay. Our results on MT concentrations were compared to other so far published data on MT levels in natural populations of organisms measured by electrochemical method in order to get an overview on MT levels in different freshwater fish and gammarid species (Table 3). For the purposes of correct comparison, MT levels (mg g⁻¹ w.w) from our study were additionally divided with the total cytosolic protein concentrations, resulting in the average concentrations in brown trout intestine of around 20.5 µg mg⁻¹ proteins and in gammarids of around 60 µg mg⁻¹ proteins. Also, MT levels (mg g⁻¹ w.w) were divided with MT molecular weight of 6600 Da, resulting in MT average concentrations in brown trout intestine of around 20 nmol g⁻¹ and in gammarids of around 40 nmol g⁻¹. So far, intestinal MT levels were only reported for the European chub from the Sava River (Croatia), which MT levels (2.9–3.1 mg g⁻¹ w.w) were twice as high as in brown trout intestine (0.8–1.5 mg g⁻¹ w.w) (Table 3). In other fish tissues electrochemically determined MTs ranged 0.3–2 mg g⁻¹ w.w and 2–7 µg mg⁻¹ prot. in gills; 2–12 mg g⁻¹ w.w and 5–18 µg mg⁻¹ prot. in liver; 9–16 mg g⁻¹ w.w and 1–10 µg mg⁻¹ prot. in kidney (Table 3). In *G. pulex* from La Bourbre River (France) and *G. fossarum* from the Sutla River (Croatia) MTs ranged 1–4 mg g⁻¹ w.w (Table 3), what is comparable to our data reported for MT levels in *G. balcanicus* and *E. acarinatus* from the Krka River (3 mg g⁻¹ w.w).

4. Conclusions

Obtained MT concentrations in the intestinal tissue of salmonid fish *S. trutta* and two gammarid species from the karst Krka River in Croatia

Table 3

Metallothionein concentrations reported in different tissues of freshwater fish (liver, kidney, gills and the intestine) and crustaceans (whole organisms) species from natural populations obtained by electrochemical methods.

Species	Tissue	MT concentration	References
Rainbow trout (<i>Salmo gairdneri</i>)	Liver	58–269 nmol g ⁻¹	Roch et al. (1982)
European eel (<i>Anguilla anguilla</i>)	Liver Kidney Gills	4.37–12.60 mg g ⁻¹ w.w 9.35–15.86 mg g ⁻¹ w.w 0.30–0.50 mg g ⁻¹ w.w	Ureña et al. (2007)
European chub – Sava River (<i>Squalius cephalus</i>)	Liver Gills Intestine	1.6–1.9 mg g ⁻¹ w.w 1.3–2.0 mg g ⁻¹ w.w 2.9–3.1 mg g ⁻¹ w.w	Podrug and Raspor (2009) Dragun et al. (2009) Filipović Marijić and Raspor (2010) and Dragun et al. (2015)
European chub – Sutla River (<i>Squalius cephalus</i>)	Liver Gills	0.80–3.73 mg g ⁻¹ w.w 0.66–2.35 mg g ⁻¹ w.w	Dragun et al. (2013)
Asp (<i>Leuciscus aspius</i>)	Liver Gills Kidney	7.4–7.5 µg mg ⁻¹ prot 3.6–3.9 µg mg ⁻¹ prot 1.4–2.3 µg mg ⁻¹ prot	
Pike-perch (<i>Sander lucioperca</i>)	Liver Gills Kidney	6.4–7.0 µg mg ⁻¹ prot 3.9–5.0 µg mg ⁻¹ prot 3.4–9.4 µg mg ⁻¹ prot	
Perch (<i>Perca fluviatilis</i>)	Liver Gills Kidney	4.8–8.3 µg mg ⁻¹ prot 4.0–5.5 µg mg ⁻¹ prot 1.3–2.8 µg mg ⁻¹ prot	
Pike (<i>Esox lucius</i>)	Liver Gills Kidney	11.0–18.1 µg mg ⁻¹ prot 2.4–5.4 µg mg ⁻¹ prot 3.3–6.8 µg mg ⁻¹ prot	
Bream (<i>Abramis brama</i>)	Liver Gills Kidney	5.3–10.1 µg mg ⁻¹ prot 4.0–4.7 µg mg ⁻¹ prot 2.5 µg mg ⁻¹ prot	Sevcikova et al. (2013)
Chub (<i>Squalius cephalus</i>)	Liver Gills Kidney	4.8–7.1 µg mg ⁻¹ prot 2.0–2.9 µg mg ⁻¹ prot 2.9–4.4 µg mg ⁻¹ prot	
Roach (<i>Rutilus rutilus</i>)	Liver Gills Kidney	5.7–12.3 µg mg ⁻¹ prot 3.4–4.3 µg mg ⁻¹ prot 1.7–2.3 µg mg ⁻¹ prot	
Silver bream (<i>Blicca bjoerkna</i>)	Liver Gills Kidney	7.5 µg mg ⁻¹ prot 4.5 µg mg ⁻¹ prot 8.9 µg mg ⁻¹ prot	
Common carp (<i>Cyprinus carpio</i>)	Liver Gills Kidney	8.5 µg mg ⁻¹ prot 4.1 µg mg ⁻¹ prot 5.0 µg mg ⁻¹ prot	
Rudd (<i>Scardinius erythrophthalmus</i>)	Liver Gills Kidney	9.6 µg mg ⁻¹ prot 6.5 µg mg ⁻¹ prot 10.3 µg mg ⁻¹ prot	
Brown trout (<i>Salmo trutta</i>)	Intestine	0.85–1.5 mg g ⁻¹ w.w 18–25 µg mg ⁻¹ prot 21–38 nmol g ⁻¹	This study
<i>Gammarus pulex</i>	Whole organism	1.25–3.25 mg g ⁻¹ w.w	Geffard et al. (2007)
<i>Gammarus fossarum</i>	Whole organism	1.55–3.65 mg g ⁻¹ w.w	Filipović Marijić et al. (2016)
<i>Gammarus balcanicus</i>	Whole organism	2.43–3.39 mg g ⁻¹ w.w 52–70 µg mg ⁻¹ prot 37–51 nmol g ⁻¹	This study
<i>Echinogammarus acarinatus</i>	Whole organism	2.53–2.94 mg g ⁻¹ w.w 55–60 µg mg ⁻¹ prot 38–45 nmol g ⁻¹	This study

revealed that anthropogenic impact near the wastewater outlets was evident, although not significantly in all cases. Wastewater impact was also confirmed regarding metal concentrations in all organisms, and comparison of total and cytosolic metal levels in fish intestine showed that As, Cd, Cs, Mo, Se, K, Mg, and Na were present mostly in the cytosolic fraction (over 67%), pointing that these metals are present in metabolically available intestinal fraction, where they can be bound to important biomolecules (enzymes) or might be detoxified by MT.

Electrochemically obtained MT levels in vertebrate and invertebrate organisms were species specific, showing higher MT concentrations in the gammarids than in the fish intestine. Despite variable MT levels, both bioindicator organisms pointed to the same trend, with higher MT values in the organisms from the contaminated compared to the reference site. Therefore, in freshwater salmon fish and gammarids MTs reflected metal contamination in the aquatic environment, so electrochemical method was confirmed as a sensitive tool in biomonitoring studies of metal exposure. Comparison of MT levels from our study with the literature data pointed to variability in MT concentrations among native fish and gammarid species, as well as among different fish tissues. Thus, proposed electrochemical method can be applied in biomonitoring studies as a tool for detecting MT changes in relation to anthropogenic impact on aquatic ecosystems and biota, but the interpretation should be done with caution knowing all the factors affecting MT levels. Advantage of the used electrochemical method is that it requires a small amount of the sample, but it also needs specialized and sensitive laboratory equipment. Presented results indicated that MT levels are species- and tissue-specific, so the comparison between MT levels should always be performed for the same species, tissue and measurement method.

Acknowledgements

This work was supported by the Croatian Science Foundation, within the project "Accumulation, subcellular mapping and effects of trace metals in aquatic organisms" AQUAMAPMET (IP-2014-09-4255). Authors are also grateful for the valuable help in the field work to the members of the Laboratory for Aquaculture and Pathology of Aquatic Organisms from the Ruđer Bošković Institute.

References

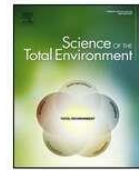
- Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquat. Toxicol.* 76 (2), 160–202. <https://doi.org/10.1016/j.aquatox.2005.08.015>.
- Barišić, J., Filipović Marijić, V., Mijošek, T., Čož-Rakovac, R., Dragun, Z., Krasnići, N., Ivanković, D., Kružličová, D., Erk, M., 2018. Evaluation of architectural and histopathological biomarkers in the intestine of brown trout (*Salmo trutta* Linnaeus, 1758) challenged with environmental pollution. *Sci. Total Environ.* 642, 656–664. <https://doi.org/10.1016/j.scitotenv.2018.06.045>.
- Baudrimont, M., Metivaud, J., Maury-Bracher, R., Ribeyre, F., Boudou, A., 1997. Bioaccumulation and metallothionein response in the Asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. *Environ. Toxicol. Chem.* 16 (10), 2096–2105. <https://doi.org/10.1002/etc.5620161016>.
- Caron, A., Rosabal, M., Drevet, O., Couture, P., Campbell, P.G., 2018. Binding of trace elements (Ag, Cd, Co, Cu, Ni, and Tl) to cytosolic biomolecules in livers of juvenile yellow perch (*Perca flavescens*) collected from lakes representing metal contamination gradients. *Environ. Toxicol. Chem.* 37, 576–586. <https://doi.org/10.1002/etc.3998>.
- Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. *J. Exp. Biol.* 203, 2455–2466.
- Correia, A.D., Sousa, A., Costa, M.H., Moura, I., Livingstone, D.R., 2004. Quantification of metallothionein in whole body *Gammarus locusta* (Crustacea: Amphipoda) using differential pulse polarography. *Toxicol. Environ. Chem.* 86 (1), 23–36. <https://doi.org/10.1080/02772240410001665472>.
- Couture, P., Rajotte, J.W., 2003. Morphometric and metabolic indicators of metal stress in wild yellow perch (*Perca flavescens*) from Sudbury, Ontario: a review. *J. Environ. Monit.* 5, 216–221. <https://doi.org/10.1039/b210338a>.
- Cukrov, N., Cmuć, P., Mlakar, M., Omanović, D., 2008. Spatial distribution of trace metals in the Krka River, Croatia: An example of the self-purification. *Chemosphere* 72, 1559–1566. <https://doi.org/10.1016/j.chemosphere.2008.04.038>.
- Dabrio, M., Rodríguez, A.R., Bordin, G., Bebbiano, M.J., De Ley, M., Sestáková, I., Vasák, M., Nordberg, M., 2002. Recent developments in quantification methods for metallothionein. *J. Inorg. Biochem.* 88, 123–134. [https://doi.org/10.1016/S0162-0134\(01\)00374-9](https://doi.org/10.1016/S0162-0134(01)00374-9).
- 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.
- Dedju, I.L., 1980. Amfipody Presnykh i Solonovatykh vod Juzgozapada SSSR. Kishinev, Shtiintsa, pp. 223.
- Demirak, A., Yilmaz, F., Tuna, A.L., Özdemir, N., 2006. Heavy metals in water, sediment and tissues of *Leuciscus cephalus* from a stream in southwestern Turkey. *Chemosphere* 63, 1451–1458. <https://doi.org/10.1016/j.chemosphere.2005.09.033>.
- Dragun, Z., Podrug, M., Raspor, B., 2009. The assessment of natural causes of metallothionein variability in the gills of European chub (*Squalius cephalus* L.). *Comp. Biochem. Physiol. Part C* 150 (2), 209–217. <https://doi.org/10.1016/j.cbpc.2009.04.011>.
- Dragun, Z., Filipović Marijić, V., Kapetanović, D., Valić, D., Vardić Smrzlić, I., Krasnići, N., Strižak, Ž., Kurtović, B., Teskeredžić, E., Raspor, B., 2013. Assessment of general condition of fish inhabiting a moderately contaminated aquatic environment. *Environ. Sci. Pollut. Res.* 20, 4954–4968. <https://doi.org/10.1007/s11356-013-1463-x>.
- Dragun, Z., Filipović Marijić, V., Vuković, M., Raspor, B., 2015. Metal bioavailability in the Sava River water. In: Milačić, R., Ščančar, J., Paučević, M. (Eds.), *The Sava River*. Springer-Verlag, Berlin Heidelberg, *The Handbook of Environmental Chemistry*, pp. 123–155.
- 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. *Ecotoxicol. Environ. Saf.* 147, 537–549. <https://doi.org/10.1016/j.ecoenv.2017.09.005>.
- Eisler, R., 1993. Zinc Hazard to Fish, Wildlife, and Invertebrates: A Synoptic Review." Contaminant Hazard Reviews, US Department of the Interior, Fish and Wildlife Service 10, p. 106. http://www.pwrc.usgs.gov/infobase/eisler/chr_26_zinc.pdf.
- Erk, M., Ivanković, D., Raspor, B., Pavičić, J., 2002. Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins. *Talanta* 57 (6), 1211–1218. [https://doi.org/10.1016/S0039-9140\(02\)00239-4](https://doi.org/10.1016/S0039-9140(02)00239-4).
- Fabrik, I., Ruferova, Z., Hilscherova, K., Adam, V., Trnkova, L., Kizek, R., 2008. A determination of metallothionein in larvae of freshwater midges (*Chironomus riparius*) using bdricka reaction. *Sensors* 8 (7), 4081–4094. <https://doi.org/10.3390/s8074081>.
- Fiket, Ž., Roje, V., Mikac, N., Kniewald, G., 2007. Determination of arsenic and other trace elements in bottled waters by high resolution inductively coupled plasma mass spectrometry. *Croat. Chem. Acta* 80, 91–100.
- Filipović Marijić, V., Raspor, B., 2007. Metallothionein in intestine of red mullet, *Mullus barbatus* as a biomarker of copper exposure in the coastal marine areas. *Mar. Pollut. Bull.* 54, 935–940. <https://doi.org/10.1016/j.marpollbul.2007.02.019>.
- Filipović Marijić, V., Raspor, B., 2010. The impact of the fish spawning on metal and protein levels in gastrointestinal cytosol of indigenous European chub. *Comp. Biochem. Physiol.* 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 Pollut.* 223, 4769–4783. <https://doi.org/10.1007/s11270-012-2>.
- Filipović Marijić, V., Dragun, Z., Sertić Perić, M., Matoničkin Kepčija, R., Gulini, V., Velki, M., Ećimović, S., Hackenberger, B.K., Erk, M., 2016. Investigation of the soluble metals in tissue as biological response pattern to environmental pollutants (*Gammarus fossarum* example). *Chemosphere* 154, 300–309. <https://doi.org/10.1016/j.chemosphere.2016.03.058>.
- Filipović Marijić, V., Kapetanović, D., Dragun, Z., Valić, D., Krasnići, N., Redžović, Z., Grgić, I., Žunić, J., Kružličová, 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. *Environ. Sci. Pollut. Res.* 25, 4715–4727. <https://doi.org/10.1007/s11356-017-0789-1>.
- Geffard, A., Amiard-Triquet, C., Amiard, J.C., 2005. Do seasonal changes affect metallothionein induction by metals in mussels, *Mytilus edulis*? *Ecotoxicol. Environ. Saf.* 61 (2), 209–220. <https://doi.org/10.1016/j.ecoenv.2005.01.004>.
- Geffard, A., Quéau, H., Dedouge, O., Biagiatti-Risboug, S., Geffard, O., 2007. Influence of biotic and abiotic factors on metallothionein level in *Gammarus pulex*. *Comp. Biochem. Physiol. Part C* 145 (4), 632–640. <https://doi.org/10.1016/j.cbpc.2007.02.012>.
- Giguère, A., Campbell, P.G.C., Hare, L., McDonald, D.G., Rasmussen, J.B., 2004. Influence of lake chemistry and fish age on cadmium, copper, and zinc concentrations in various organs of indigenous yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* 61 (9), 1702–1716. <https://doi.org/10.1139/f04-100>.
- Giguère, A., Campbell, P.G.C., 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. *Aquat. Toxicol.* 77 (2), 178–189. <https://doi.org/10.1016/j.aquatox.2005.12.001>.
- Gottstein, S., Žganec, K., Maguire, I., Kerovec, Jalžić B., 2007. Viši rakovi slatkih i boćatih voda porječja rijeke Krke, in Marguš, D., (ed.), *Zbornik radova sa simpozija Rijeka Krka i Nacionalni park Krka, Šibenik*, pp. 421–431.
- Handy, R.D., 1996. Dietary exposure to toxic metals in fish. In: Taylor, E.W. (Ed.), *Toxicology of Aquatic Pollution: Physiological, Molecular, and Cellular Approaches*. Society of Experimental Biology Seminar Series 57. Cambridge University Press, Cambridge, pp. 29–60.
- Hinton, D.E., Lauren, D.J., 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. *Am. Fish. Soc. Symp.* 8, 51–66.
- HRN EN 14011, 2005. Fish sampling by electric power (In Croatian). Croatian Standard Institute, Zagreb.
- Hylland, K., Nissen-Lie, T., Christensen, P.G., Sandvik, M., 1998. Natural modulation of

- hepatic metallothionein and 584 cytochrome P4501A in flounder, *Platichthys flesus* L. Mar. Environ. Res. 46, 51–55.
- Isani, G., Andreani, G., Kindt, M., Carpena, E., 2000. Metallothioneins (MTs) in marine molluscs. Cell. Mol. Biol. 46 (2), 311–330.
- Ivanković, D., Pavičić, J., Erk, M., Filipović Marijić, V., Raspor, B., 2005. Evaluation of the *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term field study: Seasonal and spatial variability. Mar. Pollut. Bull. 50 (11), 1303–1313. <https://doi.org/10.1016/j.marpolbul.2005.04.039>.
- Jarić, I., Višnjić-Jeftić, Ž., Cvijanović, G., Gačić, Z., Jovanović, L.J., 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. Microchem. J. 98, 77–81. <https://doi.org/10.1016/j.microc.2010.11.008>.
- Jenkins, J.A., 2004. Fish Bioindicators of Ecosystem Condition at the Calcasieu Estuary, Louisiana. National wetlands research center USGS, Lafayette.
- Krizkova, S., Adam, V., Kizek, R., 2009a. Study of metallothionein oxidation by using of chip CE. Electrophoresis 30 (23), 4029–4033. <https://doi.org/10.1002/elps.200900226>.
- Krizkova, S., Fabrik, I., Adam, V., Hrabeta, J., Eckschlager, T., Kizek, R., 2009b. Metallothionein—A promising tool for cancer diagnostics. Bratisl. Lek. Listy (Bratisl. Med. J.) 110, 93–97.
- Kunz, P.Y., Kinle, C., Gerhardt, A., 2010. Gammarus spp. in aquatic ecotoxicology and water quality assessment: toward integrated multilevel tests. Rev. Environ. Contam. Toxicol. 205, 1–76.
- 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*)? Can. J. Fish. Aquat. Sci. 54, 104–112. <https://doi.org/10.1139/cjfas-54-S1-104>.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
- MacNiel, C., Dick, J.T.A., Elwood, R., 1997. The trophic ecology of freshwater Gammarus (Crustacea: Amphipoda): problems and perspectives concerning the Functional Feeding Group concept. Biol. Rev. 72 (3), 349–364. <https://doi.org/10.1111/j.1469-185X.1997.tb00017.x>.
- Maddock, D.M., Burton, M.P.M., 1999. Gross and histological observations of ovarian development and related condition changes in American plaice. J. Fish Biol. 53, 928–944. <https://doi.org/10.1111/j.1095-8649.1998.tb00454.x>.
- Mathews, T., Fisher, N.S., 2008. Trophic transfer of seven trace metals in a four-step marine food chain. Mar. Ecol. Prog. Ser. 367, 23–33. <https://doi.org/10.3354/meps07536>.
- Mijošek, T., Erk, M., Filipović Marijić, V., Krasnići, N., Dragun, Z., Ivanković, D., 2018. Electrochemical determination of metallothioneins by the modified Brdička procedure as an analytical tool in biomonitoring studies. Croat. Chem. Acta 91 (4), 475–480. <https://doi.org/10.5562/cca3444>.
- Mijošek, T., Filipović Marijić, V., Dragun, Z., Krasnići, N., Ivanković, D., Erk, M., 2019. Evaluation of multi-biomarker response in fish intestine as an initial indication of anthropogenic impact in the aquatic karst environment. Sci. Total Environ. 660, 1079–1090. <https://doi.org/10.1016/j.scitotenv.2019.01.045>.
- 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. Parasit. Vectors 9, 300–308. <https://doi.org/10.1186/s13071-016-1576-4>.
- Podrug, M., Raspor, B., 2009. Seasonal variation of the metal (Zn, Fe, Mn) and metallothionein concentrations in the liver cytosol of the European chub (*Squalius cephalus* L.). Environ. Monit. Assess. 157, 1–10. <https://doi.org/10.1007/s10661-008-0509-x>.
- Rainbow, P.S., Moore, P.G., 1986. Comparative metal analyses in amphipod crustaceans. Hydrobiologia 141, 273–289. <https://doi.org/10.1007/BF00014222>.
- Raspor, B., Pačić, M., Erk, M., 2001. Analysis of metallothioneins by the modified Brdička procedure. Talanta 55, 109–115. [https://doi.org/10.1016/S0039-9140\(01\)00399-X](https://doi.org/10.1016/S0039-9140(01)00399-X).
- Raspor, B., Dragun, Z., Erk, M., Ivanković, D., Pavičić, J., 2004. Is the digestive gland of *Mytilus galloprovincialis* a tissue of choice for estimating cadmium exposure by means of metallothioneins? Sci. Total Environ. 333, 99–108. <https://doi.org/10.1016/j.scitotenv.2004.05.008>.
- Rätz, H.J., Lloret, J., 2003. Variation in fish condition between Atlantic cod (*Gadus morhua*) stocks, the effect on their productivity and management implications. Fish. Res. 60, 369–380. [https://doi.org/10.1016/S0165-7836\(02\)00132-7](https://doi.org/10.1016/S0165-7836(02)00132-7).
- Roch, M., McCarter, J.A., Matheson, A.T., Clark, M.J.R., Olafson, R.W., 1982. Hepatic metallothionein in rainbow trout (*Salmo gairdneri*) as an indicator of metal pollution in the Campbell river system. Can. J. Fish. Aquat. Sci. 39, 1596–1601. <https://doi.org/10.1139/B82-215>.
- Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. 22, 81–113. [https://doi.org/10.1016/0166-445X\(92\)90026-J](https://doi.org/10.1016/0166-445X(92)90026-J).
- Schlekat, C.E., Kidd, K.A., Adams, W.J., Baird, D.J., Farag, A.M., Maltby, L., Stewart, A.R., 2005. Toxic effects of dietborne metals: field studies. In: Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M. (Eds.), Toxicity of Dietborne Metals to Aquatic Organisms. Society of environmental toxicology and chemistry (SETAC), Brussels, pp. 113–152.
- Sertić Perić, M., Matonićkin 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. Ecotoxicol. Environ. Saf. 163, 125–135. <https://doi.org/10.1016/j.ecoenv.2018.07.068>.
- Sevcikova, M., Modra, H., Kruzikova, K., Zitka, O., Hynek, D., Vojtech, A., Celechovska, O., Svoboda, Z., 2013. Effect of metals on metallothionein content in fish from skalka and želivka reservoirs. Int. J. Electrochem. Sci. 8, 1650–1663.
- Shobikhuliatul, J.J., 2013. Some aspect of reproductive biology on the effect of pollution on the histopathology of gonads in *Puntius javanicus* from Mas River, Surabaya, Indonesia. J. Biol. Sci. 4 (2), 191–205.
- Sorensen, E.M., 1991. Metal poisoning in fish. CRC Press, Boca Raton.
- Staniskiene, B., Matusevicius, P., Budreckiene, R., Skibniewska, K.A., 2006. Distribution of heavy metals in tissues of freshwater fish in Lithuania. Pol. J. Environ. Stud. 15 (4), 585–591.
- Sures, B., Steiner, W., Rydlo, M., Taraschewski, H., 1999. Concentrations of 17 elements in the zebra mussel (*Dreissena polymorpha*), in different tissues of perch (*Perca fluviatilis*), and in perch intestinal parasites (*Acanthocephalus lucii*) from the subalpine Lake Mondsee, Austria. Environ. Toxicol. Chem. 18, 2574–2579. <https://doi.org/10.1002/etc.5620181126>.
- Ternej, I., Mihaljević, Z., Ivković, M., Previšić, A., Stanković, I., Maldini, K., Željčić, D., Kopjar, N., 2014. The impact of gypsum mine water: a case study on morphology and DNA integrity in the freshwater invertebrate, *Gammarus balcanicus*. Environ. Pollut. 189, 229–238. <https://doi.org/10.1016/j.envpol.2014.03.009>.
- Ureña, R., Peri, S., del Ramo, J., Torreblanca, A., 2007. Metal and metallothionein content in tissues from wild and farmed *Anguilla anguilla* at commercial size. Environ. Int. 33 (4), 532–539. <https://doi.org/10.1016/j.envint.2006.10.007>.
- van Cleef, K.A., Kaplan, L.A.E., Crivello, J.F., 2000. The relationship between reproductive status and metallothionein mRNA expression in the common killifish, *Fundulus heteroclitus*. Environ. Biol. Fishes 57, 97–105.
- Vašák, M., 2005. Advances in metallothionein structure and functions. Trace Elem. Med. Biol. 19, 13–27. <https://doi.org/10.1016/j.temb.2005.03.003>.
- Viarengo, A., Burlando, B., Dondero, F., Marro, A., Fabri, R., 1999. Metallothionein as a tool in biomonitoring programmes. Biomarkers 4, 455–466. <https://doi.org/10.1080/135475099230615>.
- Vijver, M.G., Van Gestel, C.A., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W.J., 2004. Internal metal sequestration and its ecotoxicological relevance: a review. Environ. Sci. Technol. 38, 4705–4712.
- Wallace, W.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM). Mar. Ecol. Prog. Ser. 257, 125–137. <https://doi.org/10.3354/meps257125>.
- Yeltekina, A.C., Sağlam, E., 2019. Toxic and trace element levels in *Salmo trutta macrostigma* and *Oncorhynchus mykiss* trout raised in different environments. Pol. J. Environ. Stud. 28 (3), 1613–1621.
- Zhelev, Zh., Mollova, D., Boyadziev, P., 2016. Morphological and hematological parameters of *Carassius gibelio* (Pisces: Cyprinidae) in conditions of anthropogenic pollution in Southern Bulgaria. Use hematological parameters as biomarkers. Trakia J. Sci. 14 (1), 1–15.
- Zhelev, Zh.M., Tsonev, S.V., Boyadziev, P.S., 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 Zool. Bulg. 70 (4), 547–556.
- Zieliński, D., 1995. Life History of *Gammarus balcanicus* Schäferna, 1922 from the Bieszczady Mountains (Eastern Carpathians, Poland). Crustaceana 68, 61–72. <https://doi.org/10.1163/156854095X00386>.
- Zorita, I., Strogyloudi, E., Buxens, A., Mazón, L.I., Papanthassiou, E., Soto, M., Cajaraville, M.P., 2005. Application of two SH-based methods for metallothionein determination in mussels and intercalibration of the spectrophotometric method: laboratory and field studies in the Mediterranean Sea. Biomarkers 10 (5), 342–359. <https://doi.org/10.1080/13547500500264645>.
- Žganeč, K., Lunko, P., Stroj, A., Mamos, T., Grabowski, M., 2016. Distribution, ecology and conservation status of two endemic amphipods, *Echinogammarus acarinus* and *Fontogammarus dalmatinus*, from the Dinaric karst rivers, Balkan Peninsula. Ann. Limnol. 52, 13–26. <https://doi.org/10.1051/limn/2015036>.



Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Evaluation of multi-biomarker response in fish intestine as an initial indication of anthropogenic impact in the aquatic karst environment



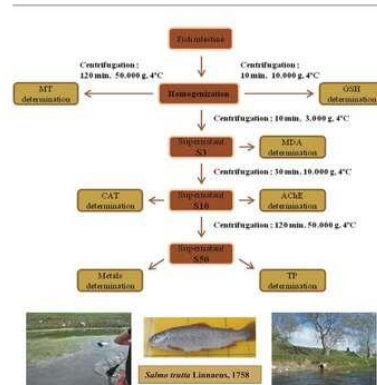
Tatjana Mijošek*, Vlatka Filipović Marijić, Zrinka Dragun, Nesrete Krasnići, Dušica Ivanković, Marijana Erk

Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička c. 54, 10000 Zagreb, Croatia

HIGHLIGHTS

- Multi-biomarker approach was assessed as an early sign of pollution in karst river.
- Fish intestine was evaluated as an indicator organ responsible for dietborne uptake.
- Wastewater impact was evident from biomarker and cytosolic metal responses.
- Intestinal biomarkers pointed to rising need of strict monitoring of water quality.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 13 November 2018
 Received in revised form 4 January 2019
 Accepted 5 January 2019
 Available online 07 January 2019

Editor: Henner Hollert

Keywords:

Karst Krka River
 Brown trout
 Biomarkers
 Wastewaters
 Biomonitoring
 Cytosolic metals

ABSTRACT

In order to assess the extent of existing anthropogenic influence on biota of the vulnerable karst ecosystem of the Krka River, multi-biomarker approach was applied in the intestinal tissue of brown trout *Salmo trutta* Linnaeus, 1758. Biomarkers of the general stress (total cytosolic proteins), oxidative stress (malondialdehyde), antioxidant capacity (catalase activity, total glutathione) and of an exposure and effect of contaminants, especially metals (metallothionein) and organophosphorous pesticides and metals (acetylcholine esterase activity) were compared in the intestine of fish from the reference site (river source) and downstream of the technological and municipal wastewater impacted site (town of Knin) in two seasons, October 2015 and May 2016. Biological response was additionally evaluated by metal/metalloid concentrations in intestinal cytosol. Site-specific differences were observed as significantly higher As, Ca, Co, Cu, Se and Sr concentrations in intestinal cytosol of fish from the contaminated compared to the reference site. Significant seasonal differences existed for Ni, Cd, Mo, Cs and Na, with higher levels in autumn, following the trend of most of the dissolved metal levels in the river water. Impact of improperly treated wastewaters was also confirmed by significantly increased levels of glutathione, total proteins and Foulton condition indices, with 1.5, 1.13 and 1.12 times higher average values in fish from that site compared to the river source, respectively. The other biomarkers showed similar trend and pointed to specific biological changes regarding oxidative stress or metal exposure in fish from the anthropogenically impacted site, especially in autumn, but without significant differences. Thus, the anthropogenic impact still seems to be

* Corresponding author at: Laboratory for Biological Effects of Metals, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička c. 54, 10000 Zagreb, Croatia.
 E-mail addresses: tmijosek@irb.hr (T. Mijošek), vfilip@irb.hr (V. Filipović Marijić), zdragun@irb.hr (Z. Dragun), nkrasnic@irb.hr (N. Krasnići), djuric@irb.hr (D. Ivanković), erk@irb.hr (M. Erk).

only moderate, although cytosolic metals and most of the biomarkers in fish intestine were confirmed as initial indicators of pollution impact, which pointed to the need of continuous monitoring of the Krka River in order to protect this natural karst world phenomenon.

© 2019 Elsevier B.V. All rights reserved.

1. Introduction

Among aquatic environments, karst systems represent the most sensitive areas from both ecological and conservation points of view (Dossi et al., 2007). Specific geological and hydrological constructions contribute to formation of complex ecosystems, which are vulnerable to contaminants due to their ability to be introduced in the karst area through underground and transported rapidly over large distances in the aquifer. In addition, processes of contaminant retardation and attenuation often do not work effectively in karst systems (Brinkmann and Parise, 2012). Although only 10% of the Earth's surface is covered with karst rocks, groundwater from karst aquifers is among the most important drinking water resources for humanity and in this sense, protection of karst hydrologic systems is essential worldwide (Bakalowicz, 2005; Calò and Parise, 2009).

Example of obvious anthropogenic disturbances in the karst systems can be observed in the Krka River, one of the longest rivers in Croatia (72.5 km) situated in karst Dinaric area of the Republic of Croatia. As a result of the constant process of tufa-deposition, the Krka River represents a unique karst phenomenon worldwide and most of its watercourse was proclaimed national park in 1985 (Cukrov et al., 2012). However, only 2 km upstream of the border of the Krka National Park, technological wastewaters of the screw factory and municipal wastewaters of the town of Knin, are released without proper treatment into the river watercourse. Therefore, anthropogenic impact is represented by direct influence of the mentioned pollution sources, as well as indirect influence of agricultural runoff from the surrounding fields, with special emphasis on metals/metalloids contamination from the nearby screw factory and fertilizers (Cukrov et al., 2008; Filipović Marijić et al., 2018). Trace metals are naturally present in the aquatic ecosystems in very low concentrations, even extremely low in karst rivers (Cukrov et al., 2008). Previous studies already reported low natural metal levels in the river water from the Krka River source, while 2–400 times higher Al, Co, Fe, Li, Mn, Ni, Sr, Ti, and Zn levels were recorded in the technological/municipal wastewaters and the Krka River water under the anthropogenic influence downstream of the town of Knin (Cukrov et al., 2008; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Wastewater impact was also confirmed by higher densities and diversity of benthic organisms dominated by contamination-tolerant taxa (Sertić Perić et al., 2018). In order to evaluate the extent of anthropogenic influence on the aquatic organisms in the vulnerable karst ecosystem, brown trout (*Salmo trutta* Linnaeus, 1758) was selected as a bioindicator organism, as a typical representative of the Krka River ichthyofauna and moreover, widely spread species in European rivers, which provides the possibility and opportunity for comparison between different regions.

Biological responses to contaminant exposure were for the first time evaluated in the karst area by application of multi-biomarker approach in fish intestine, due to its importance in dietary metal uptake, digestion and nutrient absorption. Most of the biomonitoring studies regarding metal exposure usually involved liver, kidneys and gills as typical indicator organs in fish, while data on the intestinal tissue is still limited, especially regarding fish from the karst rivers. Our previous study involved histological alterations in brown trout intestine and pointed to specific histopathological biomarkers as an indication of pollution impact in the Krka River (Barišić et al., 2018). In the present study we expanded previous findings to demonstrate exposure to and/or effects of

environmental contaminants by application of the multi-biomarker approach involving biomarkers of the general stress (total cytosolic proteins, TP), antioxidant defense (catalase activity, CAT and total glutathione, GSH as markers of antioxidant capacities), oxidative stress (malondialdehyde, MDA as an indicator of oxidative damage), and of an exposure and effect of contaminants (metallothioneins (MT) as biomarkers of metal exposure and acetylcholine esterase activity (AChE) as biomarker of effect on nervous system following exposure to organophosphate and carbamate pesticides, but also other contaminants like metals). The use of multi-biomarker approach is necessary in environments with complex mixtures of contaminants, for the assessment of different biological responses that reflect the environmental quality and for the identification of exposure to contaminants present at low levels in the environment (Monserrat et al., 2007; Cravo et al., 2009).

Biological effects may also link the bioavailability of compounds of interest with their concentrations in target organs and intrinsic toxicity (van der Oost et al., 2003). Therefore, besides biomarker approach, additional biological response was evaluated by the measurement of metal/metalloid concentrations in the intestinal cytosol of brown trout, in order to evaluate metal accumulation in fish since trace elements represent directly introduced contaminants in the Krka River water. After entering the organism, trace metals usually undergo a series of metabolic processes and incorporate into various cellular components. In general, partitioning of metals among subcellular fractions might be grouped in two categories: a) metal-sensitive fractions (MSF): heat-denaturable proteins (HDP), mitochondria and lysosomes and microsomes; b) biologically detoxified metals (BDM): insoluble metal-rich granules (MRG) and heat-stable proteins (HSP) like metallothioneins (MT) and metallothionein-like peptides (MTLP) (Wallace and Luoma, 2003; Urien et al., 2018). HSP fraction was indicated as the most responsive fraction to increased metal exposure (Caron et al., 2018; Urien et al., 2018). In order to evaluate concentrations of metals bound to cytosolic biomolecules, which represent soluble and metabolically available metal fraction (Wallace and Luoma, 2003; Rainbow et al., 2011; Caron et al., 2018; Urien et al., 2018), our research involved metal and metalloid analyses in the cytosolic fraction of fish intestine.

Hence, our main goals were: 1) to examine the impact of the direct pollution sources (technological and municipal wastewaters) on the karst region and biota using the multi-biomarker approach and cytosolic metals levels as bioindicators in the intestine of brown trout; 2) to evaluate the potential of the intestinal tissue as a novel bioindicator organ in environmental risk assessment due to its importance in food and metal uptake.

2. Materials and methods

2.1. Study area and fish sampling

The study was carried out in the Krka River which is nowadays threatened by the influence of the technological and municipal wastewater inputs. Sampling was performed at two locations, reference (Krka River source) and anthropogenically impacted site, which is situated near the town of Knin and only 2 km upstream of the border of the Krka National Park. This part of the watercourse is the recipient of the technological wastewaters from the screw factory and of municipal wastewaters from the town of Knin (15,000 inhabitants), so fish sampling was performed downstream of both outlets (Fig. 1). Previous studies indicated that



Fig. 1. Sampling locations of the brown trout in the Krka River: 1 reference location - Krka River source; 2 anthropologically impacted location downstream of the technological wastewater input from the screw factory (2a) and municipal wastewater outlet from the town of Knin (2b).

water ecological status was deteriorated and concentrations of many investigated metals/metalloids were increased at the location under the wastewaters impact compared to the reference site, the Krka River source (Filipović Marijić et al., 2018; Sertić Perić et al., 2018).

Brown trouts (*Salmo trutta* Linnaeus, 1758) were collected in the autumn 2015 (October) (16 specimens from the reference and 20 from the contaminated site) and in the spring 2016 (May) (16 specimens per each site). Fish sampling was performed by electro fishing, according to the Croatian standard HRN EN 14011 (2005). Captured fish were kept alive in an opaque tank with aerated river water until further processing in the laboratory. The biometric data involved measurement of fish total length and body mass, as well as calculation of fish indices: 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 body mass (g), L is the total length (cm), LW is the liver mass (g) and GW is the gonad mass (g). Intestine, liver and gonads were dissected after the fish were anesthetized with tricaine methane sulphonate (MS 222, Sigma Aldrich) in accordance to the Ordinance on the protection of animals used for scientific purposes (NN 55, 2013) and then sacrificed. Priborsky et al. (2015) confirmed that exposure of barbell (*Barbus barbus*) to MS 222 for 10 min. does not have a significant impact on haematological profiles, oxidative stress biomarkers and antioxidant enzymes. Accordingly, fish were anesthetized in groups of 5 in order to shorten the exposure period to <10 min., carefully applying the dosage of anaesthetic according to Topić Popović et al. (2012). The whole digestive tract was removed on ice, intestinal part was cut off and cleaned of exterior fat. Afterwards, intestinal fish parasites, acanthocephalans, and the gut content were removed from the intestine and tissue was rinsed with MQ water. Tissues were weighed and then stored in liquid nitrogen until transported to the laboratory, where samples were kept at $-80\text{ }^{\circ}\text{C}$ until further analyses.

2.2. Tissue preparation and homogenization

Each sample of intestinal tissue was divided in three parts appropriate for homogenization procedure related to the GSH measurement, MT measurement and measurement of other biomarkers and metals. Prior to GSH measurement, intestinal tissues were homogenised in five volumes of ice-cold 5% sulfosalicylic acid (SSA) and then centrifuged at $10,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ (Biofuge Fresco, Heraeus, Germany). Prior to MT measurement, fish intestinal samples were homogenised in five volumes of 20 mM Tris-HCl buffer, pH 8.6 with 0.5 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.006 mM leupeptine, and 0.01% β -mercaptoethanol as a reducing agent. The homogenates were afterwards centrifuged at $50,000 \times g$ for 2 h at $4\text{ }^{\circ}\text{C}$. Samples of fish intestine used for measurement of other biomarkers and metals were homogenised in five volumes of cooled homogenization buffer containing 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at $4\text{ }^{\circ}\text{C}$) with 1 mM DTT (Sigma, USA) as a reducing agent, 0.5 mM PMSF (Sigma, USA) and 0.006 mM leupeptin (Sigma) as protease inhibitors (Filipović Marijić and Raspor, 2010). In all cases homogenization was performed in an ice cooled tube using Potter-Elvehjem homogenizer (Glas-Col, USA) and the resulting homogenates were afterwards centrifuged in the Avanti J-E centrifuge (Beckman Coulter, USA) at different settings depending on biomarker analyses. For MDA analyses, homogenates were centrifuged at $3000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, for analyses of AChE and CAT activity remaining homogenates were centrifuged at $10,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$ to get post-mitochondrial fraction. Lastly, obtained supernatants at $50,000 \times g$ for 2 h at $4\text{ }^{\circ}\text{C}$ represented cytosolic tissue fraction and were used for metal and TP analyses. All obtained supernatants were separated and stored at $-80\text{ }^{\circ}\text{C}$ for subsequent analyses.

2.3. Digestion of cytosolic fractions and determination of total dissolved macro and trace elements

Cytosolic fractions were digested in duplicates by addition of oxidation mixture (v/v 1:1), which contained concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany) (v/v 3:1). Homogenization buffer was used as a blank and treated the same way as the samples. Digestion was performed in the laboratory dry oven at $85\text{ }^{\circ}\text{C}$ for 3.5 h. Following digestion, samples were diluted with Milli-Q water by dilution factor 20 for Na, K, and Mg, and 5 for the remaining elements. Indium ($1\text{ }\mu\text{g L}^{-1}$, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to all solutions as an internal standard to correct the changes in peak intensities due to instrumental drift and matrix suppression (Fiket et al., 2007). During the analyses, the validation of acid digestion efficiency of cell cytosolic fraction was performed by the digestion of dogfish muscle certified reference material for trace metals (DORM-2, National Research Council of Canada, NRC, Canada). The recovery means ($\pm\text{SD}$, $n = 5$) of the trace elements studied from the reference material (As, Cd, Co, Cu, Fe, Mn, Ni, Se, Tl and Zn) are presented in Table 1.

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 to analyze 20 macro and trace elements. Measurements of ^{82}Se , ^{85}Rb , ^{98}Mo , ^{111}Cd , ^{133}Cs , and ^{205}Tl were operated in low resolution mode; of ^{23}Na , ^{24}Mg , ^{42}Ca , ^{47}Ti , ^{51}V , ^{55}Mn , ^{56}Fe , ^{59}Co , ^{60}Ni , ^{63}Cu , ^{66}Zn , and ^{86}Sr in medium resolution mode; and of ^{39}K and ^{75}As in high resolution mode. The external calibration was performed using 2 calibration solutions. For macro elements, multielement stock standard solution containing $\text{Ca } 2.0\text{ g L}^{-1}$, $\text{Mg } 0.4\text{ g L}^{-1}$, $\text{Na } 1.0\text{ g L}^{-1}$, and $\text{K } 2.0\text{ g L}^{-1}$ (Fluka, Germany) was used for preparation of calibration standards. Calibration solution for the trace elements was prepared by dilution of 100 mg L^{-1} multielement stock standard solution (Analitika, Czech Republic) supplemented with Rb (Sigma-Aldrich, Germany) and Cs (Fluka, Germany). The accuracy and the precision of HR ICP-MS measurements was tested using quality control sample for macro-elements (QC

Table 1

Validation of acid digestion efficiency presented as certified and measured metal values (mean \pm S.D., n = 5) in certified reference material (DORM-2, National Research Council, Canada) and calculated recoveries.

Metal	Certified value (DORM-2)	Measured value (DORM-2)	Recovery (%)
	mg kg ⁻¹		
As	18.0 \pm 1.7	18.6 \pm 1.4	103
Cd	0.043 \pm 0.008	0.044 \pm 0.003	105
Co	0.182 \pm 0.031	0.18 \pm 0.012	99
Cu	2.34 \pm 0.16	2.35 \pm 0.073	100
Fe	142 \pm 10	142.9 \pm 7.13	101
Mn	3.66 \pm 0.34	3.71 \pm 0.23	101
Ni	19.4 \pm 3.1	19.11 \pm 1.11	99
Se	1.4 \pm 0.09	1.43 \pm 0.07	102
Tl	0.004	0.004 \pm 0.0005	100
Zn	25.6 \pm 2.3	24.28 \pm 2.08	95

Minerals, Catalog number 8052, UNEP GEMS, Burlington, Canada) and for trace elements (QC trace metals, catalog no. 8072, UNEP GEMS, Burlington, Canada). A generally good agreement was observed between our data and certified values, with the following recoveries (%) (based on two measurements in control sample for trace elements and two measurements for macro elements): As (100.7 \pm 6.7), Ca (95.5 \pm 1.6), Cd (95.1 \pm 0.7), Co (98.3 \pm 0.2), Cu (97.9 \pm 0.0), Fe (99.7 \pm 2.6), K (92.5 \pm 4.2), Mg (93.5 \pm 4.9), Mn (98.1 \pm 0.0), Na (96.2 \pm 3.4), Ni (94.1 \pm 5.0), Se (100.8 \pm 6.1), Sr (100.8 \pm 0.6), Ti (80.3 \pm 0.5), Tl (96.0 \pm 0.8), V (101.1 \pm 0.3), and Zn (96.0 \pm 1.3). Limits of detection (LOD) were calculated as three standard deviations of ten consecutive trace element determinations in the blank sample (100 mM Tris-HCl/Base, 1 mM dithiothreitol) digested according to the procedure for cytosols. LOD for macro elements, in $\mu\text{g g}^{-1}$, were as follows: Ca, 1.07; K, 0.112; Mg, 0.024; and Na, 0.320, and LOD for trace elements, in ng g^{-1} , were as follows: As, 6.72; Cd, 0.430; Co, 0.266; Cs, 0.102; Cu, 13.5; Fe, 141; Mn, 0.810; Mo, 0.680; Ni, 8.55; Rb, 0.339; Se, 2.93; Sr, 1.09; Ti, 4.76; Tl, 0.001; V, 2.86; and Zn, 635.

2.4. Biomarkers determination

2.4.1. Determination of AChE and CAT activities

The AChE and CAT activities were determined in postmitochondrial fraction (S10). AChE was analyzed according to the method described by Ellman et al. (1961). The reaction mixture consisted of the sample, 100 mM Tris-HCl buffer (pH 7.5 at 25 °C) and 1.6 mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid). After incubation in dark for 15 min, measurement of the enzyme activity was initiated by the addition of 20 mM acetylthiocholine iodide. The increase in absorbance at 412 nm was monitored immediately following the addition of acetylthiocholine iodide. The enzymatic activity was expressed as nmol of acetylthiocholine hydrolysed per min per mg of protein, using the absorption coefficient of 13.6 $\text{mM}^{-1} \text{cm}^{-1}$ for calculations (Stepić et al., 2013).

Measurement of the CAT activity was performed spectrophotometrically at 240 nm and 25 °C following the method by Claiborne (1985). According to the procedure, sodium phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (30%) were used to prepare 15.8 mM H_2O_2 , which was added to 10 times diluted sample. The specific enzyme activity was expressed as μmol of degraded H_2O_2 per min per mg of protein calculated with a molar extinction coefficient of 43.6 $\text{M}^{-1} \text{cm}^{-1}$. Protein concentrations in S10 fractions were determined by the method of Lowry et al. (1951).

2.4.2. Determination of GSH levels

Total GSH concentration was measured in ten-times diluted supernatants using a spectrophotometric DTNB-GSSG reductase recycling assay (Tietze, 1969). The procedure for the microtiter plate assay is adapted from the protocol described by Rahman et al. (2006). All

solutions were made in 0.1 M potassium phosphate buffer with added 1 mM EDTA disodium salt, pH 7.5. Volume of 150 μL of a solution containing DTNB (3.79 mM) and GR (glutathione reductase; 6 U/mL) was added to the sample in the plate. The plate was mixed and left in dark for 5 min. Following, 50 μL of NADPH (0.192 mM) solution was added and the absorbance at 412 nm was measured in intervals of 1 min for 5 min. GSH standards (3.125–25 nM mL^{-1}) were prepared in 0.5% SSA and a calibration curve was used to calculate the GSH levels. The values were expressed as nmol of GSH per g of wet tissue mass.

2.4.3. Determination of the MDA concentration

Determination of MDA concentration was performed spectrophotometrically after the reaction of MDA with 2-thiobarbituric acid (TBA) according to Botsoglou et al. (1994) and Ringwood et al. (2003). Firstly, a mixture of 1% butylated hydroxytoluene (BHT, Sigma-Aldrich, USA) dissolved in ethanol (CARLO ERBA Reagents, Italy) and 10% trichloroacetic acid (TCA, Kemika, Croatia) dissolved in Milli-Q water (BHT/ TCA = 1:100) was added to sample supernatant. Samples were then vortexed and cooled for 15 min. Next, these mixtures were centrifuged in the Biofuge Fresco centrifuge (Heraeus, Germany) at 4000 $\times\text{g}$ for 15 min at 4 °C and obtained supernatants were transferred to 1.5-mL Eppendorf® tubes. Following, TBA (Alfa Aesar, Germany) dissolved in Milli-Q water was added. Tubes were then heated for 30 min at 100 °C producing a pink, fluorescent product. Samples were left to cool and transferred into microplate. The absorbance was set to 535 nm wavelength and values were read at the spectrophotometer/fluorometer Infinite M200 (Tecan, Switzerland). To calculate the MDA values, the calibration curve was constructed using 8 concentrations (2–100 μM) of MDA (Aldrich, USA) which was dissolved in 1 N HCl (Kemika, Croatia). Homogenization buffer was used as a blank and treated in the same way. Values were obtained as μM and finally calculated as nmol of MDA per gram of wet tissue mass.

2.4.4. Determination of MT concentrations

MT determination involves ethanol/chloroform precipitation steps. Afterwards obtained pellets were washed with 87% ethanol and 1% chloroform in homogenizing buffer, centrifuged at 6000 $\times\text{g}$ for 12 min and dried under nitrogen gas stream. Pellets containing MT were dissolved by addition of 35 μL of both 0.25 M NaCl and a solution of 4 mM EDTA/1 M HCl. The thiol group content was analyzed using DTNB dissolved in 0.2 M Na-phosphate/2 M NaCl, pH 8. The absorbance was read at 412 nm at spectrophotometer/fluorometer (Infinite M200, Tecan, Switzerland). The reduced glutathione (GSH) was used as a reference standard (2.5–30 μg GSH) and obtained calibration curve was used to calculate the values. MT concentrations were expressed per total cytosolic proteins ($\mu\text{g MT mg}^{-1}$ proteins) which were determined by the method of Lowry et al. (1951).

2.4.5. Determination of total cytosolic proteins concentrations

The concentrations of total proteins were measured according to Lowry et al. (1951). Reagent A (copper tartrate, Bio-Rad) and Reagent B (Folin reagent, Bio-Rad) were added to 20 times diluted S50 samples. After 15 min waiting and appearance of a blue color, total proteins were measured on a spectrophotometer at 750 nm wavelength (Infinite M200, Tecan, Switzerland). Calibration was accomplished using a bovine serum albumin (BSA) (Serva, Germany) as a reference standard (0.25–2 mg mL^{-1} BSA).

2.5. Statistical analyses

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, USA). Data are presented as mean \pm standard deviation (S.D.). Variability of metal concentrations and biomarker values in fish intestine between two seasons and two sites were tested by Mann-Whitney *U* test, since assumptions of normality and homogeneity of variance were not always met. Correlation among different parameters

was performed using Spearman correlation analysis. Levels of significance of certain statistical test are indicated in the text.

3. Results

3.1. Fish biometric characteristics

Average total length and body mass of *S. trutta* specimens from the karst Krka River did not show spatial but pointed to seasonal differences, with significantly higher fish biometric parameters in the autumn season at both locations (Table 2). Gonadosomatic indices followed the same trend, with significantly higher levels in the autumn season at both locations, while HSI and FCI had higher values in spring samples. In addition, FCI of fish from the Krka River downstream of Knin were significantly higher compared to fish from the Krka River source in both seasons (Table 2).

3.2. Cytosolic metal/metalloid concentrations in fish intestine

The results on metal/metalloid concentrations in the metabolically available intestinal cytosolic fraction are the first of this kind for *S. trutta*. Descending order of metal/metalloid levels with concentrations higher than $100 \mu\text{g kg}^{-1}$ are shown in Fig. 2a (the highest levels of Zn and Fe in intestinal cytosol), while the ones with the concentrations lower than $100 \mu\text{g kg}^{-1}$ are shown in Fig. 2b (the lowest levels of Cs and V in intestinal cytosol). Concentrations of macroelements in the intestinal cytosol of brown trout were the highest for K and Na, as presented in Fig. 3.

Total cytosolic metal/metalloid concentrations in brown trout intestine were significantly higher in fish from the contaminated compared to the reference site, in both seasons for Co and Se, in autumn for As and Cu, and in spring for Ca and Sr (Figs. 2, 3). Average levels of these metals were 2–4 times higher in fish from the contaminated compared to the reference site. The same trend of higher accumulation in fish from the contaminated site was valid for V, Ti and Zn concentrations, but these differences were not significant in any season. On the other hand, significantly higher metal levels in the intestinal cytosols of fish from the Krka River source compared to the contaminated location were evident for Cd (2–27 times), Cs and Tl (2 to 3 times) in both seasons, and for Ni (6 times) only in autumn season (Fig. 2). Among 20 measured cytosolic metals/metalloids, there were no unique patterns observed for Fe, K, Mg, Mn, Mo, Na and Rb, which levels were mostly comparable or slightly higher either at contaminated or reference site but without any significant differences (Figs. 2, 3).

Few cytosolic intestinal metals/metalloids showed seasonal differences, which were significant for As, Cs, Na and Ni in brown trout from the river source and for Mo and Cd in fish caught downstream of the town of Knin. Each of these elements followed the same trend of significantly higher concentrations observed in autumn than spring campaign (1.2–10 times), with exception of As which levels were higher in spring than autumn for 13 times (Figs. 2, 3).

3.3. Biomarker responses in brown trout intestine

3.3.1. Biomarker of exposure to organophosphorous pesticides and metals - AChE

In the present research, average values of AChE activity in brown trout intestine did not show any spatial or temporal significant differences. However, AChE activity was decreased in fish dwelling at the pollution impacted site and this difference was more pronounced in spring indicating possible pesticide or metal exposure. Average values of AChE activity found in our study ranged from 7.52 ± 1.66 to $9.36 \pm 3.49 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ if both seasons and both locations were considered (Fig. 4a).

3.3.2. Biomarkers of antioxidative capacity - CAT and GSH

There was no unique spatial or seasonal pattern in CAT activity in brown trout intestinal tissue (Fig. 4b). Slightly higher values were observed in fish from the contaminated site compared to the reference location in autumn, while seasonal differences showed higher CAT activity in spring compared to autumn season in fish from the river source. Average values of CAT activity ranged from 13.51 ± 5.64 to $18.34 \pm 6.87 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ prot.}$ if both seasons and both locations are considered and there were no significant season- or site-specific differences observed.

GSH levels in the brown trout intestinal tissue showed both spatial and seasonal significant differences (Fig. 4c). Spatial differences were significant in autumn, when 1.5 times higher GSH levels were recorded in fish from the contaminated site compared to the reference site. Seasonal difference was observed only at the contaminated site with significantly higher values obtained in autumn ($1642.3 \pm 256.6 \text{ nmol g}^{-1} \text{ w. w.}$) than in spring ($1277.7 \pm 289.7 \text{ nmol g}^{-1} \text{ w. w.}$), while levels observed in fish from the Krka source were almost the same in both seasons (Fig. 4c).

3.3.3. Biomarker of oxidative stress - MDA

MDA concentrations showed slightly higher average values in fish originating from the location downstream of wastewater discharges, especially in autumn, but still not significantly. Also, seasonal differences were not significant although average MDA concentrations were higher in autumn (152.97 ± 58.36 and $166.1 \pm 41.19 \text{ nmol g}^{-1} \text{ w. w.}$ at the reference and anthropogenically impacted site, respectively) than in spring (147.11 ± 36.71 and $148.7 \pm 44.63 \text{ nmol g}^{-1} \text{ w. w.}$ for the reference and anthropogenically impacted site, respectively) (Fig. 4d).

3.3.4. Biomarker of metal exposure - MT

Significantly higher MT levels were evident in the intestinal tissue of fish from the Krka River source in the spring compared to the autumn season. As seen in Fig. 4e, average MT concentrations in spring ($7.03 \pm 2.07 \mu\text{g MT mg}^{-1} \text{ prot.}$) were almost two times higher than in October ($4.26 \pm 0.53 \mu\text{g MT mg}^{-1} \text{ prot.}$) in fish from the reference location. Spatial differences were not significant, although higher MT levels were evident in spring in fish from the reference location compared to the contaminated one.

Table 2

Biometric parameters (mean \pm S.D. (min.-max.)) of *S. trutta* caught in the Krka River at the reference (Krka River source) and contaminated site (Krka downstream of Knin) in two sampling campaigns (autumn- October 2015 and spring- May 2016). 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).

	Krka River source		Krka downstream of Knin	
	Autumn 2015 n = 16	Spring 2016 n = 16	Autumn 2015 n = 20	Spring 2016 n = 16
Total length (cm)	24.15 \pm 4.29* (18–30.8)	18.36 \pm 1.94* (15.2–22.1)	23.16 \pm 5.49* (13–31.8)	19.64 \pm 3.19* (13.8–26.7)
Body mass (g)	152.71 \pm 78.64* (59.53–303.7)	66.09 \pm 19.64* (36.6–107.2)	165.45 \pm 108.96* (22.15–424.3)	96.01 \pm 45.49* (31.45–200.7)
FCI ($\text{g cm}^{-3} \times 100$)	1.00 \pm 0.08 ^A (0.84–1.13)	1.04 \pm 0.06 ^A (0.94–1.15)	1.12 \pm 0.10 ^B (0.98–1.38)	1.19 \pm 0.09 ^B (1.05–1.37)
HSI (%)	0.92 \pm 0.25* (0.53–1.36)	1.27 \pm 0.30* (0.88–1.97)	0.97 \pm 0.12* (0.76–1.21)	1.50 \pm 0.47* (1.05–3.04)
GSI (%)	3.72 \pm 2.49* (0.11–8.07)	0.40 \pm 0.33 ^A (0.13–1.40)	2.30 \pm 2.61* (0.02–7.05)	0.15 \pm 0.06 ^B (0.07–0.25)
Sex (M/F/ND)	10/5/1	10/6/0	10/10/0	8/8/0

HSI –hepatosomatic index; GSI –gonadosomatic index; FCI – Fulton condition index, ND – not determined.

3.3.5. Biomarker of a general stress - TP

Site specific differences in protein levels pointed to higher values in fish caught near the town of Knin than the reference location (Fig. 4f). These differences were significant only in autumn, with 1.13 times

higher average TP levels in fish from the wastewater impacted site (54.1 mg g⁻¹ w. w.) than the reference site (47.8 mg g⁻¹ w. w.). At both locations, TP levels were a bit higher in spring season than in autumn, but not significantly (Fig. 4f).

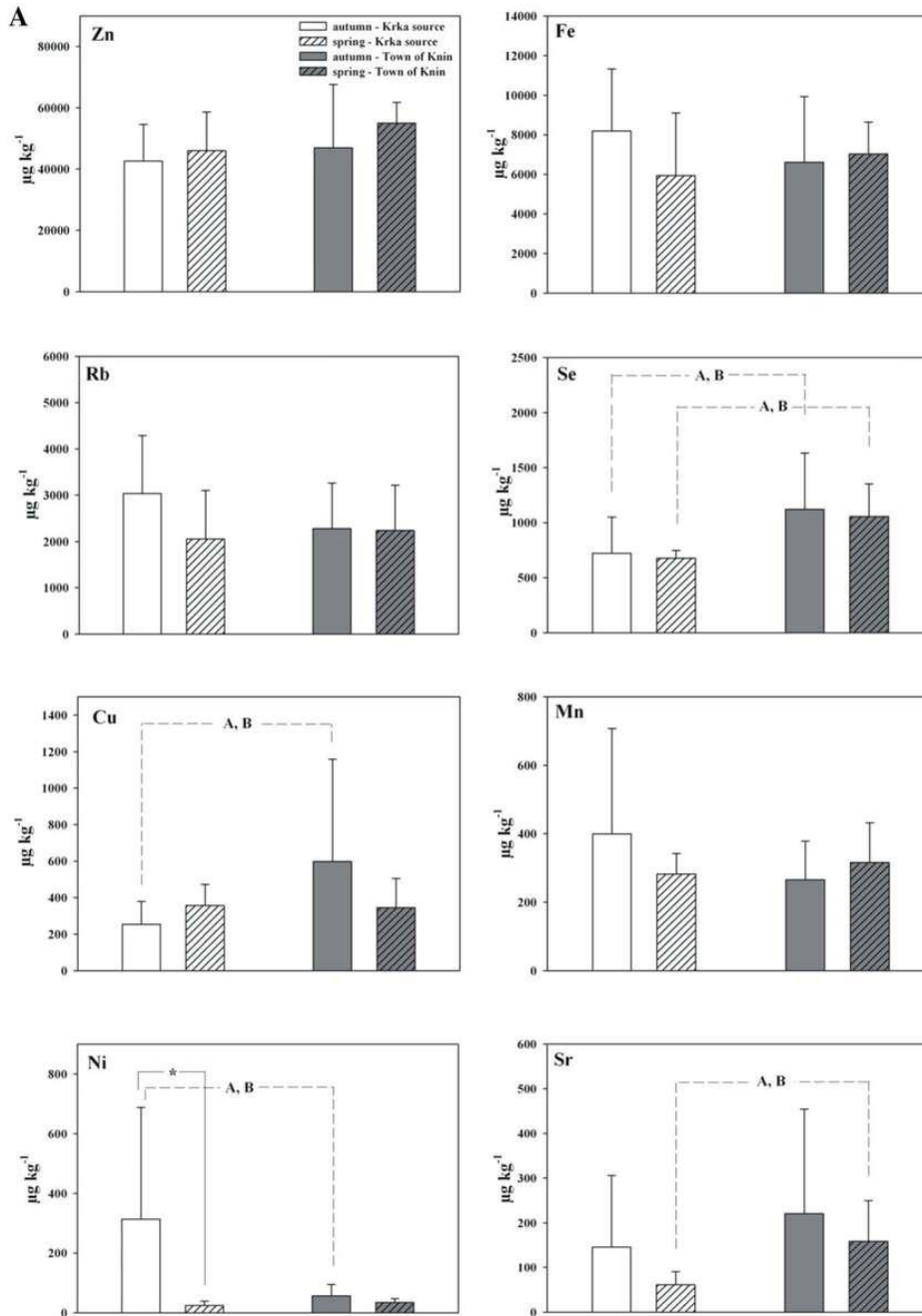


Fig. 2. Cytosolic trace metals concentrations (mean \pm S.D., $\mu\text{g kg}^{-1}$) in intestinal tissue of *S. trutta* from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka downstream of Knin) in two sampling campaigns (autumn- October 2015 and spring- May 2016); a) elements with average concentrations above $100 \mu\text{g kg}^{-1}$, b) elements with average concentrations below $100 \mu\text{g kg}^{-1}$. 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 are assigned with different superscript letters (A and B) and dashed line. Site legend: white - Krka River source, autumn season; dashed-white - Krka River source, spring season; grey - Krka downstream of Knin, autumn season; dashed-grey - Krka downstream of Knin, spring season.

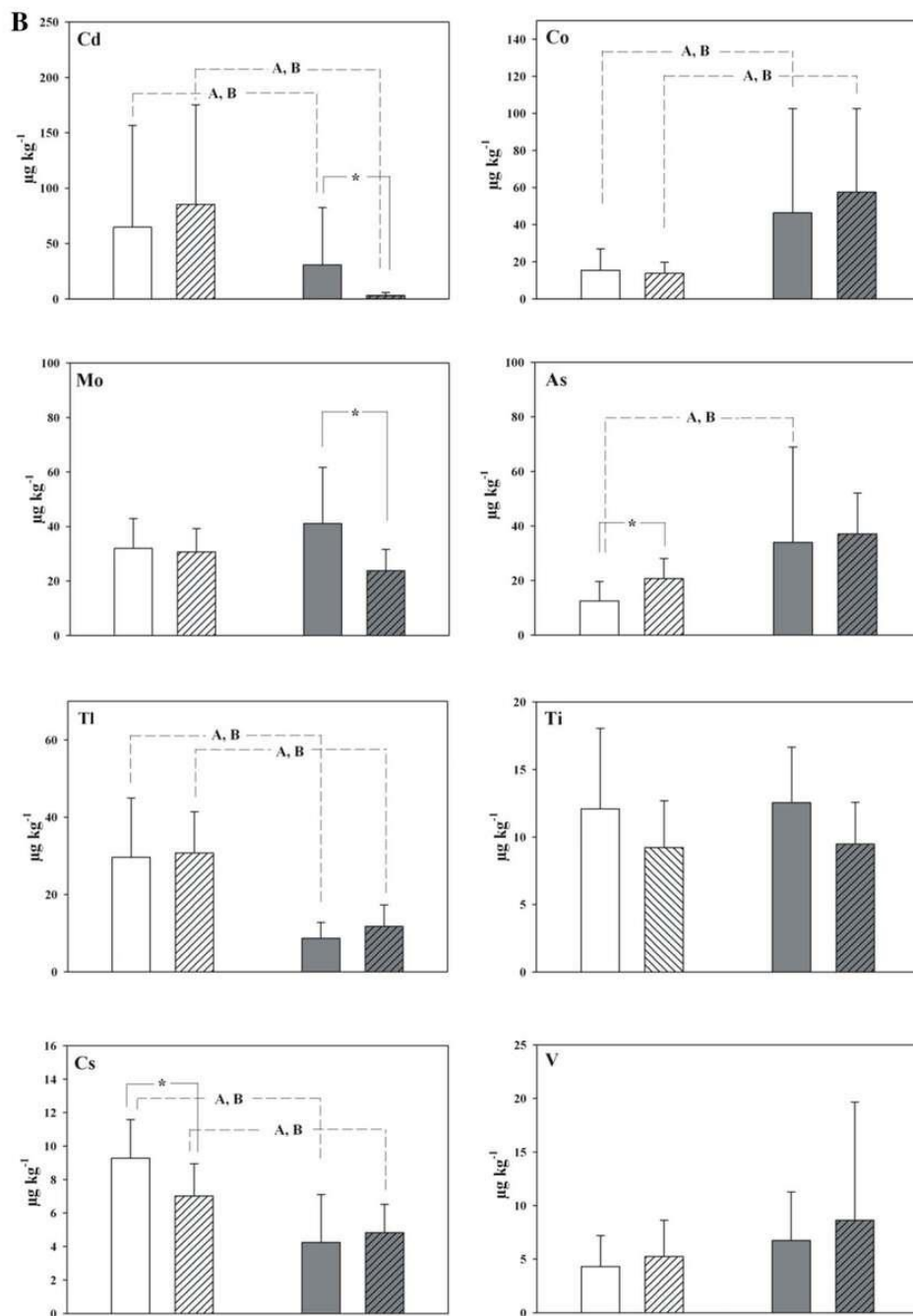


Fig. 2 (continued).

4. Discussion

4.1. Biometric characteristics

Sampled population of brown trout from the karst river confirmed seasonality of biometric parameters in relation to fish physiology. Higher values of total length, body mass and GSI observed in autumn

season at both locations are in accordance with brown trout biology and spawning period which occurs in late autumn (Mrakovčić et al., 2006; Hajirezaee et al., 2012). On the other hand, the opposite trend of HSI and FCI is probably a result of the mobilization of energy reserves needed for reproductive development, as well as of higher food supply during the spring period (Moddock and Burton, 1999). In both seasons, significant site specific differences suggested the influence of pollution

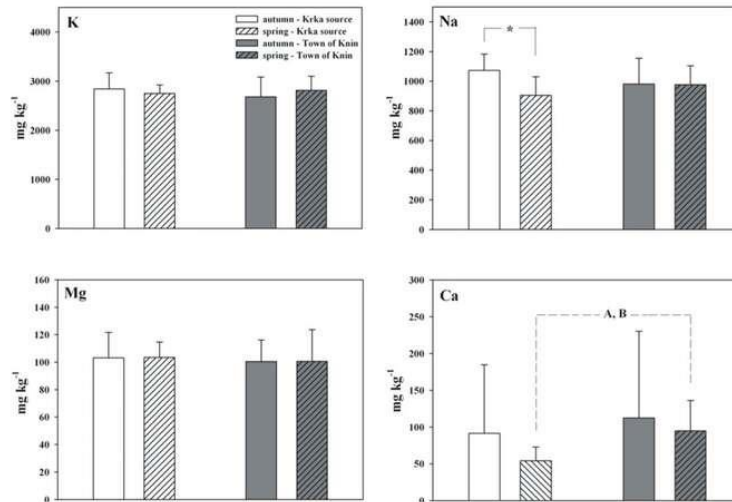


Fig. 3. Cytosolic macroelements concentrations (mean \pm S.D., mg kg⁻¹) in intestinal tissue of *S. trutta* from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka downstream of Knin) in two sampling campaigns (autumn– October 2015 and spring– May 2016). 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 are assigned with different superscript letters (A and B) and dashed line. Site legend: white – Krka River source, autumn season; dashed-white – Krka River source, spring season; grey – Krka downstream of Knin, autumn season; dashed-grey – Krka downstream of Knin, spring season.

gradient on FCI, i.e. higher FCI levels downstream of the town of Knin compared to the Krka River source might be associated to higher concentrations and consequently better availability of nutrients at the anthropogenically impacted site (Lambert and Dutil, 1997; Couture and Rajotte, 2003). In the literature data the opposite trend of lower FCI values in metal polluted locations is also frequently observed (Laflamme et al., 2000; Rajotte and Couture, 2002; Couture and Rajotte, 2003), rising to conclusion that the wastewater impact near the town of Knin did not induce defense mechanism of fish in a way to require a lot of energy which would result in decreased FCI.

4.2. Cytosolic metal/metalloid concentrations in fish intestine

Intestinal metal/metalloid levels in fish cytosol reflect soluble metal fraction which might be bound to cytosolic biomolecules and

correspond to the dietary metal uptake route. Previous studies have already reported ecological status and total dissolved metal/metalloid concentrations in the river water from the same locations (Filipović Marijić et al., 2018; Sertić Perić et al., 2018). In these studies, few physico-chemical water parameters (temperature, conductivity, total dissolved solids and total water hardness) indicated slightly degraded ecological conditions at the anthropogenically impacted site and increased dissolved metal levels in water at the same site compared to the river source, especially for Fe, Li, Mn, Mo, Sr, Rb and Ca. The highest increase was recorded for Fe and Mn, which levels were 17 times and 38 times higher near town of Knin compared to the reference site, respectively, while other metals showed the increase in average levels from 1.2 to 2.2 times (Filipović Marijić et al., 2018; Sertić Perić et al., 2018).

However, despite these differences, metal levels along the Krka River watercourse were rather low and mostly comparable to metal levels

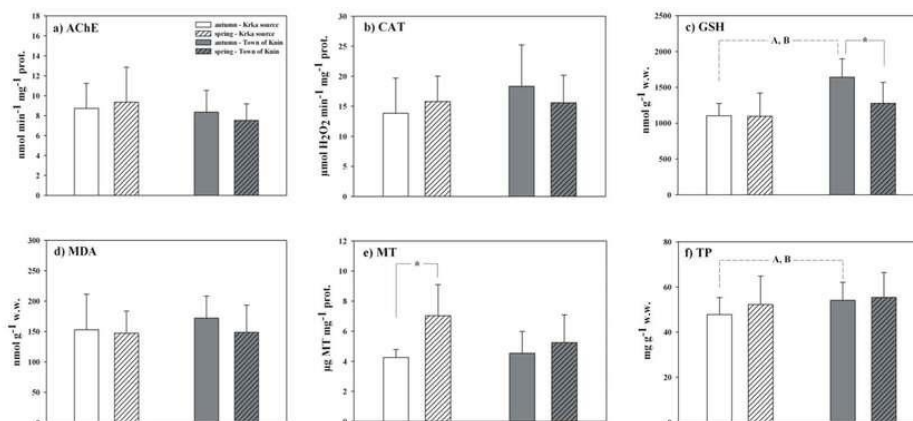


Fig. 4. Biomarker levels in intestinal tissue of *S. trutta* from the Krka River at two sampling sites presented as mean values \pm S.D. (reference site: Krka River source; contaminated site: Krka downstream of the town of Knin) in two sampling campaigns (autumn– October 2015 and spring– May 2016) ($n = 16$, except at Krka downstream of the town of Knin in autumn where $n = 20$). 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 are assigned with different superscript letters (A and B) and dashed line. Site legend: white – Krka River source, autumn season; dashed-white – Krka River source, spring season; grey – Krka downstream of Knin, autumn season; dashed-grey – Krka downstream of Knin, spring season.

reported for other karst ecosystems (Dossi et al., 2007; Cukrov et al., 2008, 2012) or lower compared to anthropogenically impacted world rivers (Filipović Marijić et al., 2018). This can be explained by effective self-purification process of the Krka River, which is contributed by the input of underground water, sinking of contaminants in lake sediments and changes in water levels (Cukrov et al., 2008; Filipović Marijić et al., 2018). Obtained results on environmental conditions in the river water were compared with the metal/metalloid accumulation in fish intestine, which mostly reflected the similar pattern as already recorded for total dissolved metals/metalloids in the river water (Filipović Marijić et al., 2018; Sertić Perić et al., 2018) and therefore, indicated bioavailability and dietary intake of these metals in fish intestinal tissue. Accumulation of metals in fish from the location influenced by technological and municipal wastewaters was significant and over 3 times increased for Co, As and Sr when compared to their levels in fish from the river source (Figs. 2a, b). However, few elements showed the opposite trend with significantly higher concentrations in fish from the Krka River source, like Cd, Cs, Tl. Such results are in accordance with total and cytosolic concentrations of metals/metalloids in the liver of the same *S. trutta* from the reference location (Dragun et al., 2018), while levels of these elements in water were uniform along the Krka River watercourse, meaning that their concentrations in the water of the river source were comparable to those in the polluted area (Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Increased metal levels in fish tissues from the Krka River source might be of natural origin, which is in the case of Cd mobilization of naturally occurring Cd, especially from dolomites in the karst area (Cukrov et al., 2008). Diet content might be an important source of Tl as already observed in juvenile fathead minnows by Lapointe and Couture (2009), although the bioaccumulation of waterborne Tl was shown to be more rapid than dietborne but both exposure routes were suggested as a risk of toxicity. However, the cause of higher metal concentrations in fish from the reference location cannot be definitely explained without further investigations, which should involve metal measurement in fish food and river sediment as their possible sources, especially if considering intestine as an organ of food uptake and its importance in fish digestion and nutrient absorption. Analysis of metals in the gut content of some fish species like European chub (*Squalius cephalus*) (Filipović Marijić and Raspor, 2012), carp (*Cyprinus carpio*) (Kraal et al., 1995), pike (*Esox lucius*) and bream (*Abramis brama*) (Rajkowska and Protasowicki, 2013) and rainbow trout (*Oncorhynchus mykiss*) (Kamunde et al., 2002) have already showed the importance of dietborne metal intake.

Observed seasonal differences in cytosolic intestinal metal levels might be linked to fish physiology. Most metals/metalloids showed higher levels in autumn than in spring, probably as a result of fish physiological changes related to the reproductive period of brown trout in late autumn. Dependence of metal levels upon fish reproductive period can be explained by the fact that essential metals have important roles in fish metabolism, as constitutive part of proteins and other important biological molecules (Miramand et al., 1991; Filipović Marijić and Raspor, 2010, 2014). In addition, Sertić Perić et al. (2018) reported that concentrations of total dissolved metal levels in the river water were also higher in autumn than spring period.

Comparison of metal/metalloid concentrations in cytosolic fraction of brown trout intestine (Figs. 2, 3) with other literature data was possible only for cytosolic metal levels in intestine of European chub from the Sava River, which showed the same descending order of metal levels and even comparable concentrations for Zn > Fe > Cu > Mn > Cd (Filipović Marijić and Raspor, 2012).

4.3. Biomarker responses in brown trout intestine

Combined use of set of different biomarkers enables a more comprehensive and integrative assessment of environmental quality (Broeg and Lehtonen, 2006; Humphrey et al., 2007). In the present study multi-biomarker approach was applied, in order to assess biological

responses of native fish exposed to the mixture of contaminants in the karst aquatic environment.

Inhibition of AChE activity is commonly used as a biomarker of organophosphorous and carbamate exposure in both aquatic and terrestrial environments (Lionetto et al., 2011). However, inhibition of AChE activity might be caused by other contaminants such as heavy metals, polycyclic aromatic hydrocarbons or detergents (Elumalai et al., 2007; Richetti et al., 2011), which also might play important role in AChE activity in the present study. Our results on decreased AChE activity, although not significantly, in fish intestine from the area near the town of Knin in both seasons (Fig. 4a), might indicate metal and fertilizer influence on AChE activity inhibition in fish caught downstream of the polluted area. Although this decrease was not significant in the fish intestine (Fig. 4a), it was discernible especially in spring, as period of crop germination and increased usage of fertilizers. In addition, many cytosolic metals in fish intestine had higher levels at the contaminated site than at the reference site (Figs. 2, 3), possibly affecting enzyme inhibition as well. Correlation analysis confirmed significantly negative correlation of AChE with Zn ($r = -0.59, p < 0.05$), Fe ($r = -0.63, p < 0.01$), Mn ($r = -0.70, p < 0.01$) and Sr ($r = -0.89, p < 0.001$) levels in intestinal cytosol, indicating metal influence on AChE inhibition. Szabó et al. (1991) reported that intestine of rainbow trout was an organ with the lowest AChE activity in comparison to the brain, muscle and heart. In the same research, trout was described as a species with the lowest AChE activity in the intestinal tissue in comparison to 11 other fish species, which average value was $10 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ In the present research, average values of AChE activity in fish from the reference site were around $9 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ (Fig. 4a) which is in agreement with the mentioned literature values.

Pollution impact near the town of Knin was also evaluated by two biomarkers of the antioxidant capacities, CAT and GSH. Our results suggest that fish were subjected to oxidative stress according to slightly higher CAT activities (Fig. 4b), as well as by the significantly higher values of GSH in autumn at contaminated compared to the reference site, respectively (Fig. 4c). CAT activity has already been measured *in vitro* and *in vivo* in the intestine of freshwater fish *Oreochromis niloticus* (Atli et al., 2006) and the values in control group ($161.7 \pm 15.3 \text{ } \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ prot.}$) and in fish exposed to Ag, Cd, Cr, Cu and Zn (mostly ranging from 25 to $225 \text{ } \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ prot.}$ depending on the metals and their concentrations) were higher compared to brown trout from the karst Krka River (ranging from 13.51 to $18.34 \text{ } \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ prot.}$, Fig. 4b). In our study, significant correlation between CAT activity and cytosolic metal levels was confirmed for Mo ($r = 0.71, p < 0.01$) and Co ($r = 0.88, p < 0.05$) in the intestine of fish from the location downstream of the wastewater outlets.

GSH is involved in different metabolic and transport processes, the protection of cells against toxic effects of different compounds, including oxygen reactive species and heavy metals (Meister and Anderson, 1983; Canesi et al., 1999). In fish from the Krka River, the significant increase in GSH levels was 1.5 times in fish from the contaminated compared to the reference site in autumn and also 1.16 times in spring but without significant difference (Fig. 4c). GSH showed significant seasonal differences, with significantly higher levels in autumn than spring in fish from the wastewater impacted site (Fig. 4c). Such results are in accordance with the higher metal concentrations in intestinal cytosol of fish from the contaminated site in autumn, and therefore suggested possible impact of metals on oxidative stress (Figs. 2, 3). There are many literature data confirming that metals affect the cell antioxidant system efficiency, like for example Cu, Se and Mo. Liu et al. (2005) reported that longer exposure to different concentration of Cu induced a significant increase of GSH content in liver of freshwater fish *Carassius auratus*. Study on fish *Piaractus mesopotamicus* showed that Se supplementation helped lessen free radical damage and boosts immune system function (Biller-Takahashi et al., 2015). GSH levels and CAT activity at the contaminated site were also in accordance with Mo

concentrations pattern, which could be due to the formation of molybdate oxoanion which is known to cause the increase in the activities of antioxidant enzymes like super oxide dismutase (SOD), glutathione peroxidase (GPOX) and catalase (CAT) (Panneerselvam and Govindasamy, 2004). To our knowledge, there is no literature data on GSH levels in the fish intestinal tissue and our results can only indicate that GSH levels in the intestine of brown trout were in range of the values observed by Otto and Moon (1996) in the liver ($1539 \pm 238 \text{ nmol g}^{-1} \text{ w. w.}$) and kidney ($1993 \pm 66 \text{ nmol g}^{-1} \text{ w. w.}$) of the adult rainbow trouts.

The elevated concentration of MDA directly reflects oxidative stress in the organism as a consequence of lipid damage caused by free radicals (Banerjee et al., 1999; Dragun et al., 2017). In our study intestinal MDA levels did not show significant site- or season-specific differences. Slightly higher MDA concentrations were only observed in fish caught near the town of Knin in autumn (Fig. 4d). Such results are in accordance with CAT and GSH results which pointed to moderate evidence of oxidative stress, therefore oxidative stress damages by means of MDA production were not observed. Metal catalyzed formation of reactive ROS capable of damaging tissues such as DNA, proteins and lipids has already been documented. For example, significant effect of dietary Fe on MDA levels in the intestine and liver of rainbow trouts was observed, which was reflected as small but persistent elevation of intestinal MDA values positively correlated with increasing Fe levels in the gut (Carriguirborde et al., 2004). On the other hand, a research on dietary Cu and Cd in Atlantic salmon revealed that no significant increase in tissue MDA levels was observed in the intestine of fish exposed to dietary Cd, while dietary Cu had a direct effect on lipid peroxidation even at relatively low concentrations (Berntssen et al., 2000). Greani et al. (2017) investigated the effect of chronic As exposure under environmental conditions on oxidative stress in wild trout and significant increase of MDA levels was observed in muscles, kidney, liver and fins of exposed trouts. In our study, levels of As in intestinal cytosol of brown trouts were higher at contaminated site than at the reference site in both seasons, even significantly in autumn (Fig. 2b), while correlation analysis confirmed significantly positive relation of MDA and Fe ($r = 0.70$, $p < 0.01$) and Ni ($r = 0.71$, $p < 0.01$) in fish from the contaminated location. However, MDA levels were not significantly higher near the town of Knin compared to the river spring, so the existing contamination in investigated area was not high enough to induce sufficient oxidative damage in fish and was probably counteracted by antioxidant defense mechanisms (CAT, GSH).

Spatial differences were also observed for TP levels, with significantly higher values recorded in fish caught near the town of Knin in autumn, but only slightly higher levels in spring (Fig. 4f), following the trend of biomarkers of antioxidant capacities and pointing to more stressful conditions for brown trouts at the site under the wastewater impact. Additionally, significantly positive correlation between TP levels and metal levels was observed for Mg ($r = 0.50$, $p < 0.01$), Cu ($r = 0.69$, $p < 0.05$), Mn ($r = 0.74$, $p < 0.05$) and Zn ($r = 0.69$, $p < 0.05$) in fish from the contaminated site. However, temperature, oxygen levels and salinity are also known as important factors influencing the protein turnover rates in active tissues, but protein synthesis can also be correlated to feeding habits (Peragón et al., 1994). Thus, higher protein content observed in spring at both locations might also suggest that there were more available food sources in spring, especially near the town of Knin, which would be in accordance with the higher FCI and fish masses from that site (Table 2).

The opposite response compared to other biomarkers was obtained only for MT, which showed higher levels in fish from the reference than polluted location in spring, but without significant differences (Fig. 4e). Metallothionein induction has been widely considered as efficient biomarker for metal pollution in a variety of animal species (Ivanković et al., 2005; Mosleh et al., 2006; Filipović Marijić and Raspor, 2010; Calisi et al., 2013). As one of the main MT roles is the regulation of essential metals like Zn and Cu, and detoxification of nonessential metals like

Cd, Hg and Ag, some of these metals might contribute to the higher MT values in brown trout intestine in spring at both sites. At the Krka River source, concentrations of Cd, Cu, and Zn were higher in the spring campaign, although without significant differences, possibly affecting higher levels of MT at this site in spring. MT induction in the intestine of different fish species has already been confirmed by Handy et al. (1999) and Berntssen et al. (1999) after dietary Cu exposure, by Ptashynski and Klaverkamp (2002) after Ni exposure and by Berntssen et al. (2001), Chowdhury et al. (2005) and Roesijadi et al. (2009) after dietary Cd uptake. However, in polluted environment fish are exposed to a mixture of different metals, and even when MT induction is shown, it is generally impossible to connect this elevated synthesis to specific elements. In addition, MT levels may also be affected by other parameters such as season, temperature, fish size, gender or nutritional status (Hylland et al., 1998; Filipović Marijić and Raspor, 2010). Therefore, higher FCI, as well as higher protein content, in the spring campaign at both sites, indicated the enhanced feeding during that period which also might cause higher MT concentrations, which increase was even significant at the reference location (Fig. 4e).

5. Conclusions

Biological responses in the intestinal tissue of *S. trutta* from two sites of the karst Krka River in Croatia revealed that anthropogenic impact downstream of the technological and municipal wastewater input was evident for biomarker of antioxidant capacities (GSH) and general stress (TP) and for numerous metals/metalloids measured in cytosolic intestinal fraction. Concentrations of As, Ca, Co, Cu, Se and Sr were significantly higher at the contaminated site near the town of Knin compared to the reference location and pointed to a rising need of strict monitoring of water quality and health of aquatic organisms in the Krka River. Cadmium, cesium and thallium levels were elevated in the intestinal cytosol of fish from the Krka River source, but further investigation on metal levels in food sources and sediment is needed to explain such pattern. Therefore, intestinal tissue was shown as a useful indicator organ which may reflect metal uptake and biological responses to contaminant effect or exposure caused by dietary pathways from food sources.

Significant biomarker responses in fish intestine, reflected as higher GSH and TP levels, revealed that fish from the polluted area experienced oxidative and general stress. But comprehensive evaluation of the multi-biomarker response, also involving CAT, MDA, AChE and MT, suggested that in fish living downstream from the wastewaters outlets no significant indication of oxidative damage occurred, neither significant correlation with most cytosolic metals/metalloids. Hence, the impact of contaminants on the Krka River still seems to be only moderate but it is of growing concern that both metals and some biomarkers indicated anthropogenic impact on water and organisms near the town of Knin. Thus, with the time, without the proper and continuous monitoring and protection plan of the region, the consequences might be more ruinous for the whole biota of the Krka River and the national park itself.

Acknowledgments

The financial support of the Croatian Science Foundation, Croatia for the project no. IP-2014-483 09-4255 Accumulation, Subcellular Mapping and Effects of Trace Metals in Aquatic Organisms (AQUAMAPMET) is gratefully acknowledged. Authors are also grateful for the valuable help in the field work to the members of the Laboratory for Aquaculture and Pathology of Aquatic Organisms from the Ruđer Bošković Institute.

References

- Atli, G., Alptekin, I., Tukek, S., Canli, M., 2006. Response of catalase activity to Ag^+ , Cd^{2+} , Cr^{6+} , Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus*. *Comp. Biochem. Physiol. C* 143, 218–224. <https://doi.org/10.1016/j.cbpc.2006.02.003>.
- Bakalowicz, M., 2005. Karst groundwater: a challenge for new resources. *Hydrogeol. J.* 13 (1), 148–160. <https://doi.org/10.1007/s10040-004-0402-9>.

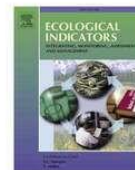
- Banerjee, B.D., Seth, V., Bhattacharya, A., 1999. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicol. Lett.* 107, 33–47. [https://doi.org/10.1016/S0378-4274\(99\)00029-6](https://doi.org/10.1016/S0378-4274(99)00029-6).
- Barišić, J., Filipović Marijić, V., Mijošek, T., Čož-Rakovac, R., Dragun, Z., Krasnići, N., Ivanković, D., Kružličová, D., Erk, M., 2018. Evaluation of architectural and histopathological biomarkers in the intestine of brown trout (*Salmo trutta* Linnaeus, 1758) challenged with environmental pollution. *Sci. Total Environ.* 642, 656–664. <https://doi.org/10.1016/j.scitotenv.2018.06.045>.
- Berntssen, M.H.G., Hylland, K., Wendelaar Bonga, S.E., Maage, A., 1999. Toxic levels of dietary copper in Atlantic salmon (*Salmo salar* L.) parr. *Aquat. Toxicol.* 46, 87–99. [https://doi.org/10.1016/S0166-445X\(98\)00117-9](https://doi.org/10.1016/S0166-445X(98)00117-9).
- Berntssen, M.H.G., Lundebye, A., Hamre, K., 2000. Tissue lipid peroxidative responses in Atlantic salmon (*Salmo salar* L.) parr fed high levels of dietary copper and cadmium. *Fish Physiol. Biochem.* 23, 35–48. <https://doi.org/10.1023/A:1007894816114>.
- Berntssen, M.H.G., Aspholm, O.O., Hylland, K., Wendelaar Bonga, S.E., Lundebye, A.K., 2001. Tissue metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo salar* L.) parr fed elevated dietary cadmium. *Comp. Biochem. Physiol. C* 128, 299–310. [https://doi.org/10.1016/S1532-0456\(00\)00204-0](https://doi.org/10.1016/S1532-0456(00)00204-0).
- Biller-Takahashi, J.D., Takahashi, L.S., Mingatto, F.E., Urbinati, E.C., 2015. The immune system is limited by oxidative stress: dietary selenium promotes optimal antioxidative status and greatest immune defense in pacu *Piaractus mesopotamicus*. *Fish Shellfish Immunol.* 47 (1), 360–367. <https://doi.org/10.1016/j.fsi.2015.09.022>.
- Botsoglou, N.A., Fletouris, D.J., Papageorgiou, G.E., Vassilopoulos, V.N., Mantis, A.J., Trakatellis, A.G., 1994. A rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissues, food, and feedstuff samples. *J. Agric. Food Chem.* 42, 1931–1937. <https://doi.org/10.1021/jf00045a019>.
- Brinkmann, R., Parise, M., 2012. Karst environments: problems, management, human impacts, and sustainability. *J. Caves Karst Stud.* 74 (2), 135–136. <https://doi.org/10.4311/2011JKS0253>.
- Broeg, K., Lehtonen, K.K., 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. *Mar. Pollut. Bull.* 53, 508–522. <https://doi.org/10.1016/j.marpolbul.2006.02.004>.
- Calisi, A., Zaccarelli, N., Lionetto, M.G., Schettino, T., 2013. Integrated biomarker analysis in the earthworm *Lumbricus terrestris*: application to the monitoring of soil heavy metal pollution. *Chemosphere* 90, 2637–2644. <https://doi.org/10.1016/j.chemosphere.2012.11.040>.
- Calò, F., Parise, M., 2009. Waste management and problems of groundwater pollution in karst environments in the context of a post-conflict scenario: the case of Mostar (Bosnia Herzegovina). *Habitat Int.* 33 (1), 63–72. <https://doi.org/10.1016/j.habitatint.2008.05.001>.
- Canesi, L., Viarengo, A., Leonzio, C., Filipelli, M., Gallo, G., 1999. Heavy metals and glutathione metabolism in mussel tissue. *Aquat. Toxicol.* 46, 67–76. [https://doi.org/10.1016/S0166-445X\(98\)00116-7](https://doi.org/10.1016/S0166-445X(98)00116-7).
- Caron, A., Rosabal, M., Drevet, O., Couture, P., Campbell, P.G., 2018. Binding of trace elements (Ag, Cd, Co, Cu, Ni, and Ti) to cytosolic biomolecules in livers of juvenile yellow perch (*Perca flavescens*) collected from lakes representing metal contamination gradients. *Environ. Toxicol. Chem.* 37, 576–586. <https://doi.org/10.1002/etc.3998>.
- Carriquiriborde, P., Handy, R.D., Davies, S.J., 2004. Physiological modulation of iron metabolism in rainbow trout (*Oncorhynchus mykiss*) fed low and high iron diets. *J. Exp. Biol.* 207, 75–86. <https://doi.org/10.1124/jeb.00712>.
- Chowdhury, M.J., Baldissarotto, B., Wood, C.M., 2005. Tissue-specific cadmium and metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. *Arch. Environ. Contam. Toxicol.* 48, 381–390. <https://doi.org/10.1007/s00244-004-0068-2>.
- Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), *CRC Handbook of Methods for Oxygen Radical Research*. CRC Press, Boca Raton FL, pp. 283–284.
- Couture, P., Rajotte, J.W., 2003. Morphometric and metabolic indicators of metal stress in wild yellow perch (*Perca flavescens*) from Sudbury, Ontario: a review. *J. Environ. Monit.* 5, 216–221. <https://doi.org/10.1039/b210338a>.
- Cravo, A., Lopes, B., Serafim, A., Company, R., Barreira, L., Gomes, T., Bebianno, M.J., 2009. A multi-biomarker approach in *Mytilus galloprovincialis* to assess environmental quality. *J. Environ. Monit.* 11, 1673–1686. <https://doi.org/10.1039/b909846a>.
- Cukrov, N., Cmuk, P., Makar, M., Omanović, D., 2008. Spatial distribution of trace metals in the Krka River, Croatia. An example of the self-purification. *Chemosphere* 72, 1559–1566. <https://doi.org/10.1016/j.chemosphere.2008.04.038>.
- Cukrov, N., Tepić, N., Omanović, D., Lojen, S., Bura-Nakić, E., Vojvodić, V., Pižeta, I., 2012. Qualitative interpretation of physico-chemical and isotopic parameters in the Krka River (Croatia) assessed by multivariate statistical analysis. *Int. J. Environ. Anal. Chem.* 92, 1187–1199. <https://doi.org/10.1080/03067319.2010.550003>.
- Dossi, C., Ciceri, E., Giussani, B., Pozzi, A., Galgaro, A., Viero, A., Viganò, A., 2007. Water and snow chemistry of main ions and trace elements in the karst system of Monte Pelmo massif (Dolomites, Eastern Alps, Italy). *Mar. Freshw. Res.* 58, 649–656. <https://doi.org/10.1071/MF06170>.
- Dragun, Z., Filipović Marijić, V., Krasnići, N., Ramani, S., Valić, D., Rebok, K., Kostov, V., Jordanova, M., Erk, M., 2017. Malondialdehyde concentrations in the intestine and gills of Vardar chub (*Squalius vardarensis* Karaman) as indicator of lipid peroxidation. *Environ. Sci. Pollut. Res. Int.* 24, 16917–16926. <https://doi.org/10.1007/s11356-017-9305-x>.
- 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. *Ecotoxicol. Environ. Saf.* 147, 537–549. <https://doi.org/10.1016/j.ecoenv.2017.09.005>.
- Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95. [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9).
- Elumalai, E., Antunes, C., Guilhermino, L., 2007. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NM Portugal) exposed to zinc and mercury. *Chemosphere* 66 (7), 1249–1255. <https://doi.org/10.1016/j.chemosphere.2006.07.030>.
- Fiket, Ž., Roje, V., Mikac, N., Kniewald, G., 2007. Determination of arsenic and other trace elements in bottled waters by high resolution inductively coupled plasma mass spectrometry. *Croat. Chem. Acta* 80, 91–100.
- Filipović Marijić, V., Raspor, B., 2010. The impact of the fish spawning on metal and protein levels in gastrointestinal cytosol of indigenous European chub. *Comp. Biochem. Physiol. C* 152, 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 Pollut.* 223, 4769–4783. <https://doi.org/10.1007/s11270-012-1233-2>.
- 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.). *Environ. Sci. Pollut. Res.* 21 (12), 7596–7606. <https://doi.org/10.1007/s11356-014-2666-5>.
- Filipović Marijić, V., Kapetanović, D., Dragun, Z., Valić, D., Krasnići, N., Redžović, Z., Grgić, I., Žunić, J., Kružličová, 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. *Environ. Sci. Pollut. Res.* 25, 4715–4727. <https://doi.org/10.1007/s11356-017-0789-1>.
- Greani, S., Loukisti, R., Berti, L., Marchand, B., Giannettini, J., Santini, J., Quilichini, Y., 2017. Effect of chronic arsenic exposure under environmental conditions on bioaccumulation, oxidative stress, and antioxidant enzymatic defenses in wild trout *Salmo trutta* (Pisces, Teleostei). *Ecotoxicology* 26 (7), 930–941. <https://doi.org/10.1007/s10646-017-1822-3>.
- Hajrezaee, S., Amiri, B.M., Mehrpoosh, M., Jafaryan, H., Mirrasuli, E., Gelpour, A., 2012. Gonadal development and associated changes in gonadosomatic index and sex steroids during the reproductive cycle of cultured male and female Caspian brown trout, *Salmo trutta caspius* (Kessler, 1877). *J. Appl. Anim. Res.* 40, 154–162. <https://doi.org/10.1080/09712119.2011.645035>.
- Handy, R.D., Sims, D.W., Giles, A., Campbell, H.A., Musonda, M.M., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquat. Toxicol.* 47, 23–41. [https://doi.org/10.1016/S0166-445X\(99\)00004-1](https://doi.org/10.1016/S0166-445X(99)00004-1).
- Heidinger, R.C., Crawford, S.D., 1977. Effect of temperature and feeding rate on the liver-somatic index of largemouth bass, *Micropterus salmoides*. *J. Fish. Res. Board Can.* 34, 633–638. <https://doi.org/10.1139/f77-099>.
- HRNEN 14011, 2005. Fish Sampling by Electric Power (in Croatian). Croatian Standard Institute, Zagreb.
- Humphrey, C.A., Codi King, S., Klumpp, D.W., 2007. A multi-biomarker approach in barramundi (*Lates calcarifer*) to measure exposure to contaminants in estuaries of tropical North Queensland. *Mar. Pollut. Bull.* 54 (10), 1569–1581. <https://doi.org/10.1016/j.marpolbul.2007.06.004>.
- Hylland, K., Nissen-Lie, T., Christensen, P.G., Sandvik, M., 1998. Natural modulation of hepatic metallothionein and 584 cytochrome P4501A in flounder, *Platichthys flesus* L. *Mar. Environ. Res.* 46, 51–55.
- Ivanković, D., Pavičić, J., Erk, M., Filipović Marijić, V., Raspor, B., 2005. Evaluation of the *Mytilus galloprovincialis* Lam. Digestive gland metallothionein as a biomarker in a long-term field study: seasonal and spatial variability. *Mar. Pollut. Bull.* 50, 1303–1313. <https://doi.org/10.1016/j.marpolbul.2005.04.039>.
- Kamunde, C.N., Grosell, M., Higgs, D., Wood, C.M., 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne Cu uptake. *J. Exp. Biol.* 205, 279–290.
- Kraal, M.H., Kraak, M.H., de Groot, C.J., Davids, C., 1995. Uptake and tissue distribution of dietary and aqueous cadmium by carp (*Cyprinus carpio*). *Ecotoxicol. Environ. Saf.* 31 (2), 179–183. <https://doi.org/10.1006/eesa.1995.1060>.
- Lafamme, J.S., Couillard, Y., Campbell, P.G.C., Hontela, A., 2000. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. *Can. J. Fish. Aquat. Sci.* 57, 1692–1700. <https://doi.org/10.1139/f00-118>.
- 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*)? *Can. J. Fish. Aquat. Sci.* 54, 104–112. <https://doi.org/10.1139/cjfas-54-51-104>.
- 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*). *Arch. Environ. Contam. Toxicol.* 57, 571–580. <https://doi.org/10.1007/s00244-009-9298-7>.
- Lionetto, M.G., Caricato, R., Calisi, A., Schettino, T., 2011. Acetylcholinesterase inhibition as a relevant biomarker in environmental biomonitoring: new insights and perspectives. In: Visser, J.E. (Ed.), *Ecotoxicology Around the Globe*. Nova Science Publishers, Hauppauge (USA), pp. 87–115.
- Liu, H., Zhang, J.F., Shen, H., Wang, X.R., Wang, W.M., 2005. Impact of copper and its EDTA complex on the glutathione-dependent antioxidant system in freshwater fish (*Carassius auratus*). *Bull. Environ. Contam. Toxicol.* 74, 1111–1117. <https://doi.org/10.1007/s00128-005-0696-x>.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Meister, A., Anderson, M.A., 1983. Glutathione. *Annu. Rev. Biochem.* 52, 711–760. <https://doi.org/10.1146/annurev.bi.52.070183.003431>.
- Miramand, P., Lafaurie, M., Fowler, S.W., Lemaire, P., Guary, J.C., Bentley, D., 1991. Reproductive cycle and heavy metals in the organs of red mullet, *Mullus barbatus* (L.), from

- the northwestern Mediterranean. *Sci. Total Environ.* 103, 47–56. [https://doi.org/10.1016/0048-9697\(91\)90352-F](https://doi.org/10.1016/0048-9697(91)90352-F).
- Moddock, D.M., Burton, M.P.M., 1999. Gross and histological observations of ovarian development and related condition changes in American plaice. *J. Fish Biol.* 53, 928–944. <https://doi.org/10.1111/j.1095-8649.1998.tb00454.x>.
- Monserrat, J.M., Martínez, P.E., Geracitano, L., Amado, L.L., Gaspar Martins, C.M., Leães Pinho, G.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. *Comp. Biochem. Physiol. C* 146, 221–234. <https://doi.org/10.1016/j.cbpc.2006.08.012>.
- Mosleh, Y.Y., Paris-Palacios, S., Biagiatti-Risbourg, S., 2006. Metallothioneins induction and antioxidative response in aquatic worms *Tubifex tubifex* (Oligochaeta, Tubificidae) exposed to copper. *Chemosphere* 64, 121–128. <https://doi.org/10.1016/j.chemosphere.2005.10.045>.
- Mrakovčić, M., Brigić, A., Buj, I., Čaleta, 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).
- NN 55, 2013. Ordinance on the Protection of Animals Used for Scientific Purposes [Pravilnik o zaštiti životinja koje se koriste u znanstvene svrhe].
- Otto, D.M.E., Moon, T.W., 1996. Endogenous antioxidant systems of two teleost fish, the rainbow trout and the black bullhead, and the effect of age. *Fish Physiol. Biochem.* 15 (4), 349–358. <https://doi.org/10.1007/BF02112362>.
- Panneerselvam, S., Govindasamy, S., 2004. Effect of sodium molybdate on the status of lipids, lipid peroxidation, and antioxidant systems in alloxan-induced diabetic rats. *Clin. Chim. Acta* 345, 93–98. <https://doi.org/10.1016/j.cccn.2004.03.005>.
- Peragón, J., Barroso, J.B., García-Salguero, L., de la Higuera, M., Lupiáñez, J.A., 1994. Dietary protein effects on growth and fractional protein synthesis and degradation rates in liver and white muscle of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 124, 35–46. [https://doi.org/10.1016/0044-8486\(94\)90352-2](https://doi.org/10.1016/0044-8486(94)90352-2).
- Priborsky, J., Stara, A., Rezabek, J., Zuskova, E., Lepic, P., Velisek, J., 2015. Comparison of the effect of four anaesthetics on haematological profiles, oxidative stress and antioxidant enzymes in barbel (*Barbus barbus*). *Neurosci. Lett.* 36 (Suppl. 1), 141–146.
- Plashynski, M.D., Klaverkamp, J.F., 2002. Accumulation and distribution of dietary nickel in lake whitefish (*Coregonus clupeaformis*). *Aquat. Toxicol.* 58, 249–264. [https://doi.org/10.1016/S0166-445X\(01\)00231-4](https://doi.org/10.1016/S0166-445X(01)00231-4).
- Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* 1, 3159–3165. <https://doi.org/10.1038/nprot.2006.378>.
- Rainbow, P.S., Luoma, S.N., Wang, W.X., 2011. Trophically available metal – a variable feast. *Environ. Pollut.* 159, 2347–2349. <https://doi.org/10.1016/j.envpol.2011.06.040>.
- Rajkowska, M., Protasowicki, M., 2013. Distribution of metals (Fe, Mn, Zn, Cu) in fish tissues in two lakes of different trophy in Northwestern Poland. *Environ. Monit. Assess.* 185 (4), 3493–3502. <https://doi.org/10.1007/s10661-012-2805-8>.
- Rajotte, J.W., Couture, P., 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* 59, 1296–1304. <https://doi.org/10.1139/F02-095>.
- Richetti, S.K., Rosemberg, D.B., Ventura-Lima, J., Monserrat, J.M., Bogo, M.R., Bonan, C.D., 2011. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. *NeuroToxicology* 32, 116–122. <https://doi.org/10.1016/j.neuro.2010.11.001>.
- Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. B. Fish. Res. Board Can. 191, 1–382.
- Ringwood, A.H., Hoguet, J., Keppler, C.J., Gielazyn, M.L., Ward, B.P., Rourk, A.R., 2003. Cellular biomarkers (lysosomal destabilization, glutathione & lipid peroxidation). Three Common Estuarine Species: A Methods Handbook. Marine Resources Research Institute South Carolina Department of Natural Resources, pp. 1–45.
- Roesijadi, G., Rezvankhah, S., Perez-Matus, A., Mittelberg, A., Torruellas, K., Van Veld, P.A., 2009. Dietary cadmium and benzo(a)pyrene increased intestinal metallothionein expression in the fish *Fundulus heteroclitus*. *Mar. Environ. Res.* 67 (1), 25–30. <https://doi.org/10.1016/j.marenvres.2008.10.002>.
- Sertić Perić, M., Matonićkin Kepčija, R., Miliša, M., Gottstein, S., Lajtner, J., Dragun, Z., Filipović Marijić, V., Krasnić, 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. *Ecotoxicol. Environ. Saf.* 163, 125–135. <https://doi.org/10.1016/j.ecoenv.2018.07.068>.
- Stepić, S., Hackenberger Kutuzović, B., Velki, M., Lončarić, Ž., Hackenberger Kutuzović, D., 2013. Effects of individual and binary-combined commercial insecticides endosulfan, temephos, malathion and pirimiphos-methyl on biomarker responses in earthworm *Eisenia andrei*. *Environ. Toxicol. Pharmacol.* 36, 715–723. <https://doi.org/10.1016/j.etap.2013.06.011>.
- Szabó, A., Nemcsók, J., Kása, P., Budai, D., 1991. Comparative study of acetylcholine synthesis in organs of freshwater teleosts. *Fish Physiol. Biochem.* 9, 93–99. <https://doi.org/10.1007/BF02265124>.
- Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27, 502–522. [https://doi.org/10.1016/0003-2697\(69\)90064-5](https://doi.org/10.1016/0003-2697(69)90064-5).
- Topić Popović, N., Strunjak-Perović, I., Čož-Rakovac, R., Barišić, J., Jadan, M., Peršin Beraković, A., Sauerborn Klobučar, R., 2012. Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. *J. Appl. Ichthyol.* 28, 553–564. <https://doi.org/10.1111/j.1439-0426.2012.01950.x>.
- Urien, N., Cooper, S., Caron, A., Sonnenberg, H., Rozon-Ramilo, L., Campbell, P.C.G., 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. *Aquat. Toxicol.* 202, 105–116. <https://doi.org/10.1016/j.aquatox.2018.07.001>.
- Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* 13, 57–149. [https://doi.org/10.1016/S1382-6689\(02\)00126-6](https://doi.org/10.1016/S1382-6689(02)00126-6).
- Wallace, W.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM). *Mar. Ecol. Prog. Ser.* 257, 125–137. <https://doi.org/10.3354/meps257125>.
- Wootton, R.J., 1990. Ecology of teleost fishes. Chapman and Hall, Fish and Fisheries Series 1. London, New York https://doi.org/10.1007/978-94-009-0829-1_9 (404 pp).



Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Intestine of invasive fish Prussian carp as a target organ in metal exposure assessment of the wastewater impacted freshwater ecosystem

Tatjana Mijošek^{*}, Vlatka Filipović Marijić, Zrinka Dragun, Dušica Ivanković, Nesrete Krasnići, Zuzana Redžović, Marijana Erk

Ruder Bosković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička Cesta 54, 10000 Zagreb, Croatia

ARTICLE INFO

Keywords:

Metal contamination
Wastewaters
Biomarkers
Oxidative stress
Subcellular metal distribution
Monitoring

ABSTRACT

The application of invasive fish Prussian carp (*Carassius gibelio* Bloch, 1782) as bioindicator organism, using intestine as bioindicator tissue of anthropogenic influence in the lowland Ilova River was estimated. Intestinal tissue enables the investigation of dietborne metal uptake, so the first record on intestinal metal levels in Prussian carp was presented, as total and cytosolic fraction, which indicates the proportions of potentially toxic and bioavailable metals. Pollution impact was also estimated by analyses of biomarkers of oxidative stress (malondialdehyde), antioxidative capacity (catalase and glutathione) and of metal exposure (metallothioneins). All analyzed parameters were compared in the intestine of fish from the reference site and contaminated site impacted by technological and municipal wastewaters in two seasons. Both total and cytosolic As, Ca, Cd, Cs, Cu, Mg, Na and Rb levels were significantly higher at contaminated than the reference site in at least one season, whereas Mn and V had higher concentrations at the reference site. Despite differences in concentrations, average proportions of total metal levels in cytosolic fraction were comparable at two sites, i.e. over 70% for Na, K, Rb, Se, Cd, Cs, As and Mo, indicating their high possibility of binding to important biomolecules. In addition, higher levels of malondialdehyde in both seasons and enhanced catalase activity in spring, indicated disturbed environmental conditions near the contaminated site and need of continuous monitoring of this region. Finally, our research represents successful application of widely distributed invasive species in ecotoxicological studies, whereas intestine was shown as a suitable bioindicator tissue, clearly reflecting dietary metal uptake.

1. Introduction

Prussian carp (*Carassius gibelio* Bloch, 1782), a cyprinid fish species nowadays widely distributed in Europe and Asia, has a high invasion potential and can tolerate unfavorable environmental conditions including low oxygen levels, variable temperatures and high levels of anthropogenic pollution (De Boeck et al., 2004). Accordingly, it is an appropriate bioindicator in pollution assessment studies (De Boeck et al., 2004; Falfushynska et al., 2011; Tsangaris et al., 2011), including metal contamination. As a highly dominant fish species in the Ilova River, it was chosen as a bioindicator organism to evaluate the extent of existing anthropogenic impact on the biota of that ecosystem. Ilova River is a lowland river in the continental part of the Republic of Croatia, significant as a part of protected wetland area Lonjsko Polje Nature Park but it is under the influence of municipal (Town of Kutina) and industrial wastewaters (fertilizer factory) (Radić et al., 2013; Mijošek et al.,

2020a). Effluents of industrial and municipal wastewaters contain a wide variety of pollutants depending on the type of activities, but high concentrations of trace metals have been often reported in wastewaters of those types (Mendiguchía et al., 2007).

Monitoring of trace and macro elements accumulation in Prussian carp in existing studies was usually carried out by measuring their total concentrations in commonly used target organs such as muscle, liver, kidney and gills (Andreji et al., 2006; Has-Schön et al., 2008; Falfushynska et al., 2011; Yabanli et al., 2014; Milošević and Simić, 2015; Dikanović et al., 2016; Zhelyazkov et al., 2018), but to our knowledge, the intestinal tissue of Prussian carp has not yet been applied as indicator tissue in metal exposure assessment. Despite its crucial role in fish digestion and nutrient absorption, as well as dietborne metal uptake (Clearwater et al., 2000), intestinal tissue is generally rarely applied as a bioindicator tissue. Existing studies mostly reported on only total metal concentrations in the intestine of different fish species (Dallinger and

^{*} Corresponding author.

E-mail address: tmijosek@irb.hr (T. Mijošek).

<https://doi.org/10.1016/j.ecolind.2020.107247>

Received 5 August 2020; Received in revised form 10 November 2020; Accepted 2 December 2020

Available online 22 December 2020

1470-160X/© 2020 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Kautzky, 1985; Staniskiene et al., 2006; Filipović Marijić and Raspor, 2010, 2012; Jarić et al., 2011; Nachev and Sures, 2016; Yeltekin and Sağlam, 2019), with only few exceptions by Filipović Marijić and Raspor (2012) and Mijošek et al. (2019a,b) who reported cytosolic metal concentrations in European chubs and brown trouts, respectively. Total concentrations, however, do not reflect biologically and metabolically available metal content since in organisms, metals are involved in many metabolic processes and end up incorporated in different cellular components (Wallace et al., 2003), such as metal-rich granules or metallothioneins (detoxified metal forms) and sensitive biomolecules (nontoxified metal forms) (Urien et al., 2018). Therefore, in the present study, to get more information on subcellular partitioning of metals and their potentially toxic levels and effects, we have measured both total and cytosolic concentrations in the intestine of invasive fish Prussian carp. Metals in cytosolic fraction can bind and interact with either biologically available part containing microsomes and heat sensitive proteins (e.g., enzymes) or detoxified part involving heat-stable proteins (e.g., metallothioneins) (Bonneris et al., 2005; Urien et al., 2018).

Moreover, many environmental contaminants, including organic compounds and metals lead to extensive formation of reactive oxygen species (ROS) which consequently cause oxidative damage to cellular biomolecules including DNA, proteins and unsaturated lipids in cell membranes of organisms (Martínez-Álvarez et al., 2005). To overcome adverse effects of ROS, fish have an efficient antioxidant defense system involving both non-enzymatic compounds (vitamins E and C, glutathione (GSH) and other thiols) and enzymatic compounds (catalase, CAT; superoxide dismutase, SOD; and glutathione-S-transferase, GST). Besides by directly increasing the cellular concentration of ROS, metals promote oxidative damage also by lowering the cellular antioxidant capacity (Pinto et al., 2003). Thus, in order to evaluate the extent of oxidative stress and efficiency of antioxidant system in fish from the Ilova River, levels of malondialdehyde (MDA) as biomarker of oxidative damage and CAT activity as enzymatic antioxidant and GSH as non-enzymatic antioxidant, as two biomarkers of antioxidative capacity, were measured in their intestine. Additionally, biomarkers of metal exposure, metallothioneins (MTs), were used, because their induction is considered as a direct response to the elevated intracellular metal concentrations. Since levels of most biomarkers are known to be affected by various biotic and abiotic factors (size, age, feeding behavior, oxygen levels, pH, temperature, presence of contaminants) and organisms are almost always exposed to multiple contaminants, using the multi-biomarker approach, as in our research, better reflects real environmental state and presence of certain contaminants (Martínez-Álvarez et al., 2005).

Thus, the overall aim of the present study was to evaluate the potential threats for the organisms and for the protected area of Lonjsko Polje Nature Park by presenting, for the first time, metal cytosolic concentrations and proportions of potentially toxic metal fractions in the intestine of Prussian carp, and by applying multi-biomarker approach to assess the oxidative stress levels. In addition, we have estimated the potential and benefits of applying intestinal tissue as a target bioindicator organ and dietary uptake site and invasive fish species Prussian carp as bioindicator in metal risk assessment.

2. Materials and methods

2.1. Study area and fish sampling

Samplings of Prussian carps (*C. gibelio*) were conducted in the Ilova River in the continental part of Croatia. Lower part of the river watercourse is nowadays known to be threatened by technological (petrochemical processing in fertilizer factory) and municipal (Town of Kutina) wastewaters. Study was performed at two sites (reference and contaminated) and two seasons (autumn 2017 and spring 2018) in order to evaluate the application of fish intestine as a bioindicator tissue in the real environmental conditions. Reference site was located near the Ilova

village and upstream of the Town of Kutina and pollution sources. Contaminated site was located near the Trebež village, about 8 km downstream of the confluence of the Kutinica River that discharges industrial wastewater originating mostly from a fertilizer factory (Radić et al., 2013), and is a part of protected area of Lonjsko Polje Nature Park. Detailed description of the sampling sites was given by Mijošek et al. (2020a). Radić et al. (2013) previously investigated the water contamination at one sampling site of the Ilova River which was located immediately downstream of the Town of Kutina, near the fertilizer factory. They recorded higher values of Fe, Cd, Pb, Cr, Hg, Zn, Cu, and Ni at this site near the factory compared to the reference site, but only concentrations of Pb and Hg were above limits set by WHO (Radić et al., 2013). Mijošek et al. (2020a) revealed that, during the same sampling campaigns as in this study, majority of measured elements were significantly elevated in water near the Trebež village, with Al, As, Cd, Ni and V being the most concerning elements as their concentrations were several times higher in comparison to the Ilova village. Similar trends were confirmed for sediments, where average level of Cd was about 20 times higher at the Trebež village compared to the Ilova village. 2–3 times higher levels at the Trebež village were observed for As, Cu, Ni, Pb, U, V and Zn while other elements were either up to 2 times higher at that site or comparable between the two investigated sites (Mijošek et al., 2020a). Further, contamination and enrichment factors, as well as pollution load indices, calculated for sediment samples, also indicated at least slightly disrupted environmental conditions at the contaminated site near the Trebež village (Mijošek et al., 2020a).

Chosen bioindicator organism, due to the highest abundance in the Ilova River, was the invasive cyprinid fish species Prussian carp (*Carrasius gibelio* Bloch, 1782). In autumn, 20 fish specimens were sampled at each site, while in spring 23 and 20 fish individuals were sampled from the reference and the contaminated site, respectively. As a standard procedure, electro-fishing was used for the fish sampling, following the Croatian standard HRN EN 14011 (2005). Captured fish were kept alive in an aerated water tank for about 2–3 h before further processing.

2.2. Dissection and biometric parameters

All fish were euthanized using freshly prepared anesthetic tricaine methane sulphonate (MS 222, Sigma Aldrich, USA) according to the Ordinance on the protection of animals used for scientific purposes (European Union, 2010). Following the fish sacrifice, total masses and lengths were recorded, and the liver, gonads and posterior part of the intestine were dissected and weighed. Posterior part of the intestinal tissue of each fish was stored at $-80\text{ }^{\circ}\text{C}$ until further analyses. Biometric calculations involved different indices: hepatosomatic index ($\text{HSI} = (\text{LM}/\text{M}) \times 100$; Heidinger and Crawford, 1977), gonadosomatic index ($\text{GSI} = (\text{GM}/\text{M}) \times 100$; Wootton, 1990) and Fulton condition index ($\text{FCI} = (\text{M}/\text{L}^3) \times 100$; Ricker, 1975), where M is the body mass (g), L is the total length (cm), LM is the liver mass (g) and GM is the gonad mass (g).

2.3. Homogenization procedure

Homogenization was performed as already described by Mijošek et al. (2019a,b). Prior to GSH analyses, a piece of intestinal tissue was homogenized in 5 volumes of ice-cold 5% sulfosalicylic acid (SSA) using Potter-Elvehjem homogenizer (Glas-Col, USA) and then centrifuged at $10,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ (Biofuge Fresco, Heraeus, Germany). Another piece of fish intestine used for the analyses of metals and other biomarkers was homogenized in 5 volumes of ice-cold 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at $4\text{ }^{\circ}\text{C}$) supplemented with 1 mM dithiothreitol (DTT, Sigma, USA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, USA) and 0.006 mM leupeptin (Sigma, USA). Samples were homogenized by Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice cooled tube. Appropriate aliquot of each homogenate was separated and set aside for subsequent digestion and analyses of total metal levels (includes insoluble and soluble tissue fractions). The

remaining part of homogenate was centrifuged by Avanti J-E centrifuge (Beckman Coulter, USA) in few steps: supernatant obtained by centrifugation at $3000 \times g$ for 10 min at 4°C was used for MDA analyses, at $10,000 \times g$ for 30 min at 4°C for analyses of CAT activity and lastly at $50,000 \times g$ for 2 h at 4°C for the determination of cytosolic metal concentrations (includes soluble tissue fraction) and MT analyses. Obtained supernatants were all stored at -80°C until analyses.

2.4. Digestion of intestinal tissue fractions and determination of total and cytosolic trace and macro elements concentrations

To prepare the samples for the metal quantitation, intestinal homogenates and cytosols were digested by adding the oxidation mixture (v/v 1:3 for homogenates and v/v 1:1 for cytosols) of concentrated HNO_3 (Rotipur® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany) in the volume ratio of 3:1. Homogenization buffer represented a blank sample and was digested in the same procedure as samples. Digestion procedure was performed in the dry oven (FN 055, Nuve, Turkey) at 85°C for 3.5 h. Following digestions, samples were diluted with Milli-Q water, 1:20 prior to Na, K and Mg analyses and 1:5 prior to Ca and trace element analyses (Dragun et al., 2018; Mijosek et al., 2019b).

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 to determine the trace and macro elements concentrations. Determination of 5 elements (^{82}Se , ^{85}Rb , ^{98}Mo , ^{111}Cd and ^{133}Cs) was operated in low resolution mode; of 11 elements (^{23}Na , ^{24}Mg , ^{42}Ca , ^{51}V , ^{55}Mn , ^{56}Fe , ^{59}Co , ^{60}Ni , ^{63}Cu , ^{66}Zn and ^{86}Sr) in medium resolution mode; and of 2 elements (^{39}K and ^{75}As) in high resolution mode. To correct changes in peak intensities, In ($1 \mu\text{g L}^{-1}$, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was used as an internal standard.

Two external calibrations were performed, one for the macro elements using multielement standard containing Na (1.0 g L^{-1}), K (2.0 g L^{-1}), Mg (0.4 g L^{-1}) and Ca (2.0 g L^{-1}) (Fluka, Germany) and the second calibration using multielement stock standard solution for trace elements (Analitika, Czech Republic) in which standard solution of Cs (Fluka, Germany) and Rb (Sigma-Aldrich, Germany) were added. All standards were prepared in 1.3% HNO_3 (Suprapur; Merck, Germany).

Two quality control samples (QC) obtained from UNEP/GEMS were used to check the accuracy and precision of HR ICP-MS measurements: QC for trace metals (QC trace metals, catalogue no. 8072, lot no. 146142–146143; Burlington, Canada) and QC sample for macro elements (QC minerals, catalogue no. 8052, lot no. 146138–146139; Burlington, Canada). Following recoveries were obtained (%) (based on three measurements in control sample for trace elements and Ca and two measurements for K, Mg and Na): As (94.0 ± 3.7), Ca (95.6 ± 1.2), Cd (94.0 ± 0.8), Co (96.0 ± 1.9), Cu (97.2 ± 2.2), Fe (93.1 ± 4.7), K (95.8 ± 1.2), Mg (90.4 ± 2.5), Mn (93.5 ± 3.7), Na (97.7 ± 1.1), Ni (96.1 ± 0.1), Se (93.9 ± 1.9), Sr (98.2 ± 1.1), V (96.6 ± 1.0) and Zn (97.2 ± 3.6).

Limits of detection (LOD) were calculated as three standard deviations of ten consecutive metal measurements in the blank (homogenization buffer) digested according to the procedure for cytosols. LOD for trace and macro elements were already published by Dragun et al. (2018) and Mijosek et al. (2019b).

Results obtained by measurement in digested homogenates present total metal/metalloid concentrations, while the results obtained for cytosolic fractions represent soluble, cytosolic metal/metalloid concentrations. All concentrations obtained in this study are presented either as $\mu\text{g kg}^{-1}$ or mg kg^{-1} of wet tissue (w.w.) depending on the element. Proportions of total metal/metalloid present in the cytosolic fractions of the intestine of *C. gibelio* were calculated as ratios of cytosolic to total metal concentrations and finally expressed as percentages (%).

2.5. Biomarkers determination

2.5.1. Determination of the MDA concentration – biomarker of oxidative stress

MDA levels were measured by spectrophotometrical method adapted from Botsoglou et al. (1994) and Ringwood et al. (2003). Mixture of 1% butylated hydroxytoluene (BHT, Sigma-Aldrich, USA) dissolved in ethanol and 10% trichloroacetic acid (TCA, Kemika, Croatia) dissolved in Milli-Q water (BHT/TCA = 1:100) was added to the supernatants (S3), which were then put in a refrigerator at 4°C for 15 min and centrifuged at $4000 \times g$ for 15 min at 4°C . In thus obtained supernatants 2-thiobarbituric acid (TBA, Alfa Aesar, Germany) was added and the samples were then heated at 100°C for 30 min. After period of cooling, the absorbance was read at 535 nm wavelength using the spectrophotometer/fluorometer microplate reader Infinite M200 (Tecan, Switzerland). Calibration curve was constructed using 8 different concentrations of MDA (Aldrich, USA) dissolved in 1 N HCl (Kemika, Croatia). Values were expressed as nmol of MDA per gram of wet tissue mass. Detailed description was given by Mijosek et al. (2019b).

2.5.2. Determination of CAT activity and GSH levels – biomarkers of antioxidative capacity

CAT activity was measured according to the spectrophotometrical method of Claiborne (1985). 15.8 mM H_2O_2 , prepared of sodium phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (30%), was added to the ten times diluted samples. Absorbance was read at 240 nm wavelength at 25°C using the spectrophotometer/fluorometer microplate reader Infinite M200 (Tecan, Switzerland). Final CAT activity was expressed as μmol of degraded H_2O_2 per min per mL and calculated using a molar extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$.

Spectrophotometric DTNB-GSSG reductase recycling assay (Tietze, 1969) was used for the determination of total GSH levels. The procedure for the microtiter plate assay was adapted from Rahman et al. (2006). 0.1 M potassium phosphate buffer supplemented with 1 mM EDTA disodium salt, pH 7.5, was used for the preparation of all solutions. Solution containing DTNB (3.79 mM) and glutathione reductase (6 U mL^{-1}) was added to the sample and the mixture was then vortexed and kept in dark for 5 min. Next, NADPH (0.192 mM) solution was added and the absorbance was read for 5 min in 1-min intervals at 412 nm. Calibration curve, used to calculate final GSH concentrations, was made using GSH standards ($3.125\text{--}25 \text{ nmol mL}^{-1}$) which were prepared in 0.5% SSA. The results were expressed as nmol of GSH per g of wet tissue mass.

2.5.3. Determination of MT levels – biomarker of metal exposure

Prior to the electrochemical MT determination, cytosols (S50 fraction) were heat-treated to avoid possible interferences of thermosensitive high molecular mass cytosolic proteins with the electrochemical MT determination. As thermostable proteins, MTs remain in the solution after the heat-treatment. To obtain heat treated supernatants (HT S50), cytosolic fractions were firstly 10 times diluted with 0.9% NaCl (Suprapur®, Merck, Germany) and then heated at 85°C for 10 min in the Dri Block (Technique, UK). Following, samples were placed on the ice for 30 min at 4°C and then centrifuged at $10,000 \times g$ for 15 min at 4°C using Biofuge Fresco centrifuge (Kendro, USA) to get this MT rich fraction (Erk et al., 2002).

MT concentrations were measured in HT S50 by differential pulse voltammetry following the modified Brdicka procedure (Raspor et al., 2001). 797 VA Computrace voltammetric measuring stand (Metrohm, Switzerland) was used, equipped with a three-electrode system (hanging mercury drop electrode, HMDE, as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum counter electrode). Electrolyte solution consisted of 2 M $\text{NH}_4\text{Cl}/\text{NH}_4\text{OH}$ and 1.2×10^{-3} M $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (v/v 1:1), pH = 9.5, and was thermostated to 20°C and purged with the pure nitrogen. Applied measurement parameters were adapted from Mijosek et al. (2018). Straight calibration line, constructed

with the commercially available standard rabbit liver MT-2 (Enzo, USA), dissolved in 0.25 M NaCl, was used for the calculation of MT concentrations which were presented as mg MT g⁻¹ of wet tissue (w.w.).

2.6. Statistical analyses

Main statistical analyses were made in SigmaPlot 11.0 (Systat Software, USA), while Microsoft Office Excel 2007 was used for regular calculations. Considering that assumptions of normality and homogeneity of variance were not always met, Mann-Whitney *U* test was applied to test the significance of differences in metal concentrations and biomarker values in the fish intestine between two seasons and two sites. Differences were regarded as significant at $p < 0.05$. Following the nonparametric analyses, correlation between parameters was tested using Spearman correlation analysis. Data are presented as mean \pm standard deviation (S.D.).

3. Results

3.1. Fish biometry

Spatial differences were evident as higher values of all biometric parameters in fish from the Trebež village compared to the reference site, except of comparable HSI levels in autumn. Trend was even statistically significant for total length in autumn, body mass and FCI in both seasons and GSI in spring (Table 1). Seasonal differences pointed to higher levels of almost all biometric parameters in autumn than spring, being especially striking and significant for HSI at both locations, as well as significant for body mass and FCI in fish from the Ilova village. Exception was GSI which showed higher levels in spring at both locations, but statistically significant only at Trebež village (Table 1).

3.2. Total and cytosolic trace and macro elements concentrations in the fish intestine

The results on total and cytosolic trace and macro element levels in the intestine of *C. gibelio* represent the first data of this kind for this invasive fish species. They are presented in three categories: a) elements with higher concentrations at the contaminated site (Trebež village) (Fig. 1); b) elements with higher concentrations at the reference site (Ilova village) (Fig. 2); c) elements with mostly comparable concentrations at both locations (Fig. 3).

Total metal levels, which represent the combination of both soluble and insoluble metal fraction, as well as cytosolic levels, which only refer to soluble metal fraction, showed various spatial and temporal patterns among measured metals/metalloids. Total concentrations of Cd, Cs and Cu were significantly higher at the contaminated site in both seasons, and of As, Ca, Mg, Na and Rb in only one season (Fig. 1). In addition, cytosolic Ca, Cd, Cs, Cu, Fe and Rb were significantly higher at the contaminated compared to the reference site in both seasons and As, Mg

and Na in one season. Manganese and V were significantly higher at the reference site in autumn and spring considering both fractions (Fig. 2), whereas other elements (Co, K, Mo, Ni, Se, Sr and Zn) had mostly comparable concentrations in both locations (Fig. 3). Seasonal trends were not so clear, but concentrations of total and cytosolic Cd and Cs, with the addition of total K were significantly higher in autumn compared to the spring season in both locations, while the opposite trend was shown significant for total and cytosolic As, Co and Sr, total Mn and Zn and only cytosolic V. Other elements did not show clear pattern (Figs. 1–3).

3.3. Proportions of intestinal trace and macro elements present in the cytosolic fractions

Average proportions of total metal levels present in cytosolic intestinal fraction, i.e. soluble tissue fraction where metals are capable of binding to biologically important molecules, are presented in Table 2. The ratio over 70% was found for Na, K, Rb, Se, Cd, Cs, As and Mo, between 50% and 70% for Mg, Co, Zn, and Sr, while the average proportions of Cu, Ca, Mn, Fe, V and Ni were below 50%.

3.4. Biomarker responses

3.4.1. MDA - biomarker of oxidative stress

MDA levels were significantly higher in fish caught at the location near the Trebež village, showing approximately 2 to 3 times higher average values at contaminated compared to the reference site in autumn and spring, respectively (Fig. 4a). In both seasons, average MDA concentrations were around 40 nmol g⁻¹ w.w. at the Ilova village site and 87–105 nmol g⁻¹ w.w. at the Trebež village (Fig. 4a). Seasonal differences were not significant and did not show clear trend.

3.4.2. CAT and GSH - biomarkers of antioxidative capacity

Regarding CAT activity, significant spatial difference was observed in spring with elevated enzyme activity in fish from the contaminated site. Seasonally, significant difference was observed at the reference site with higher average activity in autumn ($258.8 \pm 45.1 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mL}^{-1}$) compared to spring ($200.8 \pm 51.3 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mL}^{-1}$) (Fig. 4b). There were no significant seasonal differences at the contaminated site, although average CAT activity was slightly higher in spring ($255.5 \pm 63.6 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mL}^{-1}$) compared to autumn ($239.8 \pm 51.6 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mL}^{-1}$).

GSH levels did not show significant and unique season- or site-specific differences. Average value was slightly higher at the reference site compared to the contaminated site in autumn, whereas opposite pattern was visible in spring with higher values at the contaminated location (Fig. 4c). Average values of GSH concentrations ranged from 1109.9 \pm 174.0 nmol g⁻¹ w.w. to 1329.7 \pm 299.1 nmol g⁻¹ w.w. when both locations and seasons are considered (Fig. 4c).

Table 1

Biometric parameters (mean \pm S.D.) of Prussian carp (*Carassius gibelio*) from the Ilova River at the reference (Ilova village) and contaminated site (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).

Location	Season	Total length (cm)	Body mass (g)	GSI (%)	HSI (%)	FCI (g cm ⁻³ × 100)
Ilova village	Autumn 2017 n = 20	16.2 \pm 1.6 ^A	69.82 \pm 23.17 ^{*, A}	3.11 \pm 1.44	5.87 \pm 1.78 [*]	1.59 \pm 0.09 ^{*, A}
	Spring 2018 n = 23	15.9 \pm 2.2	54.57 \pm 21.43 ^{*, A}	5.25 \pm 3.60 ^A	1.44 \pm 0.53 [*]	1.31 \pm 0.10 ^{*, A}
Trebež village	Autumn 2017 n = 20	18.8 \pm 2.9 ^B	122.34 \pm 58.13 ^B	4.67 \pm 2.68 [*]	5.44 \pm 1.52 [*]	1.70 \pm 0.12 ^B
	Spring 2018 n = 20	17.5 \pm 3.9	103.03 \pm 83.00 ^B	7.63 \pm 4.67 ^{*, B}	2.36 \pm 0.77 [*]	1.67 \pm 0.15 ^B

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

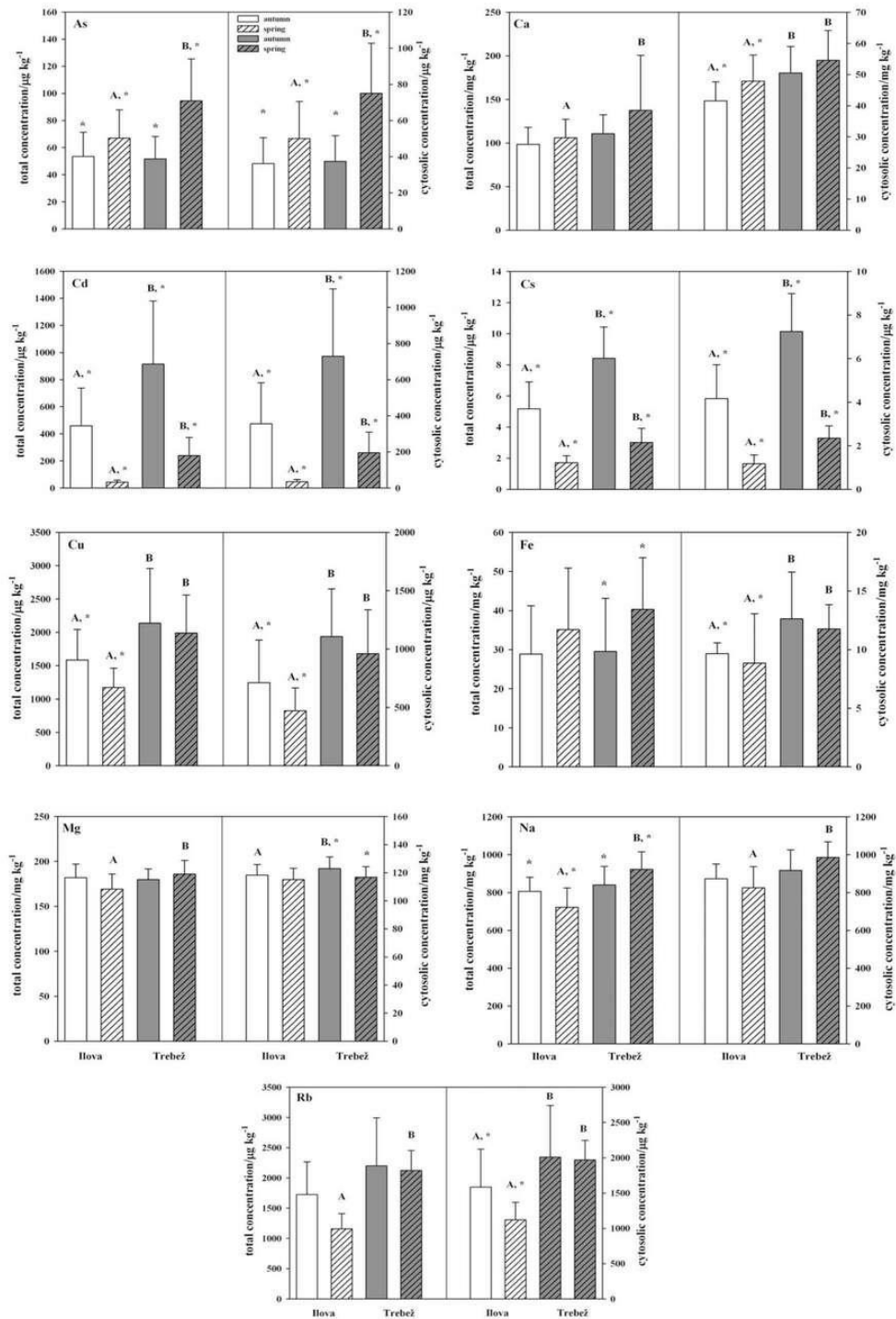


Fig. 1. Total and cytosolic concentrations of nine metals/metalloids in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons that were elevated at the contaminated site. Statistically significant differences (Mann-Whitney U test) at $p < 0.05$ levels 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).

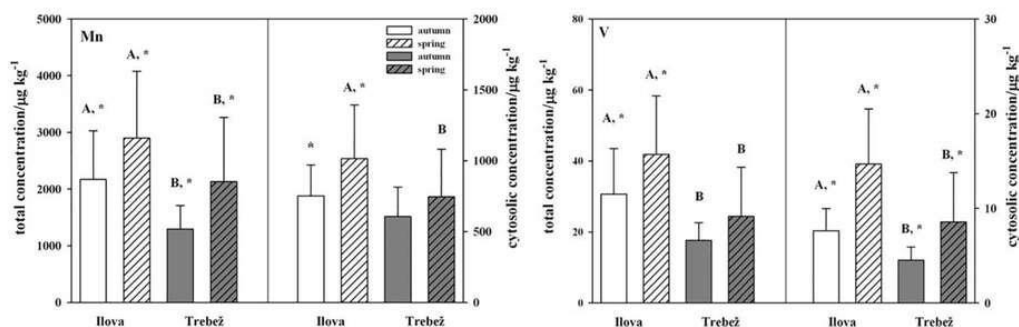


Fig. 2. Total and cytosolic concentrations of two metals in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons that were elevated at the reference site. Statistically significant differences (Mann-Whitney 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).

3.4.3. MT- biomarker of metal exposure

Significant differences in MT concentrations were not observed regarding site or season. However, slightly higher MT induction was evident at the contaminated compared to the reference site in both seasons and in autumn compared to spring at both investigated locations (Fig. 4d). Average values of MT concentrations ranged from 2.13 ± 0.80 mg g^{-1} w.w. to 2.57 ± 0.93 mg g^{-1} w.w. when both locations and seasons are considered.

4. Discussion

4.1. Fish biometry

Significantly higher biometric parameters (TL, TM and FCI) at the contaminated site indicated higher bioavailability of food and nutrients (Lambert and Dutil, 1997) at that site, possibly connected with organic matter sourcing from wastewaters discharged into the river watercourse near the contaminated location. Contrary, in many studies, FCI decline was observed at highly contaminated locations (Lafamme et al., 2000; Rajotte and Couture, 2002; Zhelev et al., 2018). Thus, contamination of the Ilova River evidently did not induce additional defense mechanisms that might cause decreased FCI values. Additionally, these three parameters were higher in autumn than in spring at both investigated locations. Opposite trend was observed for GSI, with higher values in spring which coincides with the spawning period of Prussian carp, occurring from April to July (Sasi, 2008). Due to the high energy demands during the fish reproductive development (Maddock and Burton, 1998), FCI and HSI often show the opposite trend than GSI, resulting in mostly lower values in the spawning periods of different fish species (Farkas et al., 2003; Sabrah et al., 2016; Mijošek et al., 2019b) and of Prussian carp (Leonardos et al., 2008; De Giosa et al., 2014), as also confirmed in our research.

4.2. Total and cytosolic trace and macro elements concentrations in the fish intestine

Intestinal metal levels in fish cytosol represent elements in the soluble tissue fraction which might bind to biologically important biomolecules and therefore potentially can cause toxic effects, whereas total metal concentrations refer to total metal tissue load, including both metabolically available and detoxified fractions. Our results pointed to the differences in total and cytosolic metal concentrations, i.e. higher total concentrations for several metals, but their quite similar spatial patterns (Figs. 1–3).

As studies including fish intestine as target organ in metal pollution assessments are still rare, data on total and cytosolic metal concentrations in the intestinal tissue of Prussian carp represent the first data

of such kind for this invasive fish. Descending order of total trace elements in our research mostly followed the trend: $\text{Zn} > \text{Fe} > \text{Mn} \geq \text{Rb} \geq \text{Cu} > \text{Se} \geq \text{Cd} > \text{Ni} > \text{Sr} > \text{Mo} > \text{As} > \text{Co} > \text{V} > \text{Cs}$ (Figs. 1–3). The highest total concentrations of Fe, Zn, Mn and Cu have already been observed in the intestine of other investigated freshwater fish species: rainbow trout (Dallinger and Kautzky, 1985), perch (Sures et al., 1999), starlet (Jarić et al., 2011), European chub (Filipović Marijić and Raspor, 2012), barbel (Nachev and Sures, 2016), brown trout (Mijošek et al., 2019a) and *Salmo trutta macrostigma* (Yeltekin and Sağlamer, 2019). The descending order of cytosolic elements in the intestine of Prussian carp in our research was $\text{Zn} > \text{Fe} > \text{Rb} > \text{Cu} \geq \text{Mn} > \text{Se} > \text{Cd} > \text{Sr} > \text{Ni} > \text{Mo} \geq \text{As} > \text{Co} > \text{V} \geq \text{Cs}$ (Figs. 1–3), therefore mostly following the same trend as the total metal concentrations and confirming the pattern of $\text{Zn} > \text{Fe} > \text{Cu} > \text{Cd}$ for cytosolic intestinal metals recorded in European chub from the Sava River (Filipović Marijić and Raspor, 2012) and brown trout from the Krka River (Mijošek et al., 2019a, 2019b), both in Croatia. Considering macro elements, descending concentration order was $\text{K} > \text{Na} > \text{Mg} > \text{Ca}$ in all mentioned freshwater fish species.

In order to consider our results in the wider context, we made comparison with the data on metal accumulation in liver, muscle and gills of Prussian carp reported in other studies. Andreji et al. (2006) investigated total metal concentrations in muscles of five fish species from Nitra River (Slovakia), impacted by sewage waters, power plant, chemical factory and lignite mines, and observed pattern for Prussian carp was $\text{Fe} > \text{Zn} > \text{Cu} > \text{Mn} > \text{Cd} > \text{Ni} > \text{Co}$. In gills, liver and muscle of Prussian carp from the agriculturally impacted Marmara Lake (Turkey) descending order of investigated metals was $\text{Cu} > \text{Cd} > \text{Ni} > \text{Cr} > \text{Pb} > \text{Al} > \text{As} > \text{Hg}$ (Yabanli et al., 2014). Finally, Đikanović et al. (2016) compared metal accumulation in liver, muscle and gills of nine fish species from the Meduvrše Reservoir (Serbia) which receives untreated industrial and communal waters, and Prussian carp was shown as the most effective accumulator of most metals and the highest concentrations of Fe and Zn were observed in all tissues.

Metal accumulation in the intestine of Prussian carps was compared with environmental metal concentrations in the water and sediments of the Ilova River and their spatial patterns indicated higher levels of several metals/metalloid in the area near the Trebež village. Cadmium, Cs, Cu and Rb were 2–5 times higher in fish from the contaminated than reference site depending on the season (Fig. 1), while in the water and sediment samples Cd, Cs and Rb were also considerably higher near the Trebež village (Mijošek et al., 2020a). It is already known that Cs and Cd concentration in water is one of the main factors that affect their bioaccumulation in organisms (Rowan and Rasmussen, 1994; Pinder et al., 2011; Dragun et al., 2019). However, concentrations of V in water and sediment samples were also significantly higher at the Trebež village, but that trend was not observed in fish in the present research. Quite the

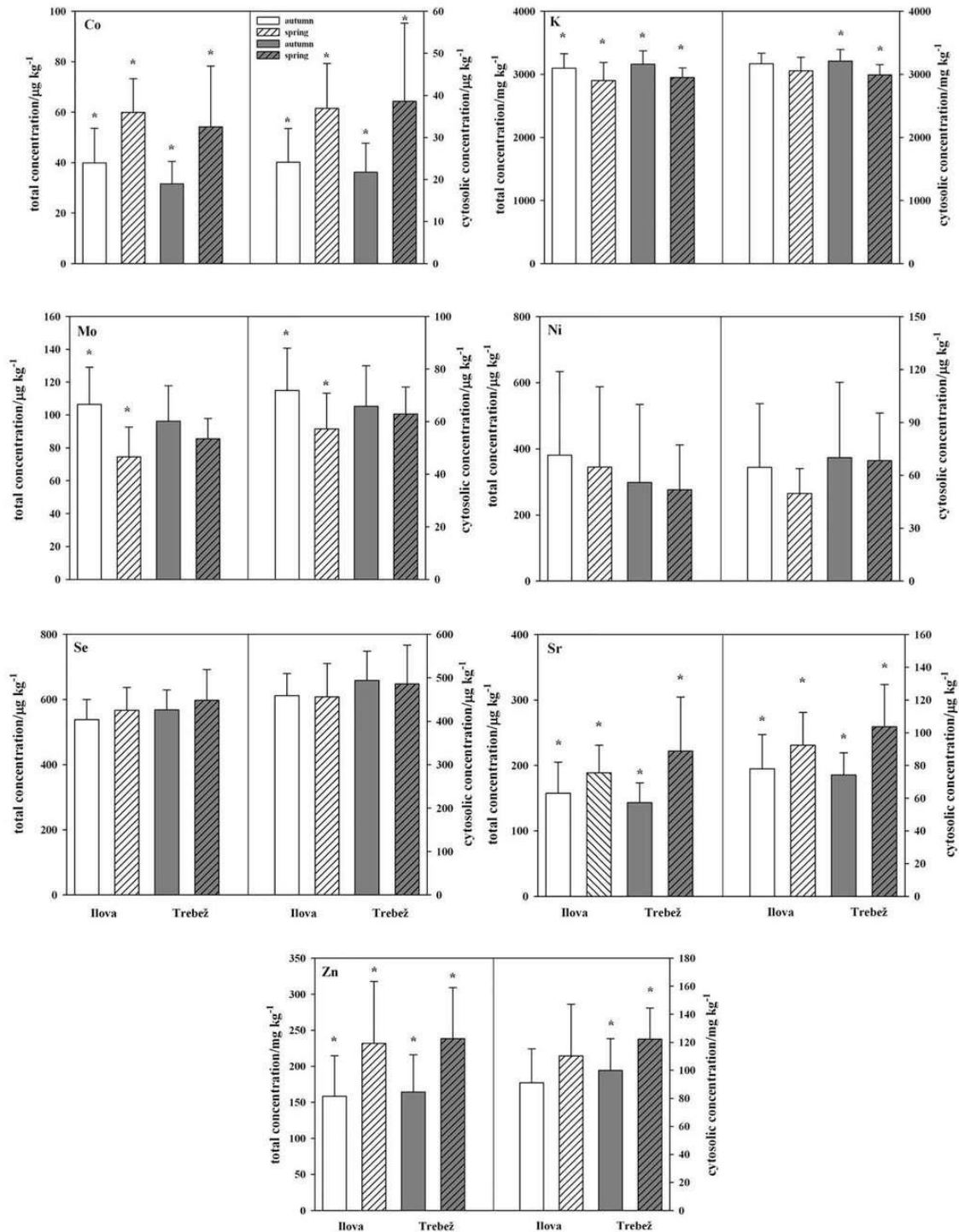


Fig. 3. Total and cytosolic concentrations of seven metals/metalloids in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons with comparable concentrations in both sites. Statistically significant differences (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).

Table 2

Average proportions of total metal/metalloid amounts present in the cytosolic fractions (%) of the intestine of *C. gibelio* from two sampling sites (reference site: Ilova village and contaminated site: Trebež village) and two sampling campaigns (autumn 2017 and spring 2018). The results are presented as mean \pm S.D. of all sites and campaigns, with average range (minima and maxima) within brackets. Metals/metalloids are listed in the descending order.

	Average proportion in cytosols
Na	109.9 \pm 7.2 (107.2–114.9)
K	102.8 \pm 5.7 (101.4–105.9)
Rb	92.9 \pm 5.2 (91.1–96.8)
Se	84.1 \pm 10.2 (80.8–87.2)
Cd	79.9 \pm 5.9 (77.5–82.2)
Cs	79.0 \pm 11.8 (69.3–86.3)
As	72.8 \pm 11.7 (67.4–78.6)
Mo	71.5 \pm 8.0 (67.6–77.4)
Mg	66.3 \pm 4.7 (63.0–68.5)
Co	65.4 \pm 10.7 (61.1–70.6)
Zn	57.1 \pm 12.4 (49.8–62.8)
Sr	50.2 \pm 6.0 (48.4–52.4)
Cu	46.1 \pm 12.4 (38.8–54.0)
Ca	44.2 \pm 5.4 (42.4–46.1)
Mn	39.0 \pm 8.2 (35.5–47.1)
Fe	34.3 \pm 14.1 (22.1–45.5)
V	30.7 \pm 8.8 (26.1–36.2)
Ni	24.7 \pm 13.8 (19.0–30.7)

opposite, V concentrations were even 1.5–2 times higher in the intestine of fish from the reference site (Fig. 2). As Ilova village is also located near the agricultural area, enhanced V accumulation in fish might be the consequence of using fertilizers, herbicides and insecticides which were reported as possible metal sources of variety of metals, including V (Dragun et al., 2011; Ramani et al., 2014). Additionally, V

concentrations in fish might reflected their feeding behavior as diet-borne metal uptake can be of equal or even higher importance than the waterborne metal uptake (Clearwater et al., 2000). Manganese levels were also significantly higher in fish from the reference site (Fig. 2), but in water samples that difference was observed only in autumn (Mijošek et al., 2020a). Hence, further research should involve determination of metal concentrations in fish food as other possible metal source, especially due to intestinal role in food digestion and nutrient absorption. Cobalt, K, Mo, Ni, Se, Sr and Zn had comparable concentrations in the fish intestine from both sites (Fig. 3), which could also not be associated to the environmental exposure from the water and sediments, indicating strong regulative role of essential elements in fish, as already reported for different species in many studies (Olsvik et al., 2000; Monna et al., 2011; Dragun et al., 2019) and possible additional impact of feeding habits on metal levels.

Although seasonal differences in our research were not so pronounced and clear, for more elements elevated total and cytosolic levels were recorded in spring at both investigated locations (Figs. 1–3). Seasonal changes of metal concentrations in fish may result from factors such as fish growth and reproductive cycle, changes in water temperature, pH or seasonal variations of metal exposure from water, food and sediments. The link of metal concentrations and reproductive stage might be due to increased metabolic needs for essential metals such as Fe, Mn and Zn as constitutive part of important biomolecules (Miramand et al., 1991; Filipović Marijić and Raspor, 2010). Therefore, our results on mostly higher metal levels in spring, the spawning period of Prussian carp, might be related to the physiological changes during reproductive period. Similar to our results, during the spawning periods of European chub mostly higher essential metal levels were observed in the gill (Dragun et al., 2007) and intestinal cytosols (Filipović Marijić and Raspor, 2010), as well as in the intestinal cytosols of brown trouts (Mijošek et al., 2019b).

Therefore, the cause of variability of metal content in the fish

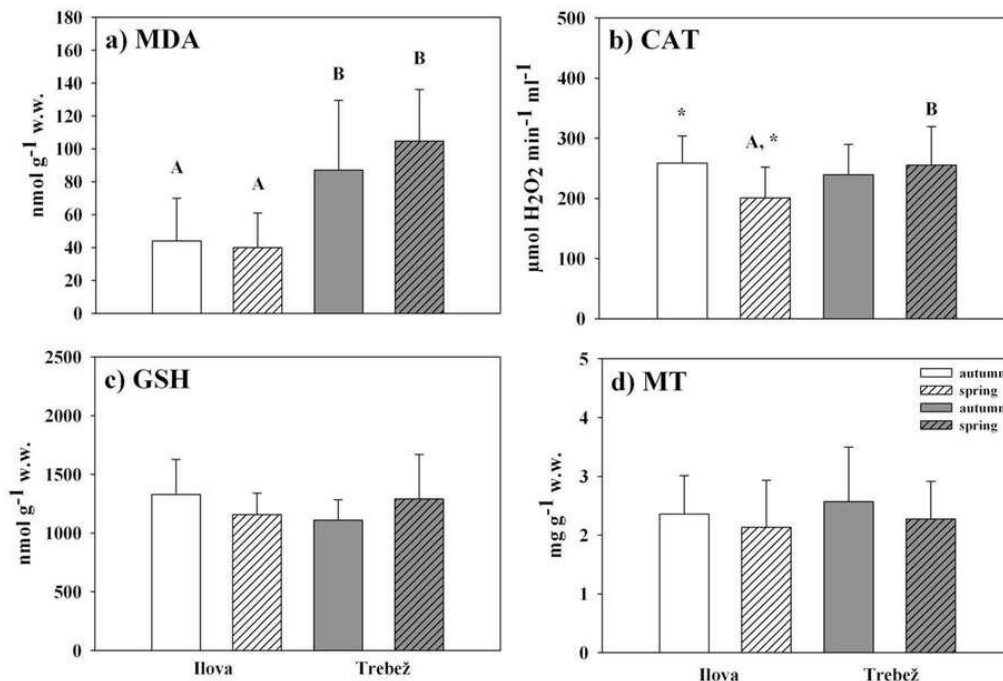


Fig. 4. Biomarker levels (a) MDA; b) CAT; c) GSH; d) MT) in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons (autumn and spring). Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ 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).

intestine cannot be completely explained by waterborne metal uptake or by sediments as alternative metal source so further research should involve determination of metal concentrations in fish food as other possible source since significance of dietary metal uptake has already been shown in variety of freshwater fish species (Clearwater et al., 2000; Lapointe and Couture, 2009; Filipović Marijić and Raspor, 2012; Rajkowska and Protasowicki, 2013).

4.3. Proportions of intestinal trace and macro elements present in the cytosolic fractions of *C. gibelio* intestine

Trace and macro elements cytosolic fraction represent their portions available to bind to physiologically important biomolecules, and therefore, might be potentially toxic (Wallace et al., 2003; Urien et al., 2018). Despite the differences in metal concentrations between the sites, the portions of metabolically available metal contents in the intestine of Prussian carps were mostly comparable at both locations. For Ca, Cu, Fe, Mn, Ni and V < 50% of total metal levels were present in the soluble cytosolic fraction, indicating their higher presence in tissue fraction which is not considered as metabolically available. We have already reported the proportion of Tl in the intestine of the same specimens to be around 40% (Mijošek et al., 2020b). Proportions of the other investigated metals (As, Cd, Co, Cs, K, Mg, Mo, Na, Rb, Se, Sr and Zn) were > 50% in cytosol, where metals have a potential to become toxic by binding to enzymes (metal-sensitive fraction), but can also be detoxified by binding to cytosolic biomolecules, such as metallothioneins (Wallace et al., 2003; Urien et al., 2018). Thus, although As, Cd, Cs, Mo, Rb and Se were present in proportions even higher than 70% (Table 2), such result does not necessarily reflect their toxic levels in organisms because it can also indicate metals detoxified by metallothioneins, which cannot cause harmful effects (Bonneris et al., 2005). As seen in Table 2, K and Na as the main cations responsible for maintaining normal cytosolic osmolarity were completely present in the cytosols.

Cytosolic proportions of intestinal metal levels in other fish species were reported only for brown trout from the karst Krka River (Mijošek et al., 2019a, 2019b) and European chub from the lowland Sava River (Filipović Marijić and Raspor, 2012). The average percentages of analyzed elements present in the soluble, cytosolic intestinal fraction of Prussian carp were comparable to the order found in brown trout: K, Na (>99%) > Se (88%) > Cs (86%) > Cd (84%) > Rb (82%) > Mo (73%) > As (69%) > Mg (67%) > Tl (60%) > Co (56%) > Fe (50%) > Sr (48%) > Zn (47%) > Ca (43%) > V (41%) > Mn (40%) > Cu (38%) > Ni (36%) (Mijošek et al., 2019a, 2019b); and in European chub: Cd (90–100%), Zn (70–80%), Cu (50–80%), Fe and Mn (30–40%) (Filipović Marijić and Raspor, 2012). Therefore, in the intestinal cytosolic fractions of different fish species metals were present in similar proportions, despite considerable differences in their concentrations. Some smaller differences in proportions are probably associated with different physiological characteristics and biology of specific fish species, as well as with different environmental conditions.

The information on cytosolic metal proportions in the intestinal tissues of some other fish species is not available, so we made the short overview of subcellular compartmentalization of metals investigated in other fish tissues. Van Campenhout et al. (2010) investigated cytosolic metal distribution in liver and kidneys of Prussian carps from metal-impacted habitats and revealed high proportion (60–70%) of the total tissue Cd, Cu and Zn concentrations in hepatic, and 50% of total Cd and 30% of total Cu and Zn concentrations in renal cytosols. For other fish species, Rosabal et al. (2015) reported the organelles and metal-sensitive fraction with heat-denaturated proteins (HDP) as main binding site for As in the liver of *Anguilla anguilla* and *Anguilla rostrata*, which indicated high probability of toxic effects. Although present in cytosol in very high proportion, as in our research, it is known that Cd mostly binds to the heat-stable proteins (HSP), metallothioneins, which therefore indicates its high detoxification level and much decreased risk of harmful effects (Kraemer et al., 2006; Rosabal et al., 2015). Although it

was recorded that Se was mostly present in cytosols in the liver of Arctic char *Salvelinus alpinus*, the same amount of Se was found in HSP and fraction containing lysosomes, microsomes and HDP, which suggested that significant part of Se is actually present in detoxified form (Barst et al., 2016). Further, Urien et al. (2018) reported results on subcellular partitioning of As, Cd, Cu, Se and Zn in liver and gonads of wild white suckers (*Catostomus commersonii*) and concluded that As, Cd, and Cu mostly bind to HSPs, contrary to Se and Zn which mostly bind to HDPs, although Zn was also found to be distributed in all the other subcellular fractions, as expected due to its essential role as a co-enzyme in many metabolically important processes (Mason and Jenkins, 1995). Low proportions of Ni in cytosols found in our research are in accordance with results from Rosabal et al. (2015) who reported that granule-like structures have the main role in detoxifying Ni in the liver of European eels. However, opposite results for Ni were also reported, specifically for wild yellow perch, in which hepatic Ni concentrations were the highest in HDP fraction within metal-sensitive fraction (Giguere et al., 2006), which enables possible interaction with physiologically important biomolecules and potential toxic effects of this element.

Hence, elements present in cytosols might only be partially toxic, due to the existing detoxification mechanisms involving their binding to HSPs (MTs and MT-like proteins), especially in conditions of only moderate metal contamination. However, some amount of metals is always found to be bound to metal sensitive HDPs showing that mechanisms of detoxification are not completely efficient. Their presence in cytosols indicates potential threats and possible toxic effects of metals/metalloids for organisms which include blocking functional groups, substitution of essential metals or modification of active sites of important biomolecules (Mason and Jenkins, 1995).

4.4. Biomarker responses

In order to estimate the application of intestine as bioindicator tissue and provide a comprehensive assessment of the environmental quality, multi-biomarker approach was applied. The impact of metal contamination on fish from the Ilova River was investigated to assess the level of oxidative stress in fish by the measurement of MDA, as a manifestation of lipid peroxidation, and of antioxidants (CAT and GSH), as components of antioxidant defense system (van der Oost et al., 2003). In addition, MTs were used as a direct link with metal contamination since these low molecular mass cysteine and metal-rich proteins have significant roles in maintaining the homeostasis of essential trace metals (Zn and Cu), removal of toxic metals (Cd, Ag and Hg) and protection against oxidative damage (Vasaák, 2005).

Increased concentrations of MDA reflect oxidative stress in organisms induced by enhanced production of ROS (Banerjee et al., 1999). In our research, significantly higher intestinal values of MDA were observed in fish at the contaminated site compared to the reference location in both seasons (Fig. 4a). Obtained MDA values ranged from 39.87 to 104.73 nmol g⁻¹ w.w., which is, especially at the reference site of the Ilova River, much lower than MDA levels reported for brown trout from the Krka River (Mijošek et al., 2019b). Although concentrations were not as high as in brown trouts, significant difference between the two sites of the Ilova River suggested that fish were exposed to higher levels of oxidative stress near the Trebež village, which might be linked to mostly elevated metal levels at that site (Mijošek et al., 2020a).

Efficiency of fish antioxidant defense system was tested by analyzing CAT activity and GSH levels. Various responses of CAT activity were observed in animals exposed to metallic contaminants in either field or laboratory experiments depending on the dose, element, the species or the route of exposure (Atli et al., 2006; Tsangaris et al., 2011; Greani et al., 2017; Mijošek et al., 2019b). CAT activity in the intestine of Prussian carp in our research was significantly elevated in fish from the contaminated compared to the reference site in spring, while there were no differences in autumn, possibly associated with the trend of higher metal levels in spring. Although average GSH levels followed the same

patterns as CAT activity, significant differences were not observed (Fig. 4b and 4c). Obtained values of CAT and GSH were either comparable or slightly lower than reported for the intestine of the brown trouts from the Krka River (Mijošek et al., 2019b). To our knowledge, there is no other available literature data on GSH levels in the intestinal tissue of any fish species.

Antioxidants responses induced by pollution vary for different species, enzymes, single or mixed contaminants. The literature data reported on either higher, unchanged or lower activities of antioxidants as the response to pollutant exposure in both laboratory and field studies (van der Oost et al., 2003) and metal caused formation of ROS and damaging tissues (DNA, proteins and lipids) has already been well investigated. For example, Berntssen et al. (2001) showed that exposure of Atlantic salmon to dietary Cu had a direct effect on lipid peroxidation of the intestine even at low concentrations, while Cd induced additional MDA synthesis only at its highest concentration. In the same research, significant differences in GSH levels in the intestine or liver were not observed among fish fed diets containing different Cd concentrations. Considering studies on Prussian carps, Tsangaris et al. (2011) investigated oxidative stress biomarkers in toxicity testing of Ukrainian polluted river waters. They measured oxidative stress biomarkers in *C. gibelio* liver after the 96 h exposure to the river water samples and found out that antioxidant enzymes, including CAT, mostly increased after the exposure, while there were no differences in MDA levels between the exposed and control fish. Further, Liu et al. (2005) reported significant induction of GSH in liver of *Carassius auratus* after Cu exposure. Considering CAT activity, Atli et al. (2006) investigated response to Ag, Cd, Cr, Cu and Zn among five tissues of freshwater fish *Oreochromis niloticus* and increasing concentrations of all elements, except Cu, caused considerable enzyme inhibition in fish intestine. Radić et al. (2013) cage-exposed *Cyprinus carpio* to the water of the Ilova River for 7- and 21-day period and observed a significant increase in lipid peroxidation in both gills and liver as bioindicator tissues, as well as decline in CAT and glutathione reductase (GR) activity in the gills, which suggested a higher sensitivity of gills and earlier failure of the antioxidant system. Correlation analysis in our research confirmed significantly positive relation of As with MDA levels ($r = 0.484$, $p < 0.05$) and with CAT activity ($r = 0.474$, $p < 0.05$) in fish from the contaminated site in autumn, as well as of CAT activity and As at the reference site in spring ($r = 0.535$, $p < 0.05$). Similar relation of oxidative stress with As exposure had already been documented by Bhattacharya and Bhattacharya (2007) who reported induced tissue lipid peroxidation and increased activity of CAT in the liver of *Clarias batrachus* after exposure to As. Greani et al. (2017) also reported significant increase in MDA concentrations in muscles, liver, kidney and fins of exposed fish, as well as an enhanced antioxidants (CAT and SOD) activities, when investigating the effect of chronic As exposure under environmental conditions on oxidative stress in wild trout (*Salmo trutta*). In our research, some of these elements such as Cd, Cu and As were significantly elevated in fish from the contaminated site, therefore, also pointing to their possible role in similar oxidative stress responses.

In addition to biomarkers related to the oxidative stress, metallothioneins (MTs) were used as widely recognized biomarkers of metal exposure. Due to their high affinity to specific metals, they are considered as efficient scavengers of ROS and contribute to the protection against oxidative injuries and many other environmental stressors (Viarengo et al., 1999). Significant MT induction was not observed in our research at any site (Fig. 4d). Comparison to other fish species revealed that average values of 2.0–2.5 mg g⁻¹ w.w. in Prussian carp were higher than in the intestine of the brown trout from the Krka River (0.8–1.5 mg g⁻¹ w.w.) (Mijošek et al., 2019a) and slightly lower than in the intestine of European chub from the Sava River (2.9–3.1 mg g⁻¹ w.w.) (Filipović Marijić and Raspor, 2010). Concentrations of the main MT inducers, Cd, Cu and Zn, were higher in fish from the contaminated than the reference site of the Ilova River (Fig. 1), possibly causing slightly higher average MT levels at that site. However, significant correlation of

MT levels was confirmed only with Zn at the reference site in autumn ($r = 0.509$, $p < 0.05$) and at the contaminated site in spring ($r = 0.506$, $p < 0.05$). MT levels are known to be dependent on numerous factors including season, temperature, pH, age and size, gender, reproductive status (Hylland et al., 1998), making it difficult to distinguish the exact cause of MT induction.

Thus, all biomarkers, to a higher or lesser degree, indicated exposure to higher levels of oxidative stress at the contaminated site, but cell antioxidant system seems to work in fish from both locations without significant decrease in levels of CAT, GSH or enhanced induction of MTs, which are usually caused by high metal concentrations in highly polluted environments. Therefore, biomarker responses in the intestinal tissue confirmed only moderate level of contamination of the studied freshwater system and indicated that fish intestine reflects environmental conditions (Mijošek et al., 2020a).

5. Conclusions

Prussian carp itself was shown as a suitable bioindicator species in aquatic environmental pollution assessment and the intestine as suitable bioindicator organ, which reflects responses to contaminants from dietary pathways. Special significance of the presented data is that they are the first data on distribution of 18 elements and on the levels of oxidative stress biomarkers in the intestine of Prussian carp, which can serve as a basis for comparison in future monitoring programmes. Total and cytosolic concentrations of many trace and macro elements, as well as some biomarker responses in the intestine of Prussian carp pointed to more disturbed environmental conditions at the contaminated site of the investigated ecosystem, the Ilova River. Presence of many analyzed elements in the cytosolic intestinal fractions was over 50%, therefore pointing that majority of metals/metalloids in the intestine can potentially cause toxic effects. Although a portion of metals present in the cytosol is still expected to be detoxified by MTs, there is a significant possibility of harmful effects of these elements for the organisms by binding to biologically important molecules such as enzymes.

Although obtained biological changes mostly indicated moderate pollution impact, significantly higher concentrations of elements such as As, Cd, Cs, Cu and Fe in combination with higher levels of oxidative stress (MDA) in fish from the contaminated site near the Trebež village highlighted the need of regular monitoring of the water quality and aquatic organisms of this region, especially knowing that this location is a part of protected wetland area of Lonjsko Polje Nature Park.

Due to its high spread potential and the fact that Prussian carp is already, naturally or introduced, a widely spread species in Europe, this data present an important contribution to the future monitoring and preservation of European freshwater systems and serve as an good example of using invasive, instead of and along with native fish species in ecotoxicological and biomonitoring studies.

CRedit authorship contribution statement

Tatjana Mijošek: Investigation, Resources, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Vlatka Filipović Marijić:** Investigation, Writing - review & editing, Validation, Resources, Funding acquisition, Supervision. **Zrinka Dragun:** Investigation, Validation, Writing - review & editing. **Dušica Ivanković:** Investigation, Writing - review & editing. **Nesrete Krasnići:** Investigation. **Zuzana Redžović:** Investigation, Resources. **Marijana Erk:** Funding acquisition, Project administration, Resources, Writing - review & editing, Supervision.

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.

Acknowledgments

This work was supported by the Croatian Science Foundation, within the project "Accumulation, subcellular mapping and effects of trace metals in aquatic organisms" AQUAMAPMET (IP-2014-09-4255). Authors are also grateful for the help in the sampling to the members of the Laboratory for Aquaculture and Pathology of Aquatic Organisms from the Ruder Bošković Institute.

References

- Andreji, J., Stranai, I., Massanyi, P., Valent, M., 2006. Accumulation of some metals in muscles of five fish species from lower nitra river. *J. Environ. Sci. Health, Part A* 41 (11), 2607–2622. <https://doi.org/10.1080/10934520600928003>.
- Atli, G., Alptekin, Ö., Tükel, S., Canli, M., 2006. Response of catalase activity to Ag⁺, Cd²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺ in five tissues of freshwater fish *Oreochromis niloticus*. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 143 (2), 218–224. <https://doi.org/10.1016/j.cbpc.2006.02.003>.
- Banerjee, B.D., Seth, V., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K., 1999. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicol. Lett.* 107 (1–3), 33–47. [https://doi.org/10.1016/S0378-4274\(99\)00029-6](https://doi.org/10.1016/S0378-4274(99)00029-6).
- Barst, B.D., Rosabal, M., Campbell, P.G.C., Muir, D.G.C., Wang, X., Köck, G., Drevnick, P. E., 2016. Subcellular distribution of trace elements and liver histology of landlocked Arctic char (*Salvelinus alpinus*) sampled along a mercury contamination gradient. *Environ. Pollut.* 212, 574–583. <https://doi.org/10.1016/j.envpol.2016.03.003>.
- Berntsen, M.H.G., Aspholm, O., Hylland, K., Wendelaar Bonga, S.E., Lundebye, A.-K., 2001. Tissue metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo salar* L.) parr fed elevated dietary cadmium. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 128 (3), 299–310. [https://doi.org/10.1016/S1532-0456\(00\)00204-0](https://doi.org/10.1016/S1532-0456(00)00204-0).
- Bhattacharya, A., Bhattacharya, S., 2007. Induction of oxidative stress by arsenic in *Clarias batrachus*: involvement of peroxisomes. *Ecotoxicol. Environ. Saf.* 66 (2), 178–187. <https://doi.org/10.1016/j.ecoenv.2005.11.002>.
- Bonneris, E., Giguère, A., Perceval, O., Buronfosse, T., Masson, S., Hare, L., Campbell, P. G.C., 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. *Aquat. Toxicol.* 71 (4), 319–334. <https://doi.org/10.1016/j.aquatox.2004.11.025>.
- Botsoglou, N.A., Fletouris, D.J., Papageorgiou, G.E., Vassilopoulos, V.N., Mantis, A.J., Trakatellis, A.G., 1994. A rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissues, food, and feeds tuff samples. *J. Agric. Food Chem.* 42, 1931–1937. <https://doi.org/10.1021/jf00045a019>.
- Claiborne, A., 1985. Catalase activity. In: Greenbook, R.A. (Ed.), *CRC Handbook of Methods for Oxygen Radical Research*. CRC Press, Boca Raton FL, pp. 283–284.
- Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. *J. Exp. Biol.* 203, 2455–2466.
- 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 (1), 82–89. <https://doi.org/10.1007/BF00378455>.
- De Boeck, G., Meus, W., Coen, W.D., Blust, R., 2004. Tissue-specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). *Aquat. Toxicol.* 70 (3), 179–188. <https://doi.org/10.1016/j.aquatox.2004.07.001>.
- De Giosa, M., Czerniejewski, P., Rybzyk, A., 2014. Seasonal changes in condition factor and weight-length relationship of invasive *Carassius gibelio* (Bloch, 1782) from Leszczynskie Lakeland, Poland. *Adv. Zool.* 2014, 1–7. <https://doi.org/10.1155/2014/678763>.
- Dragun, Z., Raspor, B., Podrug, M., 2007. The influence of the season and the biotic factors on the cytosolic metal concentrations in the gills of the European chub (*Leuciscus cephalus* L.). *Chemosphere* 69 (6), 911–919. <https://doi.org/10.1016/j.chemosphere.2007.05.069>.
- Dragun, Z., Kapetanović, D., Raspor, B., Teskeredžić, E., 2011. Water quality of medium size watercourse under baseflow conditions: the case study of River Sutla in Croatia. *Ambio* 40 (4), 391–407. <https://doi.org/10.1007/s13280-010-0119-z>.
- Dragun, Z., Filipović Marijić, V., Krasnići, N., Ivanković, D., Valić, D., Žunić, J., Kapetanović, D., Snurlić, I.V., 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. *Ecotoxicol. Environ. Saf.* 147, 537–549. <https://doi.org/10.1016/j.ecoenv.2017.09.005>.
- Dragun, Z., Tepić, N., Ramani, S., Krasnići, N., Filipović Marijić, V., Valić, D., Kapetanović, D., Erk, M., Rebok, K., Kostov, V., Jordanova, M., 2019. Mining waste as a cause of increased bioaccumulation of highly toxic metals in liver and gills of Vardar chub (*Squalius vardarensis* Karaman, 1928). *Environ. Pollut.* 247, 564–576. <https://doi.org/10.1016/j.envpol.2019.01.068>.
- Dikanović, V., Skorić, S., Gacić, Z., 2016. Concentrations of metals and trace elements in different tissues of nine fish species from the Meduvrje Reservoir (West Morava River Basin, Serbia). *Arch. Biol. Sci.* 68 (4), 811–819.
- Erk, M., Ivanković, D., Raspor, B., Pavičić, J., 2002. Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins. *Talanta* 57 (6), 1211–1218. [https://doi.org/10.1016/S0039-9140\(02\)00239-4](https://doi.org/10.1016/S0039-9140(02)00239-4).
- 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).
- Falfushynska, H.I., Gnatyshyna, L.L., Stoliar, O.B., Nam, Y.K., 2011. Various responses to copper and manganese exposure of *Carassius auratus gibelio* from two populations. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 154 (3), 242–253. <https://doi.org/10.1016/j.cbpc.2011.06.001>.
- Farkas, A., Salánki, J., Specziár, A., 2003. Age- and size-specific patterns of heavy metals in the organs of freshwater fish *Abramis brama* L. populating a low-contaminated site. *Water Res.* 37 (5), 959–964. [https://doi.org/10.1016/S0043-1354\(02\)00447-5](https://doi.org/10.1016/S0043-1354(02)00447-5).
- Filipović Marijić, V., Raspor, B., 2010. The impact of the fish spawning on metal and protein levels in gastrointestinal cytosol of indigenous European chub. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 152, 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 Pollut.* 223 (8), 4769–4783. <https://doi.org/10.1007/s11270-012-1233-2>.
- Giguère, A., Campbell, P., 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. *Aquat. Toxicol.* 77 (2), 178–189. <https://doi.org/10.1016/j.aquatox.2005.12.001>.
- Greani, S., Lourkisti, R., Berti, L., Marchand, B., Giannettini, J., Santini, J., Quilichini, Y., 2017. Effect of chronic arsenic exposure under environmental conditions on bioaccumulation, oxidative stress, and antioxidant enzymatic defenses in wild trout *Salmo trutta* (Pisces, Teleostei). *Ecotoxicology* 26 (7), 930–941. <https://doi.org/10.1007/s10646-017-1822-3>.
- Has Schöön, E., Bogut, I., Rajković, V., Bogut, S., Čačić, M., Horvatić, J., 2008. Heavy Metal Distribution in Tissues of Six Fish Species Included in Human Diet, Inhabiting Freshwaters of the Nature Park "Hutovo Blato" (Bosnia and Herzegovina). *Arch. Environ. Contam. Toxicol.* 54 (1), 75–83. <https://doi.org/10.1007/s00244-007-9008-2>.
- Heidinger, R.C., Crawford, S.D., 1977. Effect of temperature and feeding rate on the livensomatic index of largemouth bass, *Micropterus salmoides*. *J. Fish. Res. Board Can.* 34, 633–638. <https://doi.org/10.1139/f77-099>.
- HRN EN 14011, 2005. Fish sampling by electric power (In Croatian). Croatian Standard Institute, Zagreb.
- Hylland, K., Nissen-Lie, T., Christensen, P.G., Sandvik, M., 1998. Natural modulation of hepatic metallothionein and 584 cytochrome P4501A in flounder, *Platichthys flesus* L. *Mar. Environ. Res.* 46, 51–55.
- Jarić, I., Višnjić-Jeftić, Z., Cvijanović, G., Gacić, Z., Jovanović, L.J., Skorić, S., Lenhardt, M., 2011. Determination of differential heavy metal and trace element accumulation in liver, gills, intestine and muscle of starlet (*Acipenser ruthenus*) from the Danube River in Serbia by ICP OES. *Microchem. J.* 98, 77–81. <https://doi.org/10.1016/J.MICROC.2010.11.008>.
- Kraemer, L.D., Campbell, P.G.C., Hare, L., 2006. Seasonal variations in hepatic Cd and Cu concentrations and in the sub-cellular distribution of these metals in juvenile yellow perch (*Perca flavescens*). *Environ. Pollut.* 142 (2), 313–325. <https://doi.org/10.1016/j.envpol.2005.10.004>.
- Laflamme, J.S., Couillard, Y., Campbell, P.G.C., Hontela, A., 2000. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. *Can. J. Fish. Aquat. Sci.* 57, 1692–1700. <https://doi.org/10.1139/f00-118>.
- 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*)? *Can. J. Fish. Aquat. Sci.* 54, 104–112. <https://doi.org/10.1139/cjfas-54-1-104>.
- 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*). *Arch. Environ. Contam. Toxicol.* 57, 571–580. <https://doi.org/10.1007/s00244-009-9298-7>.
- Leonardos, I.D., Tsikliras, A.C., Eleftheriou, V., Cladas, Y., Kagalou, I., Chortatou, R., Papigiotti, O., 2008. Life history characteristics of an invasive cyprinid fish (*Carassius gibelio*) in Chimaditis Lake (northern Greece). *J. App. Ichthyol.* 24 (7), 213–217. <https://doi.org/10.1111/j.1439-0426.2007.01031.x>.
- Liu, H., Zhang, J.F., Shen, H., Wang, X.R., Wang, W.M., 2005. Impact of copper and its EDTA complex on the glutathione-dependent antioxidant system in freshwater fish (*Carassius auratus*). *Bull. Environ. Contam. Toxicol.* 74, 1111–1117. <https://doi.org/10.1007/s00128-005-0696-x>.
- Maddock, D.M., Burton, M.P.M., 1999. Gross and histological observations of ovarian development and related condition changes in American plaice. *J. Fish Biol.* 53, 928–944. <https://doi.org/10.1111/j.1095-8649.1998.tb00454.x>.
- Martínez-Álvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish: biotic and abiotic factors. *Rev. Fish Biol. Fisheries* 15 (1–2), 75–88. <https://doi.org/10.1007/s11160-005-7846-4>.
- Mason, A.Z., Jenkins, K.D., 1995. Metal detoxification in aquatic organisms. In: Tessier, A., Turner, D. (Eds.), *Metal Speciation and Bioavailability in Aquatic Systems*. Wiley & Sons, Chichester, UK, pp. 479–608.
- Mendiguchia, C., Moreno, C., García-Vargas, M., 2007. Evaluation of natural and anthropogenic influences on the Guadalquivir River (Spain) by dissolved heavy metals and nutrients. *Chemosphere* 69 (10), 1509–1517. <https://doi.org/10.1016/j.chemosphere.2007.05.082>.
- Mijosek, T., Erk, M., Filipović Marijić, V., Krasnići, N., Dragun, Z., Ivanković, D., 2018. Electrochemical determination of metallothioneins by the modified Brdicka procedure as an analytical tool in biomonitoring studies. *Croat. Chem. Acta* 91 (4), 475–480. <https://doi.org/10.5562/cca3444>.

- Mijosek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Erk, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matonić 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. *Ecol. Ind.* 105, 188–198. <https://doi.org/10.1016/j.ecolind.2019.05.069>.
- Mijosek, 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. *Sci. Total Environ.* 660, 1079–1090. <https://doi.org/10.1016/j.scitotenv.2019.01.045>.
- Mijosek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Sertić Perić, M., Vdović, N., Bačić, N., Dautović, J., Erk, M., 2020a. The assessment of metal contamination in water and sediments of the lowland Ilova River (Croatia) impacted by anthropogenic activities. *Environ. Sci. Pollut. Res.* 27 (20), 25374–25389. <https://doi.org/10.1007/s11356-020-08926-7>.
- Mijosek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Veseli, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matonić Kepčija, R., Erk, M., 2020b. Thallium accumulation in different organisms from karst and lowland rivers of Croatia under wastewater impact. *Environ. Chem.* 17 (2), 201–212. <https://doi.org/10.1071/EN19165>.
- Milošević, A., Simić, V., 2015. Arsenic and other trace elements in five edible fish species in relation to fish size and weight and potential health risks for human consumption. *Pol. J. Environ. Stud.* 24, 199–206. <https://doi.org/10.15244/pjoes/24929>.
- Miramanand, P., Lafaurie, M., Fowler, S.W., Lemaire, P., Guary, J.C., Bentley, D., 1991. Reproductive cycle and heavy metals in the organs of brown trout, *Mullus barbatus* (L), from the northwestern Mediterranean. *Sci. Total Environ.* 103, 47–56. [https://doi.org/10.1016/0048-9697\(91\)90352-F](https://doi.org/10.1016/0048-9697(91)90352-F).
- Monna, F., Camizuli, E., Revelli, P., Biville, C., Thomas, C., Losno, R., Scheffler, R., Brugniere, O., Baron, S., Chateau, C., Ploquin, A., Alibert, P., 2011. Wild brown trout affected by historical mining in the Cévennes National Park, France. *Environ. Sci. Technol.* 45 (16), 6823–6830. <https://doi.org/10.1021/es200755n>.
- 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. *Parasit. Vectors* 9, 300–308. <https://doi.org/10.1186/s13071-016-1576-4>.
- Olsvik, P.A., Gundersen, P., Andersen, R.A., Zachariassen, K.E., 2000. Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a run-off episode. *Aquat. Toxicol.* 50, 301–316.
- Pinder III, J.E., Hinton, T.G., Taylor, B.E., Whicker, F.W., 2011. Cesium accumulation by aquatic organisms at different trophic levels following an experimental release into a small reservoir. *J. Environ. Radioact.* 102 (3), 283–293. <https://doi.org/10.1016/j.jenvrad.2010.12.003>.
- Pinto, E., Sigaud-Kutner, T.C.S., Leitão, M.A.S., Okamoto, O.S., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. *J. Phycol.* 39, 1008–1018.
- Radić, S., Gregorović, G., Stipanović, D., Cvjetko, P., Šrut, M., Vujčić, V., Oreščanin, V., Vinko Klobučar, G.L., 2013. Assessment of surface water in the vicinity of fertilizer factory using fish and plants. *Ecotoxicol. Environ. Saf.* 96, 32–40. <https://doi.org/10.1016/j.ecoenv.2013.06.023>.
- Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* 1 (6), 3159–3165. <https://doi.org/10.1038/nprot.2006.378>.
- Rajkowska, M., Protasowicki, M., 2013. Distribution of metals (Fe, Mn, Zn, Cu) in fish tissues in two lakes of different trophy in Northwestern Poland. *Environ. Monit. Assess.* 185 (4), 3493–3502. <https://doi.org/10.1007/s10661-012-2805-8>.
- Rajotte, J.W., Couture, P., 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* 59, 1296–1304. <https://doi.org/10.1139/F02-095>.
- Ramani, S., Dragun, Z., Kapetanović, D., Kostov, V., Jordanova, M., Erk, M., Hajrulai-Mušliu, Z., 2014. Surface water characterization of three rivers in the lead/zinc mining region of Northeastern Macedonia. *Arch. Environ. Contam. Toxicol.* 66 (4), 514–528. <https://doi.org/10.1007/s00244-014-0012-z>.
- Raspor, B., Paić, M., Erk, M., 2001. Analysis of metallothioneins by the modified Brdička procedure. *Talanta* 55, 109–115. [https://doi.org/10.1016/S0039-9140\(01\)00399-X](https://doi.org/10.1016/S0039-9140(01)00399-X).
- Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. *B. Fish. Res. Board Can.* 191, 1–382.
- Ringwood, A.H., Hogue, J., Keppler, C.J., Gielazyn, M.L., Ward, B.P., Rourk, A.R., 2003. Cellular biomarkers (lysosomal destabilization, glutathione & lipid peroxidation). Three common estuarine species: a methods handbook. Marine Resources Research Institute South Carolina Department of Natural Resources, pp. 1–45.
- Rosabal, M., Pierron, F., Couture, P., Baudrimont, M., Hare, L., Campbell, P.G., 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. *Aquat. Toxicol.* 160, 128–141.
- Rowan, D.J., Rasmussen, J.B., 1994. Bioaccumulation of radiocesium by fish: the influence of physicochemical factors and trophic structure. *Can. J. Fish. Aquat. Sci.* 51, 2388–2410.
- Sabrah, M.M., Mohamedein, L.I., El-Sawy, M.A., Abou El-Naga, E.H., 2016. Biological characteristics in approaching to biochemical and heavy metals of edible fish *Terapon puta*, Cuvier, 1829 from different fishing sites along the Suez Canal, Egypt. *J. Fish. Aquat. Sci.* 11, 147–162. <https://doi.org/10.3923/jfas.2016.147.162>.
- 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). *Turk. J. Fish. Aquat. Sci.* 8, 87–92.
- Staniskiene, B., Matusевичius, P., Budreckiene, R., Skibniewska, K.A., 2006. Distribution of heavy metals in tissues of freshwater fish in Lithuania. *Pol. J. Environ. Stud.* 15, 585–591.
- Sures, B., Steiner, W., Rydlo, M., Taraschewski, H., 1999. Concentrations of 17 elements in the zebra mussel (*Dreissena polymorpha*), in different tissues of perch (*Perca fluviatilis*), and in perch intestinal parasites (*Acanthocephalus lucii*) from the subalpine lake Mondsee, Austria. *Environ. Toxicol. Chem.* 18 (11), 2574–2579. <https://doi.org/10.1002/etc.5620181126>.
- Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27 (3), 502–522. [https://doi.org/10.1016/0003-2697\(69\)90064-5](https://doi.org/10.1016/0003-2697(69)90064-5).
- Tsangaris, C., Vergolyas, M., Fountoulaki, E., Goncharuk, V.V., 2011. Genotoxicity and oxidative stress biomarkers in *Carassius gibelio* as endpoints for toxicity testing of Ukrainian polluted river waters. *Ecotoxicol. Environ. Saf.* 74 (8), 2240–2244. <https://doi.org/10.1016/j.ecoenv.2011.08.010>.
- Urien, N., Cooper, S., Caron, A., Sonnenberg, H., Rozon-Ramilo, L., Campbell, P.G.C., Couture, P., 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. *Aquat. Toxicol.* 202, 105–116. <https://doi.org/10.1016/j.aquatox.2018.07.001>.
- Van Campenhout, K., Goenaga Infante, H., Hoff, P.T., Moens, L., Goemans, G., Belpaire, C., Adams, F., Blust, R., Bervoets, L., 2010. Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (*Carassius auratus gibelio*) from metal-impacted habitats. *Ecotoxicol. Environ. Saf.* 73 (3), 296–305. <https://doi.org/10.1016/j.ecoenv.2009.10.007>.
- van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* 13 (2), 57–149. [https://doi.org/10.1016/S1382-6689\(02\)00126-6](https://doi.org/10.1016/S1382-6689(02)00126-6).
- Vasák, M., 2005. Advances in metallothionein structure and functions. *J. Trace Elem. Med. Biol.* 19 (1), 13–17. <https://doi.org/10.1016/j.jtemb.2005.03.003>.
- Viarengo, A., Burlando, B., Dondero, F., Marro, A., Fabri, R., 1999. Metallothionein as a tool in biomonitoring programmes. *Biomarkers* 4, 455–466. <https://doi.org/10.1080/135475099230615>.
- Wallace, W.G., Lee, B.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol. Prog. Ser.* 249, 183–197. <https://doi.org/10.3354/meps249183>.
- Wootton, R.J., 1990. Ecology of teleost fishes. Chapman and Hall, Fish and Fisheries Series 1. London, New York. https://doi.org/10.1007/978-94-009-0829-1_9 (404 pp).
- Yabanli, M., Yozukmaz, A., Sel, F., 2014. Bioaccumulation of heavy metals in tissues of the gibel carp *Carassius gibelio*: example of Marmara Lake, Turkey. *Russ. J. Biol. Invasions* 5 (3), 217–224. <https://doi.org/10.1134/S2075111714030126>.
- Yeltek, A.C., Sağlamer, E., 2019. Toxic and trace element levels in *Salmo trutta macrostigma* and *Oncorhynchus mykiss* trout raised in different environments. *Pol. J. Environ. Stud.* 28 (3), 1613–1621. <https://doi.org/10.15244/PJOES/90620>.
- Zhelev, Zh.M., Tsonev, S.V., Boyadziev, P.S., 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 Zool. Bulg.* 70 (4), 547–556.
- Zhelyazkov, G. I., Georgiev, D.M., Peeva, S.P., Kalcheva, S.E., Georgieva, K.Y., 2018. Chemical composition and levels of heavy metals in fish meat of the Cyprinidae family from Zhrebchevo Dam, Central Bulgaria. *Ecol. Balk.* 10, 133–140.

4. RASPRAVA

Neprestani porast onečišćenja uslijed ispuštanja organskih i anorganskih zagađivala koja potječu od antropogenih aktivnosti, poput industrijske proizvodnje, poljoprivrede, iskapanja ruda ili prometa, predstavlja jedan od vodećih problema za vodene ekosustave. Prekomjerni unos zagađivala u vodene ekosustave može imati toksičan učinak na biotu te utjecati i na bioraznolikost i funkcioniranje čitavog ekosustava. Značajno mjesto među zagađivalima zauzimaju metali koji se ne mogu ukloniti iz biogeokemijskog kruženja te se trajno zadržavaju u okolišu i akumuliraju u organizmima. Iako neki imaju i esencijalne uloge u organizmu, kao dio enzima i važnih bioloških molekula, i oni u previsokim koncentracijama uzrokuju toksične učinke na različitim sinekološkim razinama, od stanica, preko organizama pa sve do razine populacije, što posljedično može utjecati i na zdravlje ljudi koji vodene organizme, poput riba, školjkaša ili rakova, koriste u prehrani (Livingstone, 1993). Stoga je uz ispitivanje samih okolišnih uvjeta putem mjerenja koncentracija metala u vodi i/ili sedimentu važno procijeniti i utjecaj onečišćenja metalima na bioindikatorske organizme.

U ovom istraživanju su kao bioindikatorski organizmi odabrane dvije vrste riba (potočna pastrva, *S. trutta* Linnaeus, 1758; i babuška *C. gibelio* Bloch, 1782), rakušci roda *Gammarus* i *Echinogammarus* (*G. balcanicus* Schäferna, 1922; *E. acarinatus* (Karaman, 1931); *G. fossarum* Koch, 1936; i *G. roeselii* Gervais, 1835) te kukaši (*D. truttiae* Sinzar, 1955), nametnici u probavilu riba. Probavilo riba odabrano je kao indikatorsko tkivo zbog važnosti unosa metala hranom te nedostatka podataka o unosu metala probavnim putem u riba u okolišnim uvjetima, a kukaši kao organizmi s visokom učinkovitošću akumulacije metala (Sures, 2004; Filipović Marijić i sur., 2013; Nachev i Sures, 2016) te obećavajući bioindikatorski organizmi u biomonitoring studijama. Kako bi bili uključeni svi organizmi uključeni u životni ciklus kukaša, korišteni su i rakušci kao njihovi međudomadari.

Budući da toksičnost metala potječe od reakcija u citosolu, putem vezanja na fiziološki važne molekule, uz ukupne koncentracije metala u probavnom tkivu riba mjerene su i citosolske koncentracije koje daju više informacija o bioraspoloživosti, potencijalnim toksičnim učincima metala, kao i njihovoj detoksikaciji (Wallace i Luoma, 2003). Kako navedeni procesi ovise prije svega o vezanju metala na stanične biomolekule, važno je i opisati i odrediti raspone molekulskih masa biomolekula koje vežu metale u probavilu navednih vrsta riba. Nadalje, vezanje metala na osjetljive biomolekule mijenja njihovu funkciju, strukturu i/ili dinamiku u stanicama te smo stoga koristili stanične biomarkere, koji su definirani kao prve mjerljive promjene i biokemijski odgovori u organizmima izloženim

onečišćenju (Phillips i Rainbow, 1993). Kako bi se okolišni uvjeti procijenili što realnije, koristili smo multibiomarkerski pristup koji omogućava procjenu izloženosti različitim okolišnim zagađivačima, čak i pri niskim razinama (Cravo i sur., 2009).

Opisane analize metala i biomarkera u mekim tkivima riba, a u ovome istraživanju u probavilu, pokazatelji su nedavne izloženosti na koje uz okolišne uvjete utječu i fiziologija vrste, procesi detoksikacije, metaboličke transformacije i eliminacije (Bath i sur., 2000) te praćenje akumulacije metala i bioloških odgovora zahtijeva učestala uzorkovanja. Kao pokazatelji dugotrajne izloženosti onečišćenju i metalima mogu se koristiti kalcificirane strukture, poput ljusaka i otolita, za koje je specifično da daju podatak o izloženosti tijekom čitavog vijeka riba (Campana, 1999; Tzadik i sur., 2017). Uz probavilo i tvrda tkiva, koncentracije metala određene su i u kukašima i rakušcima kako bi se dobili novi i vrijedni podaci za učinkovitost akumulacije metala u vrsti *D. truttiae* za koju ne postoje podaci u znanstvenoj literaturi, iako su sami kukaši ranije opisani kao organizmi s vrlo visokom učinkovitosti akumulacije metala (Sures, 2001). Rakušci su međudomadari kukaša te su korišteni kako bismo uključili sve organizme uključene u životni ciklus kukaša, a također, upotreba većeg broja organizama, predstavnika kralješnjaka i beskrležnjaka, omogućuje pouzdaniju procjenu okoliša i utjecaja onečišćenja na živi svijet nekog ekosustava.

Stoga je glavni cilj ovoga rada bio primijeniti moderne analitičke pristupe u opisivanju i sveobuhvatnom prepoznavanju kratkotrajnih, ali i dugotrajnih promjena u odgovorima bioindikatorskih organizama pod uvjetima različite izloženosti metalima u okolišnim uvjetima. Pri tome za probavilo riba, kao i mjerene biološke odgovore u navedenim vrstama organizama nema dostupnih literaturnih podataka pa ovo istraživanje daje nove podatke ovakve vrste u znanstvenoj zajednici i okolišnoj ekotoksikologiji.

4.1. Utjecaj razine onečišćenja, reproduktivnog ciklusa (sezone) na morfometrijske i biološke pokazatelje pastrve i babuške

Reproduktivno aktivno razdoblje potočne pastrve obuhvaća razdoblje kasne jeseni (Mrakovčić i sur., 2006), dok se babuška razmnožava uglavnom u razdoblju od travnja do srpnja (Šaši, 2008). Kako bi se ispitao utjecaj na biološke i biokemijske pokazatelje te akumulaciju metala u ovim dvjema vrstama riba, uzorkovali smo ih u dvjema sezonama, jesen i proljeće, pri čemu je jedna od tih sezona predstavljala reproduktivnu fazu vrste, jesen za pastrvu, a proljeće za babušku. Također, svako uzorkovanje je provedeno na dvjema lokacijama koje su predstavljale područja bez utjecaja i pod utjecajem antropogenih aktivnosti, prema tome nazvanima referentna i onečišćena postaja. Potočne pastrve iz ovog

istraživanja uzorkovane su u rijeci Krki u jesen 2015. godine (listopad, utjecaj mrijesta) te proljeće 2016. (svibanj) iz izvora rijeke Krke (referentna postaja) te na postaji nizvodno od grada Knina (onečišćena postaja). Babuške su uzorkovane u rijeci Ilovi tijekom jeseni 2017. godine (listopad) te proljeća 2018. godine (svibanj, utjecaj mrijesta) u blizini sela Ilova (referentna postaja) te blizu sela Trebeža (onečišćena postaja). Rezultati ovog dijela istraživanja predstavljeni su u radovima 1 i 2 s popisa radova za potočne pastrve iz rijeke Krke te u radu 3 za babuške iz rijeke Ilove.

Dok masa i ukupna dužina potočnih pastrva iz rijeke Krke nisu pokazale stastistički značajne razlike između dviju lokacija niti u jednoj sezoni, babuške su imale značajno višu masu i dužinu na onečišćenoj postaji kod sela Trebeža u odnosu na referentnu postaju kod sela Ilove. To je ukazalo na moguću višu dostupnost hrane i nutrijenata kod sela Trebeža nego sela Ilove. To je potvrdila i izmjerena veća količina nitrata, nitrita i fosfata u uzorcima vode kod sela Trebeža (Mijošek i sur., 2020a), vjerojatno povezana s organskom tvari iz otpadnih voda i s ispiranjem okolnih poljoprivrednih zemljišta (Lambert i Dutil, 1997). Nadalje, količine nutrijenata općenito su bile više u rijeci Ilovi, nego u Krki pa se isti trend možda iz tog razloga nije mogao uočiti i u pastrva (Sertić Perić i sur., 2018; Mijošek i sur., 2020a).

Zajednički odgovor i karakteristika obiju vrsta bile su statistički značajno povišene vrijednosti gonadosomatskih indeksa (GSI) u razdoblju mrijesta svake vrste riba, dakle u jesen za potočnu pastrvu, a u proljeće za babušku, dok su vrijednosti Fultonovog kondicijskog indeksa (FCI) te hepatosomatskog indeksa (HSI) imale suprotan trend te su više vrijednosti zabilježene u razdobljima izvan reproduktivnih sezona. Fultonov kondicijski indeks ukazuje na uhranjenost riba (Koç i sur., 2007) te su niže vrijednosti u reproduktivnom razdoblju u oba slučaja vjerojatno rezultat velike energetske potrošnje i mobilizacije energetske rezervi tijekom reprodukcije i razvoja (Maddock i Burton, 1998), slično kao i za HSI. Posljedično, FCI i HSI često pokazuju suprotan trend od GSI što rezultira njihovim nižim vrijednostima u vrijeme mrijesta riba (Farkas i sur., 2003; Leonardos i sur., 2008; De Giosa i sur., 2014; Sabrah i sur., 2016). Što se tiče razlika između lokacija u vrijednostima indeksa, očitovale su se uglavnom samo kao povišene vrijednosti FCI na onečišćenim postajama u objema rijekama, što ukazuje na mogućnost da utjecaj onečišćenja u objema rijekama nije bio toliko jak da bi potaknuo dodatne obrambene mehanizme koji bi posljedično mogli rezultirati padom FCI vrijednosti, što je često zabilježeno u jako onečišćenim vodenim sustavima (Laflamme i sur., 2000; Couture i Rajotte, 2003; Jenkins, 2004; Zhelev i sur., 2016, 2018).

Navedene promjene uz utjecaj reproduktivnog statusa i onečišćenja mogu nastati i zbog drugih čimbenika vezanih uz sezonu ili fiziologiju riba, poput količine svjetlosti, temperature

vode, dostupnosti hrane i intenziteta hranjenja, prisutnosti nametnika te kompeticije ili međusobnog odnosa riba (Adams i McLean, 1985; Jonas i sur., 1996; Lagrue i Poulin, 2015; Lundova i sur., 2018) pa samo ovakav morfometrijski pristup nije dovoljan kako bi se procijenio utjecaj kakvoće vode na fiziološko stanje organizama.

4.2. Koncentracije metala/metaloida/nemetala u probavilima potočne pastrve i babuške

Iako se monitoring akumulacije metala/metaloida/nemetala u bioindikatorskim organizmima često provodi samo mjerenjem njihovih ukupnih koncentracija u ciljnim organima, sve je češća praksa mjeriti koncentracije i na substancičnoj razini. Naime, raspodjela metala u različitim frakcijama daje važan podatak o bioraspoloživosti i potencijalnoj toksičnosti metala (Wallace i Luoma, 2003; Barst i sur., 2016). Toksični učinci metala nisu povezani s ukupnim koncentracijama metala u organizmima jer se metal veže u različitim unutarstaničnim odjeljcima poput citosola, granula, organela ili staničnih membrana te određene količine predstavljaju detoksicirani oblik metala (McGeer i sur., 2012). Primjerice, metali prisutni u granulama bogatim metalima predstavljaju jedan od detoksiciranih oblika koji nije toksičan za organizam, dok se pretpostavlja da toksičnost metala uglavnom proizlazi iz reakcija u citosolu putem nespecifičnog vezanja na biološki važne molekule, a očituje se inaktivacijom biomolekula, promjenom njihove konformacije ili funkcije (Mason i Jenkins, 1995). Ipak, i u samoj citosolskoj frakciji, dobivenoj odvajanjem supernatanta nakon centrifugiranja tkivnih homogenata na $50000\times g$ (Giguère i sur., 2006), metali se mogu vezati, kako na osjetljive komponente poput mikrosoma i toplinski nestabilnih proteina kao što su enzimi, tako i na toplinski stabilne proteine poput metalotioneina koji predstavljaju jedan od biološki detoksiciranih oblika metala (Bonneris i sur., 2005; Urien i sur., 2018). Unutarstanična raspodjela metala je rezultat složenih i dinamičnih reakcija svakog pojedinog metala, kao i specifičnosti svakog organa i organizma, te ovisi i o uvjetima izloženosti i nizu drugih okolišnih čimbenika (Wang i Rainbow, 2006). Stoga je važno proučavati načine vezanja metala na različite komponente stanice te doprinos takvog vezanja ekotoksikološkom značaju za vodene organizme u okolišima pod različitim uvjetima izloženosti (Wang, 2013).

4.2.1. Ukupne koncentracije metala u probavilima potočne pastrve i babuške

Nastavno na morfometrijske i biološke pokazatelje kod riba, u ovom dijelu istraživanja izmjerene su ukupne koncentracije 20 metala/metaloida/nemetala u probavilima potočnih pastrva iz rijeke Krke te babuški iz rijeke Ilove u dvjema sezonama (jesen i proljeće) te na

dvjema postajama koje su predstavljale gradijent onečišćenja, kao prvi korak procjene utjecaja otpadnih voda i onečišćenja metalima na vodene organizme.

Prije objavljeni podaci o fizikalno-kemijskim čimbenicima, mikrobiološkoj analizi vode te koncentracijama metala u rijeci Krki ukazali su na lošije okolišne uvjete oko grada Knina i ispusta otpadnih voda u odnosu na referentnu postaju na izvoru rijeke Krke (Filipović Marijić i sur., 2018; Sertić Perić i sur., 2018). Naime, temperatura, vodljivost, količina nitrata, ukupne otopljene tvari te tvrdoća vode pokazali su statistički značajne razlike između postaja s višim vrijednostima na onečišćenoj postaji nizvodno od Knina. Nadalje, povećana brojnost svih tipova indikatorskih bakterija (ukupni koliformi, *Escherichia coli*, enterokoki, *Pseudomonas aeruginosa*) u odnosu na referentnu postaju na izvoru rijeke Krke zabilježena je kod grada Knina, osobito u neposrednoj blizini ispusta komunalnih otpadnih voda (Filipović Marijić i sur., 2018). Iako su razine metala bile relativno niske u čitavom sustavu, značajan porast u blizini grada Knina zabilježen je za većinu elemenata, pri čemu se najveće povećanje bilježi za Fe, Li, Mn, Mo, Ni, Sr, Rb i Ca (Filipović Marijić i sur., 2018; Sertić Perić i sur., 2018), što je ukazalo na opasnosti od potencijalno jačeg onečišćenja u ovom zasad umjereno onečišćenom području. Koncentracije otopljenih metala u vodi bile su usporedive s prethodnim istraživanjima na području izvora rijeke Krke (Cukrov i sur., 2008b, 2012), kao i s krškom rijekom Unom (Dautović, 2006) te sličnim područjima u Italiji (Dossi i sur., 2007). Unatoč vidljivom jačanju antropogenog utjecaja oko grada Knina i ispusta otpadnih voda, vapnenasta, odnosno sedrena podloga, kao i blago alkalni uvjeti vjerojatno su pogodovali adsorpciji metala na podlogu i sediment te smanjili koncentracije metala u stupcu vode (Korfali i Davies, 2004; Jensen i sur., 2009; Nunes i sur., 2014). Cukrov i sur. (2008b) su opisali da rijeka Krka ima sposobnost „samopročišćavanja“, preko manjih kaskadnih jezera, gdje sedimentacija i precipitacija rezultiraju značajnim smanjenjem koncentracija metala, čemu doprinosi i brzi podzemni utok čistih podzemnih voda koji smanjuje vrijeme zadržavanja zagađivala (Brkić i sur., 2019). U sustavu rijeke Krke „samopročišćavanje“ su potvrdili i rezultati određivanja fizikalno-kemijskih čimbenika, mikrobiološkog stanja vode i koncentracija metala, koji su ukazali na lošu kakvoću vode i povišene koncentracije metala na postajama uz same ispuste otpadnih voda grada Knina, te na poboljšanje ekološkog stanja nizvodno od ispusta, iako su količine nutrijenata i vrijednosti vodljivosti i dalje bile ispod preporučenih vrijednosti za vode dobre kakvoće. Koncentracije Co, Fe, Mn, Ni i Zn bile su još uvijek povišene i na postaji 1 km nizvodno od ispusta (Filipović Marijić i sur., 2018), ukazujući na snažan utjecaj industrijskih i komunalnih ispusta na kakvoću vode, te je pad njihovih koncentracija bio vidljiv tek na većim nizvodnim udaljenostima.

Rijeka Ilova može se smatrati slabo do umjereno onečišćenom metalima, pri čemu su rezultati analize vode ukazali na lošije okolišne uvjete i više koncentracije metala u nizvodnim dijelovima toka rijeke u blizini sela Trebeža u odnosu na uzvodne postaje (Mijošek i sur., 2020a). Lošije ekološko stanje rijeke Ilove u nizvodnijim dijelovima ranije su potvrdila i faunistička istraživanja makrozoobentosa s manjim brojem zabilježenih vrsta u nizvodnom dijelu (Delić, 1991). Najveći porast između referentne postaje u blizini sela Ilove i onečišćene postaje kod sela Trebeža zabilježen je za Al, As, Cd, Cs, Ni i V, a lošiju kakvoću vode na toj lokaciji su potvrdili i fizikalno-kemijski čimbenici, uključujući povišenu kemijsku potrošnju kisika te povišenu količinu nitrata i fosfata (Mijošek i sur., 2020a). Zabilježene koncentracije otopljenih metala u Ilovi bile su uglavnom manje u usporedbi s rijekama Savom i Sutlom u Hrvatskoj (Dragun i sur., 2009, 2011; Filipović Marijić i Raspor 2010; Filipović Marijić i sur., 2016b), za koje je također poznato da su pod utjecajem antropogenih aktivnosti. Rijeka Sava je najduža rijeka u Hrvatskoj te je u srednjem i donjem toku pod utjecajem industrije, rafinerije i ispusta komunalnih otpadnih voda; opisana je kao slabo i umjereno onečišćena metalima (Dragun i sur., 2009; Filipović Marijić i Raspor, 2010). Zabilježene koncentracije Cd, Co, Pb, Cu, Ni, Zn, Mn i Fe u Savi uglavnom su usporedive s koncentracijama zabilježenim u rijeci Ilovi u našem istraživanju, izuzev viših koncentracija Co i Mn u uzorcima vode rijeke Ilove na objema postajama. Usporedba s rijekom Sutlom, ranije okarakteriziranom kao rijekom pod značajnim utjecajem antropogenih aktivnosti koja je na pojedinim dijelovima toka izrazito onečišćena metalima podrijetlom iz tvornice stakla, ali i poljoprivrede (Dragun i sur., 2011; Filipović Marijić i sur., 2016b), ukazala je na uglavnom niže koncentracije metala u Ilovi nego u Sutli, izuzev Mn koji je bio viši u Ilovi na objema postajama, što bi mogla biti posljedica aktivne poljoprivrede na cijelom području uz rijeku Ilovu, kao i korištenja gnojiva i fungicida (IPCS, 1999). Povišene koncentracije V na onečišćenoj postaji, s druge strane, vrlo vjerojatno potječu iz fosfogipsa, kao nusprodukta prerade fosfatnih gnojiva u obližnjoj tvornici (Durgo i sur., 2009). Ukoliko se usporede dva izučavana sustava u ovome istraživanju, koncentracije metala u rijeci Ilovi bile su značajno više nego u Krki, međutim akumulacija metala u potočnoj pastrvi i babuški ovisi i o specifičnosti vrste, fiziološkim prilagodbama, načinu prehrane i života riba, pa se mogu očekivati i različite strategije s kojima se organizmi nose s onečišćenjem i povišenim razinama metala.

Kako bismo ispitali utjecaj okolišnih uvjeta na same organizme, određene su, kao prvo, ukupne koncentracije metala u probavilima riba, mjerenjem u razgrađenim tkivnim

homogenatima. Rezultati ovog dijela istraživanja objavljeni su u radovima pod rednim brojem 1 za rijeku Krku te u radu pod rednim brojem 3 za rijeku Ilovu, u popisu znanstvenih radova.

Za razliku od koncentracija otopljenih metala u vodi, ukupne koncentracije akumuliranih metala u probavilu potočnih pastrva nisu ukazale na statistički značajne razlike za većinu metala između referentne postaje na izvoru rijeke Krke i onečišćene postaje kod grada Knina, ali niti između dviju sezona. Naime, u jesen je statistički značajna razlika ukupnih koncentracija u probavilu riba između postaja bila vidljiva samo za Cs, Se i Tl, a u proljeće za Ca, Cd, Co, Mo, Se, Sr i Tl pri čemu su Cd, Cs, Mo i Tl bili viši na referentnoj postaji, a ostali navedeni elementi na onečišćenoj postaji. To samo djelomično odražava okolišne uvjete iz vode gdje je većina elemenata pokazivala značajan porast na onečišćenoj postaji (Filipović Marijić i sur., 2018; Sertić Perić i sur., 2018), čime se potvrđuje značaj unosa metala u organizme i drugim izvorima osim vode, kao što su hranai/ili sediment, kao i utjecaj fiziološke regulacije na razine esencijalnih elemenata. Utjecaj unosa hranom posebno se odnosi na elemente koji su povišeni u organizmima s referentne postaje (Cd, Cs, Mo i Tl), a niti jedan od tih elemenata nije bio značajno povišen u uzorcima vode s referentne postaje. Uz probavilo, povišene vrijednosti Cd, Cs i Tl na referentnoj postaji potvrđene su i u jetri istih jedinki riba (Dragun i sur., 2018a). Sezonske razlike u ukupnim koncentracijama metala u probavilu potočne pastrve bile su statistički značajne samo za akumulaciju As i Na na referentnoj postaji te za Cd, Mo i Na na postaji kod Knina, pri čemu je većina navedenih elemenata imala više razine u jesen, nego u proljeće, što je bio vidljiv trend i za većinu preostalih istraživanih elemenata u probavilu. Velik dio elemenata bio je povišen i u uzorcima vode u jesen u istom razdoblju kad je uzorkovana riba (Sertić Perić i sur., 2018). Iz tog razloga se nešto pojačana akumulacija metala u riba u jesen djelomično može pripisati i stvarnim uvjetima u okolišu, ali i činjenici da je jesen reproduktivno razdoblje potočne pastrve tijekom kojeg rastu i metaboličke potrebe za esencijalnim elementima (Miramand, 1991). Ukupne koncentracije elemenata u tragovima u probavnom tkivu potočne pastrve slijedile su redosljed: $Zn > Fe > Rb > Se > Mn \geq Cu > Sr > Ni > Cd > Co \geq Mo > Tl \geq As > V > Cs$, a ukupne koncentracije makroelemenata: $K > Na > Ca > Mg$. Unatoč razlici u fiziološkoj i metaboličkoj ulozi organa, ovakav redosljed koncentracija metala je usporediv s onim u jetri istih jedinki potočne pastrve iz rijeke Krke ($K > Na > Mg > Fe > Ca > Cu > Zn > Rb > Se > Mn > Tl > Mo > Sr > Cd > Co > As > V > Cs$) (Dragun i sur., 2018a), uz iznimku Zn koji je pokazao znatno veće koncentracije u probavilu, nego u jetri. Naime, probavilo je organ u kojemu se pohranjuju zalihe Zn u organizmu (Sun i Jeng, 1998) pa je moguće da se u njemu detektira i izvjesna količina Zn unesena vodom putem škruga (Dallinger i Kautzky,

1985). Stoga se često bilježe više koncentracije u ovom organu u odnosu na druge, poput jetara, bubrega, škrge, gonada ili mišića (Dallinger i Kautzky, 1985; Jarić i sur., 2011; Filipović Marijić i Raspor, 2012; Yeltekin i Sağlamer, 2019). Drugi elementi uglavnom su prisutni u višim koncentracijama u jetri nego u probavilu, što je i očekivano s obzirom na izraženu metaboličku aktivnost jetara. Ipak, uz Zn, više koncentracije elemenata u probavilu u odnosu na jetru zabilježene su i za Co, Sr te V (Dragun i sur., 2018a). Potočnu pastrvu kao bioindikatorski organizam ranije su koristili i Vitek i sur. (2007) u procjeni kakvoće okoliša rijeke Loučke u Češkoj te su usporedili akumulaciju Cu, Cd, Ni i Zn u mišiću i jetri. Dobiveni padajući nizovi koncentracija bili su $Zn > Cu > Ni > Cd$ za mišić te $Cu > Zn > Cd > Ni$ za jetru. Can i sur. (2012) su usporedili akumulaciju As, Cd, Cu i Se u jetri, škragama i mišiću pastrva iz rijeke Munzur u Turskoj te su dobiveni slijedovi $Se > Cu > As > Cd$ za škrge, $Cu > Cd > As > Se$ za jetru te $Cu > Se > As > Cd$ za mišić. Pri tome su dobivene koncentracije u jetri i škragama uglavnom bile više nego u probavilu iz našeg istraživanja, dok su koncentracije u mišiću bile najniže, ali ovdje, uz nisku metaboličku aktivnost mišića, treba uzeti u obzir i razlike uzrokovane okolišnim uvjetima riba iz različitih rijeka.

Slično kao i kod potočnih pastrva, i u našem dijelu istraživanju provedenom na babuškama u rijeci Ilovi ukupne koncentracije metala u probavnom tkivu babuški samo su se djelomično podudarale s okolišnim uvjetima, odnosno samo su neki elementi pratili porast koncentracija metala u vodi i sedimentima kod sela Trebeža u odnosu na selo Ilova (Mijošek i sur., 2020a). Statistički značajne razlike između postaja uočene su za relativno mali broj elemenata. U objema sezonama značajna je razlika između riba s dviju postaja različitog stupnja onečišćenja zabilježena za Cd, Cs, Cu i V te dodatno za As, Ca, Mg, Mn, Na i Rb u samo jednoj sezoni, pri čemu su Mn i V bili povišeni u probavilu riba s referentne lokacije, a ostali elementi u riba s onečišćene postaje kod sela Trebež. Akumulacija tih elemenata u babuški samo se za neke elemente može objasniti trendovima njihovih razina u vodi i sedimentima, kao potencijalnih izvora izloženosti organizama metalima, i to za As, Cd, Cs i Rb, koji su uz organizme bili značajno povišeni i u vodi i sedimentima s onečišćene postaje (Mijošek i sur., 2020a). Za Cd i Cs je iz literature poznato da je koncentracija u vodi jedan od glavnih čimbenika koji utječu na njihovu bioakumulaciju u vodenim organizmima (Rowan i Rasmussen, 1994; Pinder i sur., 2011; Dragun i sur., 2019). Međutim, akumulacija metala u probavilu babuški, slično kao i u potočnih pastrva, ukazala je kako moraju postojati i drugi značajni izvori osim vode. Naime, koncentracije Mn i V bile su značajno više u riba s referentne postaje kod sela Ilove, iako je V u uzorcima vode i sedimenta pokazao upravo suprotan obrazac i povišene vrijednosti kod sela Trebeža, dok je Mn u vodi u jesen bio

povišen na referentnoj postaji, međutim u proljeće je imao suprotan trend, što vrijedi i za uzorke sedimenta u objema sezonama (Mijošek i sur., 2020a). Samim time, nameće se važnost i velika vjerojatnost unosa metala putem hrane, osobito zbog važne uloge probavila u prehrani i apsorpciji hranjivih sastojaka. Sezonske razlike koncentracija metala u vodi s referentne postaje rijeke Ilove ukazale su na povišene vrijednosti većine elemenata u proljeće, dok je suprotan trend bio vidljiv na onečišćenoj postaji s višim vrijednostima u jesen (Mijošek i sur., 2020a), ali to se također nije uvijek na jednak način očitovalo i u ribama. Naime, za većinu elemenata nije dokazana statistički značajna razlika između sezona, iako razlike postoje i za većinu elemenata u probavilu babuški ukazuju na povišene razine u proljeće u odnosu na jesen na objema lokacijama, što je vjerojatno posljedica reproduktivne faze u toj sezoni. Statistički značajno povišene ukupne koncentracije As, Co, Mn, Sr i Zn u probavnom tkivu babuški bile su zabilježene u proljeće na objema lokacijama. Nasuprot tome, statistički značajno povišene ukupne koncentracije Cd, Cs i K zabilježene su u jesen. Ukupne koncentracije elemenata u tragovima u probavnom tkivu babuške slijedile su redosljed $Zn > Fe > Mn \geq Rb \geq Cu > Se \geq Cd > Ni > Sr > Mo > As > Co > V > Cs > Tl$, što je unatoč vrsno-specifičnim razlikama, kao i različitim okolišnim uvjetima potvrdilo već dokazani trend visokih koncentracija Zn, Fe, Mn i Cu u probavilima različitih vrsta riba u uvjetima različite izloženosti metalima (Dallinger i Kautzky, 1985; Sures i sur., 1999; Staniskiene i sur., 2006; Jarić i sur., 2011; Filipović Marijić i Raspor, 2012; Nachev i Sures, 2016; Yeltekin i Sağlamer, 2019). Također, slični trendovi akumulacije ukupnih metala su zabilježeni i u drugim organima babuški iz različitih geografskih područja; $Fe > Zn > Cu > Mn > Cd > Ni > Co$ u mišiću riba iz rijeke Nitre u Slovačkoj koja je izložena utjecaju otpadnih komunalnih voda, elektrana, tvornice kemikalija te rudnika lignita (Andreji i sur., 2006); $Cu > Cd > Ni > As$ u jetri, mišiću i škragama babuški iz jezera Marmara u Turskoj pod utjecajem poljoprivrede (Yabanli i sur., 2014), dok je istraživanje Đikanović i sur. (2016) potvrdilo babušku iz Međuvršja u Srbiji pod utjecajem industrijskih i komunalnih otpadnih voda kao vrstu s najučinkovitijom akumulacijom metala među 9 ribljih vrsta.

Stoga, iako je teško uspoređivati različite sustave i različite vrste, naše istraživanje je pokazalo da ukupne koncentracije metala u objema vrstama riba samo djelomično odražavaju analizirane okolišne uvjete te je ukazalo na vjerojatan značaj hrane kao važnog izvora za unos metala. Nadalje, vidljive razlike između dviju istraživanih vrsta riba su posljedica bilo razlike u izloženosti metalima ili u specifičnostima svake vrste, ali se to ne može sa sigurnošću utvrditi bez dodatnih istraživanja. Uglavnom veća izloženost metalima zabilježena je u rijeci Ilovi te su i ukupne koncentracije većine elemenata u probavilu babuške bile uglavnom više

nego u probavilu potočne pastrve iz rijeke Krke, s izuzetkom Ca, Se, Sr i Tl koji su bili viši u pastrvama što vjerojatno govori o određenim fiziološkim prilagodbama i biološkim specifičnostima svake vrste. Naime, ove vrste razlikuju se i u načinu prehrane; pastrva je omnivorna, i uglavnom predatorska vrsta (Mrakovčić i sur., 2006), dok je babuška omnivorna, ali većim dijelom herbivorna vrsta (Kottelat i Freyhof). Značajan dio prehrane pastrve stoga čine i rakušci, čiji je oklop bogat Ca i Sr te stoga vrlo vjerojatno predstavlja i izvor visoke akumulacije ovih elemenata u toj vrsti (Mertz, 1987). Što se tiče Tl, već je dokazano da u debeloglavih gavčica njegov unos putem hrane može biti od jednakog značaja kao unos vodom, odnosno predstavljati čak i veći rizik toksičnosti jer je unos putem vode bolje reguliran (Lapointe i Couture, 2009).

Kako dobivene informacije o ukupnim koncentracijama metala u probavilu riba daju samo uvid u ukupni sadržaj metala, koji uključuje i metale u staničnim strukturama i detoksicirane metale, a ne samo metabolički raspoložive koji mogu izazvati potencijalne toksične i štetne učinke u organizmima te samo djelomično potvrđuju hipoteze H1 i H2, sljedeći je korak bio mjerenje citosolskih, odnosno metabolički dostupnih, a time i potencijalno toksičnih, koncentracija metala u probavilima obiju vrsta riba.

4.2.2. Citosolske koncentracije metala u probavilima potočne pastrve i babuške

Kako bismo dobili detaljniji uvid u utjecaj okolišnih uvjeta na same organizme, izmjerene su citosolske, odnosno metabolički dostupne koncentracije metala u vodotopivoj staničnoj frakciji probavila riba, koje predstavljaju potencijalno toksičan udio metala za organizme jer određeni dio metala prisutan i u citosolu može biti detoksiciran vezanjem na metalotioneine ili njima slične proteine (Wallace i Luoma, 2003; Urien i sur., 2018). Rezultati ovog dijela istraživanja, prvi ove vrste za probavilo ovih slatkovodnih vrsta riba, objavljeni su u radovima pod rednim brojevima 1 i 2 za potočnu pastrvu te radu pod rednim brojem 3 za babušku u popisu znanstvenih radova.

Kao što se i očekivalo i u pastrvi iz rijeke Krke i babuški iz rijeke Ilove, citosolske koncentracije metala bile su niže, nego ukupne koncentracije koje predstavljaju i topivu i netopivu frakciju metala. Unatoč razlici u vrijednostima, citosolske koncentracije su imale dosta slične prostorne i vremenske trendove kao i ukupne koncentracije, odnosno odražavale su okolišne uvjete na sličan način.

U potočnih pastrva značajan porast citosolskih koncentracija metala na nizvodnoj postaji kod Knina u odnosu na referentnu postaju zabilježen je za Co i Se u objema sezonama, za As i Cu u jesen te za Ca i Sr u proljeće, dok su Cd, Cs i Tl kao i u slučaju ukupnih

koncentracija metala bili viši kod izvora rijeke Krke u objema sezonama. Kadmij, Cs i Tl također su bili povišeni i u citosolu jetara jedinki s referentne postaje u odnosu na onečišćenu (Dragun i sur., 2018a). Sezonske razlike u koncentracijama citosolskih metala su se očitovale kao više vrijednosti Cs, Na i Ni u jesen te As u proljeće na referentnoj postaji, kao i Cd i Mo u jesen na onečišćenoj postaji. Dakle, i sezonske varijacije citosolskih koncentracija imale su usporediv trend s ukupnim koncentracijama metala u probavilu. S obzirom na to da je i većina elemenata u vodi imala više razine u jesen, ovi podaci ukazuju i na utjecaj izravnog unosa metala vodom, ali kao i kod ukupnih koncentracija, varijabilnost pojedinih elemenata upućuje i na druge važne izvore poput hrane i sedimenta. Nadalje, sezonske razlike mogu biti i posljedica promjene fizioloških potreba i stanja organizma tijekom reproduktivnih razdoblja, ali i biotičkih čimbenika poput promjena temperature, pH vrijednosti te varijacija u izloženosti onečišćenju. Citosolske koncentracije elemenata u tragovima uglavnom su slijedile redosljed: $Zn > Fe > Rb > Se > Cu \geq Mn > Ni \geq Sr > Cd > Co \geq Mo \geq Tl > As > Cs > V$, a makroelemenata $K > Na > Mg > Ca$, bez većih i značajnih razlika između postaja te slično kao u homogenatima. Najviše koncentracije esencijalnih elemenata, zajedno s njihovim ujednačenim vrijednostima u organizmima s malo značajnih razlika između lokacija, potvrđuju uspješnu i jaku regulaciju esencijalnih elemenata u ribama neovisno o okolišnim uvjetima, kao što je već opisano u mnogobrojnim istraživanjima (Olsvik i sur., 2000; Monna i sur., 2011; Dragun i sur., 2019). U našem slučaju su npr. koncentracije otopljenog Fe, Mn i Zn u uzorcima vode rijeke Krke bile značajno više na onečišćenoj u odnosu na referentnu postaju, ali te razlike nisu potvrđene u probavilu potočnih pastrva.

Razlika u citosolskim koncentracijama u probavilu babuški pokazala se statistički značajnom između referentne postaje kod sela Ilove te onečišćene postaje kod sela Trebeža za Ca, Cd, Cs, Cu, Fe, Rb i V u objema sezonama te za As, Mn, Mg i Na u jednoj sezoni. Pri tome su svi citosolski elementi, osim Mn i V, bili povišeni kod Trebeža, jednako kao i u slučaju ukupnih koncentracija. Sezonske razlike nisu bile jasno izražene, ali ponovno je nešto veći broj elementa bio povišen u ribama u proljeće, nego u jesen. Time su se opet na organizme djelomično odrazile i razlike u koncentracijama metala u vodi i sedimentu, ali i potvrdio trend viših koncentracija metala u citosolu probavila u reproduktivnom razdoblju riba, kada su povećane metaboličke potrebe (Miramand i sur., 1991), kao što je potvrđeno i u citosolu probavila potočne pastrve te citosolu škruga i probavila europskog klena (Dragun i sur., 2007; Filipović Marijić i Raspor, 2010). Padajući niz citosolskih koncentracija u probavilu babuški bio je uglavnom: $K > Na > Mg > Ca > Zn > Fe > Rb > Cu \geq Mn > Se > Cd > Sr > Ni > Mo \geq As > Co > V \geq Cs > Tl$ što je uglavnom pratilo redosljede ukupnih metala.

Uz pastrve i babuške, usporedba s koncentracijama u citosolu probavila bila je moguća za europske klenove iz rijeke Save gdje je također zabilježen redoslijed $Zn > Fe > Cu > Mn > Cd$ (Filipović Marijić i Raspor, 2012).

Stoga, unatoč razlikama u koncentraciji citosolskih i ukupnih metala, njihovi trendovi su pokazali da obje frakcije odražavaju okolišne uvjete na sličan način u objema vrstama riba.

S obzirom na primjećene razlike u ukupnim i citosolskim koncentracijama metala između lokacija i sezona uočenim kod obiju vrsta, sa značajnim porastima akumulacije na onečišćenim postajama, ovi dijelovi istraživanja o bioakumulaciji metala potvrđuju hipoteze H1 i H2 da ispusti otpadne vode dovode do narušavanja kakvoće okoliša i značajnih promjena u ribama s onečišćenih postaja u odnosu na one s referentnih postaja uslijed antropogenog utjecaja, iako sezonske razlike mogu biti i fiziološke prirode.

4.2.3. Raspodjela metala/metaloida/nemetala između topljive i netopljive frakcije u probavilima potočne pastrve i babuške

Zbog detaljnijeg uvida u raspodjelu metala/metaloida/nemetala u odabranim vrstama riba, idući korak bilo je izračunavanje omjera citosolskih i ukupnih koncentracija, odnosno udjela topljive i netopljive frakcije, što govori o bioraspoloživosti metala i detoksikacijskom potencijalu organizma za pojedini element. Rezultati ovog dijela istraživanja obrađeni su i prikazani u radu pod rednim brojem 1 za potočnu pastrvu te radu pod rednim brojem 3 za babušku, u popisu znanstvenih radova.

Koncentracije elemenata u tragovima i makroelemenata u probavilu riba razlikovale su se između postaja, međutim opadajući redoslijedi udjela metala u metabolički dostupnoj citosolskoj frakciji bili su usporedivi na referentnim i onečišćenim postajama obiju rijeka, dok su se brojčane vrijednosti udjela ponešto razlikovale u slučaju nekih elemenata. U potočnim pastrvama zastupljenost u postotku većem od 60 % u topljivoj citosolskoj frakciji zabilježena je za As, Cd, Cs, K, Mg, Mo, Na, Se, Rb i Tl pri čemu je najveći udio na svim postajama i lokacijama s rijeke Krke uvijek bio za Na, K, Se, i Cs. Ti su elementi svojim većim dijelom raspoloživi za metaboličke potrebe ili za toksične učinke u stanici. S druge strane, najmanji udio (< 41 %) u citosolskoj frakciji uglavnom su imali Cu, Mn, Ni i V, zbog čega se može pretpostaviti da se značajan udio njihove detoksikacije odvija putem granula. Međutim, postotna zastupljenost pojedinih elemenata u topljivoj frakciji probavila ipak djelomično varira ovisno o ukupnoj koncentraciji tog elementa u tkivu potočnih pastrva. Naime, nešto osjetnije razlike u raspodjeli između dviju lokacija rijeke Krke javile su se za elemente poput As, Cd, Ni ili Tl, dakle neesencijalne i potencijalno toksične elemente, čije koncentracije su

pokazivale i značajne razlike između lokacija. Arsen, koji je imao više koncentracije u pastrva s onečišćene postaje također je imao i nešto veću zastupljenost u topljivoj frakciji s te postaje što upućuje na njegovo zadržavanje u citosolu nakon povišene akumulacije te veći potencijal toksičnog djelovanja. Visoki udio As u frakciji osjetljivoj na prisutnost metala (MSF, eng. *Metal Sensitive Fraction*), koja uključuje i toplinski nestabilne proteine poput enzima, zabilježili su i Rosabal i sur. (2015) u jetri jegulja što je kao i u našem istraživanju ukazalo na veliku mogućnost toksičnih učinaka, osobito zbog činjenice da se udio u toj frakciji povećava s porastom koncentracije. Sličan obrazac vidljiv je i za Cd i Tl te Ni u jednoj sezoni, čija postotna zastupljenost u citosolu je veća u riba s referentne postaje na kojoj su bile povišene i njihove ukupne koncentracije. Dakle, zastupljenost pojedinih elemenata u topljivoj frakciji se mijenja ovisno o ukupnoj koncentraciji elementa u tkivu. S obzirom na to da je poznato da se Cd veže za metalotioneine (Viarengo, 1985; Langston i sur., 2002), njegova visoka prisutnost u citosolu, zabilježena u više vrsta riba i tkiva (Kraemer i sur., 2006; Kamunde, 2009; Rosabal i sur., 2015; Dragun i sur., 2018a), vjerojatno uključuje i uspješnu detoksikaciju, ali u slučaju As, Ni i Tl takav mehanizam nije poznat pa njihova veća prisutnost u citosolu nakon povišene akumulacije, zajedno s poznatom toksičnošću već u niskim koncentracijama, svakako može predstavljati opasnost za stanicu i organizam. Ipak, u našem istraživanju zabilježena je sveukupno niska zastupljenost Ni, slično kao i za jetru jegulja (*A. anguilla*), gdje je zabilježeno da glavnu detoksikacijsku ulogu za ovaj element imaju granule (Rosabal i sur., 2015). Međutim, u slučaju grgeča su toplinski nestabilni proteini (HDP, eng. *Heat Denaturable Proteins*) mjesto najveće akumulacije Ni (Giguere i sur., 2006). Nadalje, Lapointe i sur. (2009), Rosabal i sur. (2015) te Barst i sur. (2016) izvještavaju o dominantnom vezanju Tl za HSP (eng. *Heat Stable proteins*) u jetri jegulja te jezerskih zlatovčica što ipak upućuje na učinkovitost detoksikacijskih mehanizama za ovaj element i u slučaju vezanja za MSF. Međutim, isti autori navode i da značajno povećanje izloženosti Tl dovodi do njegovog smanjenog udjela u detoksiciranom obliku te se u tom slučaju značajna količina Tl može vezati za organele (Lapointe i sur., 2009; Barst i sur., 2016) te eliminacijski i detoksikacijski mehanizmi funkcioniraju do određene granice i u umjereno onečišćenim uvjetima. Pozitivna korelacija između porasta postotnog udjela u topljivoj frakciji i ukupnih koncentracija metala dobivena je za As, Cd, Cs i Tl i u jetri potočnih pastrva (Dragun i sur., 2018a). Povećana prisutnost u topljivoj frakciji u jedinkama s povišenim ukupnim koncentracijama metala u našem je istraživanju ponekad uočena i vezano uz sezonu. Primjerice, ukupne koncentracije Ni ili Rb u probavilu potočne pastrve s izvora rijeke Krke bile su više u jesen, nego u proljeće

te je isti trend uočen i za postotni udio tih elemenata u citosolu, odnosno s nešto višom citosolskom zastupljenošću u jesen.

S druge strane, za esencijalne elemente koji su pokazali značajno više koncentracije na onečišćenoj postaji, poput Ca ili Se, postotna zastupljenost u topljivoj frakciji ostaje jednaka ili čak i pada na toj lokaciji, pa su u nekim slučajevima njene vrijednosti niže na onečišćenoj postaji nego na izvoru. To ukazuje na učinkovitije uklanjanje tih metala iz metabolizma te njihovu uspješnu regulaciju i detoksikaciju i kada su prisutni u povišenim koncentracijama. Isto je dokazano i za Co, Fe, Mg, Mn i Sr u jetri istih jedinki pastrva te objašnjeno njihovim vezanjem za granule kao detoksicirani oblik metala u slučaju visokih koncentracija (Dragun i sur., 2018a).

Također, rezultati dobiveni za probavilo potočnih pastrva ukazali su na sličnosti, ali i neke razlike u usporedbi s jetrom u raspodjeli metala, što je i očekivano s obzirom na drugačije funkcije i strukturu organa (Dragun i sur., 2018a). Naime, jetra predstavlja glavni metabolički i detoksikacijski organ koji ima i najučinkovitiju sposobnost akumulacije raznih zagađivala (Sindayigaya i sur., 1994; Linde i sur., 1998; Giguère i sur., 2004), dok probavilo, sličnije škragama, predstavlja organ unosa metala u organizam uz manje metaboličkih aktivnosti. Padajući udjeli metala u citosolu jetara potočnih pastrva bili su: Na, K ($\geq 100\%$) > Cd, Rb, V (90–99 %) > Co, Cs, Se (80–89 %) > Cu, Fe, Mn, Mo, Tl, Zn (60–69 %) > As, Mg, Sr (50–59 %) > Ca (40 %) kod riba s referentne postaje te Na, K ($\geq 100\%$) > Rb (90–99 %) > As, Cd, Co, Cs, Se, V (80–89 %) > Cu, Mn, Mo, Tl, Zn (60–69 %) > Fe, Mg (50–59 %) > Ca, Sr (< 50 %) kod riba s onečišćene postaje kod grada Knina (Dragun i sur., 2018a). Glavne razlike između probavila i jetara odnose se na V, koji pokazuje puno veći udio u citosolu jetara nego probavila, te Co, Cu, Mn i Zn s oko 20 % višim udjelima u citosolskoj frakciji jetara nego probavila. I u jednom i u drugom organu ističu se visoki udjeli elemenata poput As, Cd, Cs i Rb u citosolu, što upućuje na veliku mogućnost toksičnih učinaka.

Kod babuški je zastupljenost u postotku većem od 60 % u topljivoj citosolskoj frakciji zabilježena za As, Cd, Co, Cs, K, Mg, Mo, Na, Se i Rb, odnosno za iste elemente kao i kod potočnih pastrva, s izuzetkom Co, koji je u pastrva imao citosolsku zastupljenost blizu 60 %. Pri tome su najveći udio na svim postajama i sezonama uvijek imali Na, K, Se i Rb, više od 80 %, dok su najmanji udio u citosolskoj frakciji (< 40 %), pa time i manji toksični potencijal, uglavnom imali Fe, Mn, Ni i V. Unatoč postojanju značajnih razlika u ukupnim i citosolskim koncentracijama nekih elemenata u babuškama s dviju lokacija, postotna zastupljenost pojedinih elemenata u topljivoj frakciji i ovdje je uglavnom bila ujednačena na objema lokacijama i u objema sezonama. Međutim, ponešto različiti udjeli zabilježeni su za Cs, Cu,

Mn, Ni i V te su potvrdili sličan obrazac kao i u potočnih pastrva, da se povećanjem ukupne koncentracije Cs ili Cu ponešto povećava i njihov udio u topljivoj frakciji, dok se odstupanja u zastupljenosti među postajama nisu mogla objasniti razlikama u ukupnim koncentracijama za Mn, Ni i V. Koncentracije Ni bile su ujednačene između lokacija, no unatoč tome veći udjeli u topljivoj frakciji zabilježeni su u ribama s onečišćene postaje kod Trebeža. Iako su ukupne i citosolske koncentracije Mn i V bile značajno više u riba kod sela Ilove, najviši postotak zastupljenosti u citosolu zabilježen je na Trebežu u jesen, odnosno proljeće, sugerirajući uspješniju detoksikaciju i regulaciju u slučaju ovih elemenata u babuški neovisno o njihovoj višoj koncentraciji. Nadalje, As i Cd, iako povišeni u riba s Trebeža, nisu pokazali veću zastupljenost u citosolu tih jedinki, što također upućuje na nešto učinkovitiju detoksikaciju nekih toksičnih elemenata u invazivnoj vrsti babuški, nego u potočnih pastrva, ali i dalje uz slične postotne udjele. Usporedbu raspodjele metala u babuška bilo je moguće napraviti s jetrom i bubrezima babuški sa šest lokacija pod utjecajem onečišćenja metalima iz Belgije, gdje je udio Cd, Cu i Zn bio 60-70 % u citosolu jetara te 50 % Cd i 30 % Cu i Zn u citosolu bubrega (van Campenhout i sur., 2010). U probavilu babuški iz rijeke Ilove u našem istraživanju udio Cd je bio oko 80 %, Cu 40-50 %, a Zn 50-60 %.

U konačnici, usporedba između dviju vrsta riba iz ovog istraživanja ipak je ukazala na slične prosječne udjele metala u citosolu probavila sa sljedećim rezultatima: Na, K (> 98 %) > Se (87 %) > Cs (85 %) > Rb (82 %) > Mo (73 %) > As (69 %) > Cd, Mg (67 %) > Tl (60 %) > Co (58 %) > Fe (50 %) > Sr (48 %) > Zn (47 %) > Ca (43%) > Mn, V (41%) > Cu (39 %) > Ni (36 %) za potočne pastrve iz rijeke Krke te Na, K (100 %) > Rb (93 %) > Se (84 %) > Cd (80 %) > Cs (79 %) > As (73 %) > Mo (71,5 %) > Mg (66 %) > Co (65 %) > Zn (57 %) > Sr (50 %) > Cu (46 %) > Ca, Tl (45 %) > Mn (39 %) > Fe (34 %) > V (31 %) > Ni (25 %) za babuške iz rijeke Ilove. Usporedba s citosolom probavila klenova iz rijeke Save ukazala je na višu citosolsku zastupljenost Cd (90-100 %), Zn (70-80 %) i Cu (50-80 %) u klenu u odnosu na potočne pastrve i babuške, te uglavnom usporedive udjele Fe i Mn (30-40 %) (Filipović Marijić i Raspor, 2012). Postojeće razlike, iako se odnose na dvije različite vrste riba i dva različita slatkovodna ekosustava, dinaridsku rijeku Krku i panonsku rijeku Ilovu, nisu bile jako izražene i govore u prilog relativno sličnim mehanizmima detoksikacije i metabolizma metala u probavilu riba općenito, dok se postojeće razlike mogu povezati s drugačijim fiziološkim karakteristikama svake vrste, njihovom biologijom, kao i različitim okolišnim uvjetima te posljedično i izloženosti metalima iz okoliša i hrane.

Metali prisutni u citosolu, osobito u uvjetima niske ili umjerene izloženosti, mogu biti detoksicirani putem vezanja za toplinski stabilne proteine, poput MT ili GSH, ali određeni dio

se ipak veže i za toplinski osjetljive proteine (HDP), što pokazuje da detoksikacija nije u potpunosti učinkovita (Kraemer i sur., 2006; Rosabal i sur., 2015). Za akumulaciju Cd, Cu ili Zn može se očekivati vrlo visoka detoksikacija putem MT (Kraemer i sur., 2006; Campbell i sur., 2008; van Campenhout i sur., 2010). Nadalje, u slučaju Cu i Zn očekuje se i raspodjela među brojnim citosolskim biomolekulama vezano uz njihovu fiziološku ulogu, kao i uspješna regulacija njihovih koncentracija u stanicama, budući da se radi o esencijalnim elementima potrebnim za normalan metabolizam i održavanje homeostaze (Kraemer i sur., 2006; Kamunde i MacPhail, 2008; Monna i sur., 2011). Međutim, akumulacija neesencijalnih elemenata u citosolu i osjetljivim odjeljcima može predstavljati prijetnju i mogućnost toksičnih učinaka koji uključuju blokiranje funkcionalnih skupina, zamjenu esencijalnih elemenata ili modifikacije aktivnih mjesta važnih biomolekula (Mason i Jenkins, 1995). Stoga visoka citosolska zastupljenost elemenata poput As, Cd, Cs ili Rb u probavilima riba u našem istraživanju upućuje na potencijal za toksične učinke u analiziranim ribama.

Razlike u akumulaciji metala, kao i u stupnju detoksikacije te zastupljenosti u topljivim frakcijama svakako su posljedica antropogenog utjecaja i različitog onečišćenja dviju lokacija, što potvrđuje hipotezu H2.

4.3. Raspodjela Cd, Co, Cu, Fe, Mo, Se i Zn među citosolskim biomolekulama različitih molekularnih masa u probavilima potočne pastrve iz rijeke Krke i babuške iz rijeke Ilove

Da bismo opisali ponašanje i metabolizam metala na substancijskoj razini u ribama, u ovome smo dijelu istraživanja istražili raspodjelu sedam elemenata među citosolskim biomolekulama različitih molekularnih masa u probavilima potočne pastrve i babuške. Utvrđeni su rasponi molekularnih masa citosolskih biomolekula koje vežu esencijalne elemente Co, Cu, Fe, Mo, Se i Zn te neesencijalni Cd u probavilima u uvjetima uglavnom niske izloženosti u okolišu, ali i opisane promjene koje se javljaju pri nešto povišenoj bioakumulaciji. Također, opisane su i specifične razlike između dviju vrsta. S obzirom na to da se radi o prvim podacima za probavilo ribe uopće, rezultati pružaju bazu za usporedbu s drugim vrstama i različitim razinama izloženosti metalima te ključni prvi korak prema točnoj identifikaciji biomolekula koje vežu metale u probavilu i koje bi potencijalno mogle biti korištene kao biomarkeri. Rezultati tog dijela istraživanja obrađeni su u pripremljenom radu kao Prilogu 1.

Za metale obuhvaćene ovim izučavanjem, koncentracije otopljenih oblika u vodi rijeke Krke u razdobljima uzorkovanja potočnih pastrva bile su većinom usporedive s nezagađenim i krškim vodotocima (Cd, 0,005-0,01 $\mu\text{g L}^{-1}$; Co 0,019-0,211 $\mu\text{g L}^{-1}$; Cu < 0,401 $\mu\text{g L}^{-1}$; Fe 0,9-

5,16 $\mu\text{g L}^{-1}$; Mo 0,21-0,515 $\mu\text{g L}^{-1}$; Zn < 7,34-20,41 $\mu\text{g L}^{-1}$). Povišene koncentracije otopljenog Fe i Mo na onečišćenoj postaji u odnosu na referentnu su omogućile praćenje promjena citosolskih raspodjela tih metala ovisno o izloženosti u vodi. Koncentracije otopljenih metala u rijeci Ilovi u vrijeme uzorkovanja babuški također su bile usporedive s čistim ili umjereno onečišćenim riječnim tokovima iako su koncentracije općenito bile više nego u Krki. Kretale su se u sljedećim rasponima: Cd 0,006-0,053 $\mu\text{g L}^{-1}$; Co 0,121-0,338 $\mu\text{g L}^{-1}$; Cu 0,401-0,716 $\mu\text{g L}^{-1}$; Fe, 17,89-21,81 $\mu\text{g L}^{-1}$; Mo 0,561-0,981 $\mu\text{g L}^{-1}$; Zn <7,34 $\mu\text{g L}^{-1}$ te su također povišene koncentracije Cd i Mo na onečišćenoj u odnosu na referentnu postaju rijeke Ilove omogućile analizu promjena u raspodjeli tih elemenata ovisno o razini izloženosti metalima.

Primjena tekućinske kromatografije visoke djelotvornosti s isključenjem po veličini (SEC-HPLC) radi razdvajanja citosola probavila na frakcije koje sadrže biomolekule odgovarajućih molekulskih masa te primjena masenog spektrometra s induktivno spregnutom plazmom (ICP-MS) radi određivanja koncentracija metala i nemetala u tako dobivenim frakcijama (Montes-Bayón i sur., 2003) čine značajan početni korak u prepoznavanju biomolekula koje vežu metale, kao i preduvjet za njihovu točnu identifikaciju. Kako bi se što jednostavnije opisali dobiveni profile raspodjele metala, prema Krasnići i sur. (2013, 2014, 2018), definirane su četiri glavne kategorije biomolekula koje vežu metale, uzimajući u obzir njihovu molekulsku masu:

1. VMM ili biomolekule visokih molekulskih masa (>100 kDa);
2. SMM ili biomolekule srednjih molekulskih masa (>30-100 kDa);
3. NMM ili biomolekule niskih molekulskih masa (10-30 kDa);
4. JNMM ili biomolekule jako niskih molekulskih masa (<10 kDa).

Kadmij je neesencijalni element koji može biti toksičan već pri vrlo niskim koncentracijama te kroz kompeticiju s nekim esencijalnim elementima dovodi do pogrešnog smatanja proteina, pojave oksidacijskog stresa i oštećenja stanica (Sedak i sur., 2015). Dva glavna mehanizma toksičnosti uključuju inhibiciju aktivnosti enzima vezanjem na -SH skupine te zamjenu Zn na veznim mjestima biološki važnih molekula (Landis i Yu, 1999). Citosolske koncentracije Cd u probavilu potočnih pastrva bile su u rasponu od 2,25-271,3 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 25,1-753,0 $\mu\text{g kg}^{-1}$. Kadmij je bio raspodijeljen unutar jednog jasnog NMM molekuskog pika čiji maksimum po vremenu eluiranja i molekulskoj masi odgovara standardima MT, čime se potvrdio visok afinitet MT za vezanje Cd kao jedan od glavnih mehanizama detoksikacije ovog elementa (Roesijedi, 1992). Porast citosolskih

koncentracija Cd doveo je do povećanog eluiranja unutar MT područja, dok vezanje Cd na dodatne biomolekule u VMM području, koje ukazuje na potencijalnu toksičnost kod visokih koncentracija, nije uočeno osim slabo u uzorcima s najvišim koncentracijama (selo Trebež u jesen). S obzirom na više citosolske koncentracije Cd u babuškama, nego u pastrvama, i visina pika, odnosno količina eluiranog Cd u MT regiji je bila viša u babuškama. Vezanje na ovo područje sugerira uspješnu i gotovu potpunu detoksikaciju Cd u probavilu obiju vrsta riba. Dominantno vezanje Cd za isto područje je opisano i za jetru potočnih pastrva (Dragun i sur., 2018b), kao i jetru i škrge babuški (Dragun i sur., 2020) iz istih uzorkovanja, kao i za druge slatkovodne ribe (Goenaga Infante i sur., 2003; Krasnići i sur., 2013, 2018; Caron i sur., 2018).

Kobalt je esencijalni element čija raspodjela je obuhvaćala četiri pika, ali je dominantno područje eluiranja ovisilo o vrsti. Citosolske koncentracije Co u probavilu potočnih pastrva bile su u rasponu od 5,31-121,3 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 11,3-39,9 $\mu\text{g kg}^{-1}$. Najveći dio citosolskog Co u probavilu potočne pastrve bio je vezan uz VMM biomolekule (85-235 kDa), dok je samo manji dio bio vezan u SMM (30-85 kDa) i JNMM (0,5-1,8 kDa i 2,4-18 kDa) područjima. I prijašnja istraživanja na raznim organima raznih vrsta riba ukazala su na visok afinitet iona Co za enzimske proteine visokih molekulskih masa što bi moglo obuhvaćati enzime poput metilmalonil CoA-mutaze ili 5- metiltetrahidrofolat - homocistein metiltransferaze (Banerjee i Ragsdale, 2003; Krasnići i sur., 2013, 2014, 2018; Paustenbach i sur., 2013; Caron i sur., 2018; Dragun i sur., 2018b). Povišenje citosolskih koncentracija dovelo je uglavnom do porasta eluiranja Co u VMM području. Iako su ista područja raspodjele Co zabilježena i u citosolu babuški, dominantno vezanje je uočeno u JNMM području. Glavnina Co u JNMM području se vezala na biomolekule u rasponu od 2,4-18 kDa, a nešto manji dio za biomolekule molekulskih masa 0,7-1,8 kDa. Vezanje za VMM i SMM bilo je vidljivo i u babuškama, ali gotovo zanemarivo. Iako je najpoznatija uloga Co vezana uz izgradnju strukture kobalamina (1,3 kDa; Blust, 2012), profili raspodjele Co u probavilu istraživanih riba samo manjim su dijelom ukazali na njegovo vezanje na područje koje obuhvaća molekularnu masu toga spoja (Kirschbaum, 1981). Dakle, uočene su neke specifične razlike ovisno o vrsti što je posljedica drugačije ekologije i biologije pojedine vrste te okolišnih uvjeta.

Bakar ima važnu ulogu u metaboličkim procesima koja uključuje njegovu katalitičku ili strukturalnu funkciju u mnogim enzimima i metaloproteinima (Harris, 2001). Ima izuzetno važnu ulogu u respiraciji, ali je potreban i za stvaranje vezivnog tkiva, održavanje mijelina i uklanjanje slobodnih radikala superoksida (Gaetke i sur., 2014). Citosolske koncentracije Cu

u probavilu potočnih pastrva bile su u rasponu od 0,21-1,348 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 0,358-1,361 $\mu\text{g kg}^{-1}$. Profili raspodjele ukazali su na dominantnu prisutnost u NMM području s maksimumima koji odgovaraju standardima MT pa se može pretpostaviti da se Cu u probavilu obiju vrsta riba, kao i Cd, uglavnom veže za MT. Dakle, može se zaključiti da MT imaju glavnu ulogu i u detoksikaciji Cu, što je potvrđeno i porastom prisutnosti Cu u području MT pri povišenju citosolskih koncentracija Cu. Ipak, u jedinkama s najvećim citosolskim koncentracijama Cu moglo se primijetiti i širenje pikova, što ukazuje na mogućnost vezanja i na druge citosolske biomolekule u slučaju visokih koncentracija. Dodatno vezanje u objema vrstama primijećeno je i u SMM regiji te pokriva biomolekule od 18-85 kDa, što obuhvaća molekulske mase raznih proteina koji sadrže Cu poput superoksid dismutaze (SOD, 32 kDa) i ugljične anhidraze (29 kDa) (Szpunar i Lobinski, 1999). Maksimum tog pika odgovarao je i vremenu eluiranja standarda SOD. Vezanje Cu uz SOD ima dvojaku ulogu te može upućivati i na esencijalnu ulogu tog elementa (Sanchez i sur., 2005), ali i na povećanu mogućnost toksičnih učinaka, odnosno potencijalne inhibicije tog enzima (Vutukuru i sur., 2006). Usporedba raspodjele u probavilu potočne pastrve s raspodjelom u jetri iste vrste ukazala je na sličnost u dominantnom vezanju u NMM području, ukazujući na važnu ulogu MT, ali u jetri je bilo vidljivo i vezanje za VMM područje (Dragun i sur., 2018b). Moguće da je ta razlika posljedica puno većih koncentracija Cu u jetri nego u probavilu, jer prema Dragun i sur. (2018b), s povećanjem koncentracije raste i količina Cu u VMM području. Usporedba u babuškama ukazala je na veću sličnost probavila s jetrom nego sa škragama, s većinskim vezanjem Cu u NMM području (Dragun i sur., 2020). Dominantno vezanje Cu za MT-frakciju opisano je u mnogim tkivima i vrstama (van Campenhout i sur., 2008, 2010; Krasnići i sur., 2013, 2014, 2018; Caron i sur., 2018; Urien i sur., 2018) što je očekivano za tkiva koja su aktivna u uzimanju, skladištenju i izlučivanju elemenata u tragovima, poput škrge, probavila ili jetara (Roesijadi i Robinson, 1994).

Esencijalna uloga Fe ogleda se u transportu kisika putem hemoglobina, sudjelovanju u DNA sintezi, metabolizmu masnih kiselina i tirozina te zaštiti od bakterijskih infekcija (Vidal i sur., 1993; Bury i sur., 2012; Kuhn i sur., 2016). Citosolske koncentracije Fe u probavilu potočnih pastrva bile su rasponu od 2,0-12,20 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 8,27-22,03 $\mu\text{g kg}^{-1}$. Profili raspodjele u probavilu ukazali su na njegovu prisutnost u dva ili tri područja citosolskih biomolekula, ovisno o vrsti. Prvi pik u VMM području (182-1088 kDa) te drugi u SMM području (18-109 kDa) bili su zajednički za obje vrste riba, dok je samo u potočnih pastrva bio vidljiv i treći pik u NMM području, odnosno u nekoliko uzoraka s izvora rijeke

Krke u VNMM području, pokrivajući područje od 1,8-14 kDa, što je upućivalo na moguće vezanje na nukleotide, aminokiseline, pirofosfate i komplekse Fe (Beard i sur., 1996). Maksimum VMM pikova obuhvaćao je vezanje uz biomolekule 400-500 kDa čime je opravdano misliti da se radi prvenstveno o vezanju za feritin (450 kDa, Szpunar i Lobinski, 1999), poznati protein za skladištenje Fe. Dominantno vezanje Fe u potočnih pastrva ipak je zabilježeno u SMM području što ukazuje na vezanje za različite proteine poput hemoglobina (65 kDa), transferina (80 kDa, Asmamaw, 2016), feroportina (63 kDa) ili podjedinica katalaze (60 kDa) (Martin-Antonio i sur., 2009). Također, prema Krasnići i sur. (2019) može se raditi i o monomerima (15,5 kDa), dimerima (31,5 kDa) i trimerima (47 kDa) podjedinica hemoglobina, kao u jetri i škrigama Vardarskog klena. Slična raspodjela među citosolskim biomolekulama zabilježena je i u jetri potočnih pastrva, osim što je dominantno bilo vezanje za VMM područje, vjerojatno kao posljedica viših koncentracija Fe u jetri nego u probavilu, kao i važnijoj skladišnoj ulozi jetre (Walker i Fromm, 1976). Kod babuške je raspodjela bila podijeljena između VMM i SMM područja pri čemu je raspodjela među VMM biomolekulama bila dominantna na objema postajama rijeke Ilove, ukazujući na vezanje na feritin. Raspodjela među citosolskim biomolekulama jetara i škriga babuški ukazala je na slične obrasce te na veću sličnost probavila s jetrom gdje je dominantno vezanje također zabilježeno u VMM području (Dragun i sur., 2020), dok je u škrigama dominantan pik smješten u SMM području. Povišene koncentracije Fe su praćene porastom prvog ili drugog pika, ovisno o uzorku. Uočili smo i sličnost raspodjele u probavilu babuške s profilima raspodjele u citosolima jetri i škriga klenova, gdje također nije zabilježeno vezanje Fe u JNMM području (Krasnići i sur., 2013, 2014, 2018) što upućuje na slične mehanizme vezanja Fe u vrstama koje pripadaju istoj porodici, šaranima.

Raspodjela Mo zabilježena je u VMM i JNMM području u objema vrstama riba. Citosolske koncentracije Mo u probavilu potočnih pastrva bile su u rasponu od 17,3-49,7 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 49,5-81,8 $\mu\text{g kg}^{-1}$. U objema vrstama dominantno vezanje je zabilježeno u JNMM području, iako je VMM područje obuhvaćalo molekulske mase enzima kojima Mo služi kao kofaktor poput Fe-Mo flavoprotein ksantin oksidaze (275 kDa, Truglio i sur., 2002), aldehid oksidaze (130 kDa, Uchida i sur., 2003) te sulfid oksidaze (120 kDa, Johnson i Rajagopalan, 1976). Raspodjela unutar VMM područja bila je dominantna u jetri potočne pastrve, babuške i klenova potvrđujući specifičnost ovih enzima za pojedina tkiva (Krasnići i sur., 2013, 2018; Dragun i sur., 2018b, 2020). Glavni pik u našem istraživanju obuhvaćao je raspon od 1,8-8 kDa s maksimumom na 5,1 kDa, slično kao u škrigama babuški (Dragun i sur., 2020). Sličnost probavila i škriga vjerojatno odražava nedavni unos Mo putem

vode ili hrane, a rast citosolske koncentracije Mo je uglavnom doveo do znatnijeg vezanja za JNMM područje.

Biološka uloga Se primarno se odnosi na njegovu ugradnju u proteine u obliku selenocisteina te čini sastavni dio glutathion peroksidaze, tioredoksin reduktaze ili vitamina E (Watanabe i sur., 1997). Citosolske koncentracije Se u probavilu potočnih pastrva bile su u rasponu od 0,362-1,784 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 0,377-0,560 $\mu\text{g kg}^{-1}$. Profili raspodjele Se u probavilu ovih dviju vrsta ukazali su na razlike među vrstama u broju pikova te području dominantnog vezanja. U potočnih pastrva Se je raspodijeljen u dva pika u JNMM području pri čemu je glavina vezanja zabilježena za područje 0,4-1,4 kDa, slično kao i u jetri potočnih pastrva (Dragun i sur., 2018). Takvo vezanje u probavilu bi moglo predstavljati oblike selenocisteina ili selenometionina u citosolu, putem kojih ribe i unose Se (Janz i sur., 2010). Uz to, može se raditi i o komponentama aktivnim u obrani organizma od oksidacijskog stresa, poput selenoneina (0,5 kDa, Yamashita i Yamashita, 2010). Povišenje citosolskih koncentracija Se u pojedinim uzorcima dovelo je povećanog vezanja za biomolekule < 1,5 kDa, što je uz pastrve, već zabilježeno i u škragama klenova (Krasnići i sur., 2014, 2018). Uz JNMM pikove koji su u babuški obuhvaćali biomolekule u rasponu od 0,4-11 kDa, potencijalno uključujući i selenoprotein SelW (~10 kDa), koji ima antioksidacijsku ulogu (Lopez Heras i sur., 2011), Se se u ovoj vrsti vezao i u VMM području u rasponu od 30-303 kDa s maksimumom oko 110 kDa. Taj raspon obuhvaća poznate selenoproteine poput glutathion peroksidaze (85 kDa, Shulgin i sur., 2008) i tioredoksin reduktaze (66 kDa). Iako su citosolske koncentracije Se u babuškama bile vrlo slične na objema lokacijama, vidljivo je da je porast koncentracije u riba s referentne postaje rezultirao porastom VMM pika, a u riba s onečišćene postaje porastom JNMM pika. U škragama babuški, Dragun i sur. (2020) su zabilježili 4 pika, s većinskim vezanjem Se u JNMM području, dok je u jetri većina Se bila vezana za VMM područje, a samo neznatan dio raspodjele bio je vezan uz dva pika prisutna u JNMM području.

Cink sudjeluje u različitim biološkim procesima uključujući metabolizam proteina ili lipida, staničnu signalizaciju, rad i zaštitu imunološkog sustava, odnosno ima i katalitičku i regulacijsku i strukturalnu funkciju (Coelman, 1992). Citosolske koncentracije Zn u probavilu potočnih pastrva bile su u rasponu od 26,2-61,5 $\mu\text{g kg}^{-1}$, a u probavilu babuški od 75,9-147,2 $\mu\text{g kg}^{-1}$ što je vjerojatno utjecalo i na uočene razlike u raspodjeli Zn između dviju vrsta. Kod potočnih pastrva iz rijeke Krke, raspodjela Zn zabilježena je u dva pika u VMM području, pri čemu je dominantno vezanje na objema postajama uočeno u području od 392-1088 kDa. Slični pikovi zabilježeni su i u jetri pastrva, iako je u jetri bilo prisutno vezanje Zn i na NMM

područje koje obuhvaća MT (Dragun i sur., 2018b). Međutim, iako je Zn-MT vezanje zabilježeno i u jetri klenova (Krasnići i sur., 2013, 2018), kao i jegulje (van Campenhout i sur., 2008), u našem istraživanju nije bilo prisutno u probavilu pastrve. Slično probavilu, kao organu unosa metala hranom, vezanje Zn za MT nije zabilježeno ni u škragama više vrsta riba što potvrđuje njihovu sličnu ulogu u unosu metala (Krasnići i sur., 2014, 2018; Dragun i sur., 2020). Vezanje za molekule 30-235 kDa, nešto slabije zastupljeno u probavilu pastrva uključuje molekulske mase albumina (66 kDa), transferina (80 kDa), Zn-SOD (32 kDa) te alkohol dehidrogenaze (150 kDa) (Szpunar i Lobinski, 1999). Kod babuški, Zn se, uz VMM područje, vezao i u JNMM području u dva pika. Ta regija je pokrivala biomolekule u rasponu od 0,7-14 kDa, čime se potvrdilo i moguće vezanje Zn na MT, ali to nije bilo jasno izraženo. Vezanje za JNMM područje zabilježeno je i u škragama, ali ne i jetri babuški (Dragun i sur., 2020), kao i u škragama Europskog klana (Krasnići i sur., 2013). Vezanje u području JNMM potencijalno ukazuje i na važnu ulogu Zn u antioksidacijskoj obrani putem vezanja za GSH (307 Da), tiol koji ima ulogu u detoksikaciji metala i ponekad čini prvu liniju obrane, čak i prije MT (Lavradas i sur., 2016). Porast citosolskih koncentracija Zn doveo je do porasta visine već postojećih pikova, a značajne promjene u raspodjeli Zn kao posljedica razlika u koncentraciji nisu bile vidljive. U većini uzoraka dominantno je bilo vezanje za VMM područje.

Rezultati ovog dijela istraživanja ukazali su kako na sličnosti, tako i na razlike u raspodjeli elemenata među citosolskim biomolekulama različitih molekulskih masa u probavilima potočnih pastrva iz rijeke Krke i babuški iz rijeke Ilove. Nije bilo značajnih razlika u citosolskoj raspodjeli Cd, Cu i Mo, dok su razlike u dominantnim ili dodatnim područjima raspodjele zabilježene za Co, Fe, Se i Zn. U objema vrstama bilo je izraženo vezanje Cd i Cu za MT, ali ne i Zn. Vezanje Fe za JNMM biomolekule je zabilježeno samo u pastrvi, a Se za VMM te Zn za JNMM biomolekule samo u babuški. Većina esencijalnih elemenata u objema vrstama bila je raspodijeljena u više pikova i područja što je u skladu s njihovom značajnom fiziološkom ulogom. Usporedba s drugim organima istih vrsta ukazala je na specifičnosti svakog organa ovisno o njegovoj biološkoj funkciji. Nadalje, elementi su većinom bili vezani uz ista molekulska područja u svim uvjetima i vrstama, samo u drugim količinama i omjerima, ovisno o višoj bioakumulaciji u određenim jedinkama te o funkciji organa i svojstvima organizma. Stoga je djelomično potvrđena hipoteza H4 da se raspodjela metala mijenja pri različitim razinama izloženosti metalima, kao i među organizmima. No, kako su strategije često bile slične u objema vrstama riba iz ovih čistih do umjereno

onečišćenih rijeka, razlike koje bi mogle nastati uslijed jačeg onečišćenja, treba dodatno istražiti.

4.4. Rakušci kao bioindikatorski organizmi

U svrhu usporedbe akumulacije metala i procjene stanja okoliša u različitim bioindikatorskim organizmima, uz ribe su uzorkovani i rakušci kao predstavnici beskralješnjaka te kao važna karika u životnom ciklusu kukaša. Budući da predstavljaju međudomadare kukaša (Kennedy, 2006), rakušci iz rijeke Krke uključeni su u istraživanje i kako bismo procijenili njih kao bioindikatore, ali i kao dio procjene kukaša kao bioindikatora izloženosti metalima. Rezultati dijela istraživanja vezanog uz akumulaciju metala u rakušcima nalaze se u radu 1 na listi radova za rakušce iz rijeke Krke te u Prilogu 2 za rakušce iz rijeke Ilove.

4.4.1. Biološki pokazatelji u rakušaca

Različiti rakušci roda *Gammarus* uzorkovani su iz rijeka Krke i Ilove za dodatnu procjenu akumulacije metala u organizmima, kao i stanja okoliša. Na izvoru rijeke Krke uzorkovali smo dvije vrste: *Gammarus balcanicus* te *Echinogammarus acarinatus*, dok je na nizvodnoj postaji kod Knina bila prisutna samo vrsta *G. balcanicus*. Od ranije je poznato da je izvor rijeke Krke stanište dviju endemskih svojti područja Dinarida, već spomenute vrste *E. acarinatus* i podvrste *Fontogammarus dalmatinus krkensis* S. Karaman, 1931 (Gottstein i sur., 2007; Žganec i sur., 2016), koja nije pronađena u našem istraživanju, vjerojatno kao posljedica načina uzorkovanja i njezinog specifičnog mikrostaništa unutar mahovina te nastanjivanja samog izvora Krke tj. zone eukrenala (Žganec i sur., 2016). Mahovina ima obrambenu ulogu od predacije i struje vode, a predstavlja i izvor hrane za rakušce jer zarobljuje čestice detritusa. Ove dvije endemske svojte moguće je zajedno pronaći i u čistim, izvorišnim i gornjim dijelovima rijeke Une dok nisu prisutne u srednjem i donjem toku (Žganec i sur., 2010). Također, odsustvo ovih dviju endemskih svojti s područja rijeke Krke pod antropogenim utjecajem kod Knina je također već ranije opisano u rijeci Krki, iako je vrstu *E. acarinatus* moguće pronaći u mnogim dijelovima toka rijeke Krke s različitim ekološkim zonacijama (Gottstein i sur., 2007; Žganec i sur., 2016) te mozaičnu rasprostranjenost ove vrste u ovom riječnom toku nije moguće objasniti samo na temelju varijabilnosti fizikalno-kemijskih čimbenika (Žganec, 2009).

Nadalje, vrsta *E. acarinatus* bila je manjih dimenzija tijela i manje mase nego *G. balcanicus*, a razlika u masi očitovala se i među jedinkama vrste *G. balcanicus* jer su sitniji

primjerci nađeni na izvoru rijeke, nego na nizvodnijoj postaji, što je vjerojatno posljedica brzine toka koja smanjuje količinu dostupnih hranjivih tvari (Suren, 1991). Vjerojatno zbog manjih energetske potrebe malih vrsta i jedinki (White i sur., 2007), sitnija vrsta *E. acarinatus* je na izvoru bila brojnija, nego vrsta *G. balcanicus*. Uz to, općenito je riječ o ekološkoj zonaciji s obzirom na termiku, tip i količinu ponuđene hrane i specifična preferirana mikrostaništa svake od navedenih vrste rakušaca. Mikrostaništa se razlikuju s obzirom na sastav supstrata, brzinu strujanja, količinu organskog materijala, prisutnost makrofita kao što su mahovine i cvjetnice te prisutnost algi. Supstrat predstavlja prostor za kretanje, odmaranje, razmnožavanje ili sakupljanje hrane te ga čini organska (komadići lišća, mrtva vodena vegetacija i uginule životinje) i anorganska komponenta (stijene, mulj, pijesak, šljunak, obluci) (Giller i Malmqvist, 1998). Mahovina, kao vrlo pogodno mikrostanište, pak omogućava zaklon od predacije ili struje vode, osobito manjim vrstama rakušaca, te služi i kao izvor hrane. Uz to, vrste roda *Gammarus* preferiraju staništa s vodenom vegetacijom u usporedbi sa šljunkom ili golim pijeskom (MacNeil i Prenter 2000), a temperatura je općenito jedan od najvažnijih čimbenika koji regulira njihovu rasprostranjenost (Žganec, 2009).

U rijeci Ilovi rakušci roda *Gammarus* su pronađeni samo na najuzvodnijoj postaji u blizini sela Maslenjača, dok je svega nekoliko jedinki ovog roda uzorkovano kod sela Ilove, a kod sela Trebeža uzorkovano je tek nekoliko jedinki invazivne vrste iz roda *Dikerogammarus*. Na Maslenjači su uzorkovane dvije vrste rakušaca - *G. fossarum* te *G. roeselii*, pri čemu je veća brojnost zabilježena za vrstu *G. fossarum*. Također, prosječno su primjerci *G. roeselii* bili veći, nego *G. fossarum*. Longitudinalna distribucija rakušaca ovisi o kompleksnim interakcijama između fizikalno – kemijskih čimbenika te o biotičkim interakcijama, a među najvažnijim fizikalno-kemijskim čimbenicima su: temperatura vode, koncentracija kisika u vodi, koncentracija kalcijevih iona, alkalinitet, tip supstrata te strujanje vode (MacNeil i sur., 2000). Mnogi autori longitudinalnu zonaciju određenih vrsta rakušaca pripisuju upravo kombiniranom utjecaju temperature i nekih drugih već spomenutih čimbenika, kao što su brzina strujanja vode, pH vrijednost, koncentracija otopljenog kisika u vodi i onečišćenje (Žganec, 2009). Uz to, na rasprostranjenost vrsta utječu i kompeticija, predacija i prisutnost parazita. Nedostatak rakušaca u donjem toku rijeke odražava i njihovu prirodnu i uobičajenu distribuciju te slijedi koncept riječnog kontinuiteta (Vannote i sur., 1980) prema kojem u gornjim tokovima rijeka, zbog pritoka alohtone organske tvari s kopna, poput otpalog lišća i komadića drveta, u zajednici makrobekralješnjaka dominiraju usitnjivači te detritivori (eng. *shredders*) koji prikupljaju sitnije čestice nastale usitnjavanjem listinca, što se smatralo i funkcionalnom grupom rakušaca (Cummins, 1973). Međutim, danas takva kategorizacija nije

toliko zastupljena jer se pokazalo da rakušci zauzimaju i puno više položaje u hranidbenim lancima te da velik udio u njihovoj prehrani mogu činiti i alge, biljni te životinjski materijal (Kelly i sur., 2002; Glazier, 2009), dok njihovu stopu usitnjavanja listinca uvelike povećava prisutnost raznolikih vrsta gljiva (Lecerf i sur., 2005; Glazier, 2009) koje time zapravo reguliraju brojnost i stopu rasta rakušaca i sudjeluju u razgradnji listinca. Sama struktura staništa i sastav supstrata su među dominantnim čimbenicima koji utječu na distribuciju svih svojti beskralješnjaka u mnogim slatkovodnim sustavima.

4.4.2. Citosolske koncentracije metala u rakušcima

Zbog postojanja hitinskog oklopa kod rakušaca, koncentracije metala mjerene su samo u citosolskoj, odnosno topljivoj i metabolički dostupnoj frakciji. Homogenate zbog viskoznosti i gustoće uzorka nije bilo moguće odvojiti u preciznom volumenu te stoga nismo mogli dobiti vrijednosti citosolskih i ukupnih metala u istim jedinkama.

Nadalje, razlike između referentne i onečišćene lokacije bilo je moguće testirati samo na rijeci Krki za vrstu *G. balcanicus*, budući da je, kako je već navedeno, vrsta *E. acarinatus* bila prisutna samo na izvoru rijeke. Također, u rijeci Ilovi je bilo moguće uzorkovati rakušce samo na jednoj lokaciji pa su stoga uspoređene sezonske razlike. Provedena je i usporedba specifičnih razlika između više vrsta ovog roda, unutar svakog istraživanog ekosustava, čiji se pripadnici često koriste kao bioindikatorski organizmi (Rinderhagen i sur., 2000; Gerhardt i sur., 2011).

Kod vrste *G. balcanicus* iz rijeke Krke zabilježene su statistički značajno više koncentracije Co, Fe, K, Mn, Mo i Na na onečišćenoj postaji kod Knina u odnosu na referentnu postaju u objema sezonama te dodatno As u jesen te Cu i Zn u proljeće. Na izvoru u odnosu na postaju kod Knina u objema sezonama bile su povišene koncentracije Cd, Cs i Tl, jednako kao i u probavilu potočnih pastrva. Dakle, više organizama je potvrdilo neobičan trend povišenih vrijednosti ovih elemenata na izvoru i ukazalo na njihov značajan izvor koji zahtijeva dodatna istraživanja. Za organizme koji se velikim dijelom hrane detritusom općenito postoje dva načina unosa metala u organizam: unos putem sedimenata bogatih metalima tijekom hranjenja te unos iz vode, pri čemu je unos putem vode značajniji tijekom presvlačenja (Luoma, 1989; Ternjej i sur., 2014). S obzirom na to da rakušci čine i važan dio prehrane potočnih pastrva, slični trendovi u oba organizma također upućuju na važnost unosa metala hranom u riba jer su upravo rakušci mogli poslužiti kao dodatni izvor tih elemenata za ribe. Također, s obzirom da se radi o karbonatnom području i blago alkalnim uvjetima u rijeci Krki na objema lokacijama, metali se adsorbiraju na suspendiranu tvar i talože u sedimentima,

što utječe na brži pad koncentracija metala u vodi (Korfali i Davies, 2004; Cukrov i sur., 2008b), ali i na njihovu dostupnost vodenim organizmima putem hrane. Sezonske razlike kod vrste *G. balcanicus* nisu bile ujednačene, ali više značajnih razlika zabilježeno je na izvoru nego kod Knina, pri čemu je veći broj elemenata na izvoru bio povišen u jesen (Ca, Cd, Cu, Fe, Mg, Mn, Mo, Sr), dok je u Kninu više elemenata bilo povišeno u proljeće. Usporediv sezonski obrazac na izvoru rijeke pokazale su i koncentracije metala u vrsti *E. acarinatus* kod koje su razine Ca, Cd, Cs, K, Mg, Mn, Mo, Sr i Tl bile više u jesen, a samo As i Na u proljeće. Padajući redoslijed koncentracija u citosolu u objema vrstama bio je sljedeći: $Ca > K > Na > Mg > Sr \geq Zn > Cu > Rb > Fe > Mn > Se > As \geq Cd > Mo > Co \geq Tl > V > Cs$. Međutim, koncentracije metala u vrsti *E. acarinatus* bile su značajno više za As, Co, Cs, Mn i Tl u objema sezonama te za Ca, Cd i Na u proljeće, nego u vrsti *G. balcanicus*. Moguće objašnjenje viših koncentracija u vrste *E. acarinatus* je to što se radi o sitnijoj vrsti, slično istraživanju Rainbow i Moore (1986) koje je pokazalo značajno višu akumulaciju metala u sitnim rakušcima. Isti autori navode i specifične izvore hrane i mikrostaništa za blisko srodne vrste rakušaca, što u konačnici dovodi i do različitog unosa metala putem hrane i konačne različite akumulacije u pojedinoj vrsti.

U vrsti *G. roeselii* u rijeci Ilovi, značajne sezonske razlike u koncentracijama zabilježene su za Cd, Rb i Zn s višim vrijednostima u proljeće te za Ca, Mo i Sr s višim vrijednostima u jesen. U vrsti *G. fossarum* razlike između sezona bile su očite za veliki broj elemenata, pri čemu su Ca, K, Mo, Na i Sr bili viši u jesen, a Cu, Se, Rb, Tl i Zn u proljeće. Ti trendovi djelomično su ukazali na unos metala iz vode te su rakušci odražavali okolišne uvjete u vodi, ali kao i kod riba, u slučaju nekih elemenata sezonske razlike između organizama nisu posljedica unosa vodom (Mijošek i sur., 2020a). Općenito su koncentracije bile ili usporedive između dviju vrsta ili više u rakušaca vrste *G. roeselii*, što se pokazalo statistički značajnim za As i Mg u objema sezonama te za Co, Cu, Mn, Na, Tl i Zn u jednoj sezoni. Redoslijed koncentracija u citosolu bio je jednak u objema vrstama: $Ca > K > Na > Mg > Sr \geq Cu > Zn > Mn > Rb > Se > As \geq Mo > Co > Cd > V > Tl$, dakle bez značajno većih razlika u odnosu na rakušce iz rijeke Krke.

Citosolske koncentracije elemenata poput Co, K, Mo, Na, Sr i V bile su slične i usporedive u svim istraživanim vrstama rakušaca iz obiju rijeka, dok su elementi poput Cu, Mn i Zn bili viši u vrstama iz rijeke Ilove, a Cd, Se, Tl, Ca i Rb u dvjema vrstama iz rijeke Krke. U oba istraživana sustava je potvrđeno i da su koncentracije mnogih elemenata u citosolu više u rakušcima nego u probavilu riba, potvrdivši da se za većinu metala ne očekuje biomagnifikacija kroz hranidbene mreže (Mathews i Fisher, 2008). U rijeci Krki najveća

razlika u koncentracijama između rakušaca i riba očitovala se za Sr, Ca, Cu i As, pri čemu su srednje vrijednosti bile 50-60 puta više u rakušcima za Sr i Ca, 15 puta više za Cu te osam puta za As. Nadalje, dvostruko više vrijednosti u rakušaca su zabilježene i za Cd i Mg, dok su neki elementi poput K, Cs, Se i Rb ipak imali dvostruko više vrijednosti u potočnim pastrvama nego rakušcima, kao i Fe i Zn koji su u riba postizali i pet puta više vrijednosti. Gotovo identični trendovi su dobiveni i na rijeci Ilovi gdje su vrijednosti Sr i Ca bile 60-70 puta, Cu i Mn 7-8,5 puta te As i Mg oko tri puta više u rakušcima nego u babuškama. Cink je imao 15 puta više vrijednosti u ribama, Rb tri puta te K i Se dvostruko više vrijednosti u ribama. Očigledno je da su unatoč različitom staništu i samim ekologijama vrsta, sve vrste odražavale okolišne uvjete na sličan način i sa sličnim strategijama akumulacije metala. Dobiveni rezultati su u skladu s istraživanjem u morskom okolišu i hranidbenim mrežama gdje se sugerira mogućnost biomagnifikacije za Cs, Se i Zn, elemente koji su i u našem istraživanju bili viši kod riba (Mathews i Fisher, 2008). Također su očekivane i puno više koncentracije Ca i Sr kod rakušaca s obzirom na postojanje njihovog egzoskeleta osobito bogatog Ca u obliku kalcijevog karbonata (Greenway, 2008). Stroncij je drugi česti element u skeletu vodenih organizama koji može poslužiti kao zamjena za Ca (Mertz, 1987). Iako prirodno visoko zastupljen element, dugotrajno i povišeno djelovanje Sr može narušiti mineralizaciju oklopa i skeleta rakušaca (Mertz, 1987; Nielsen 2004) i na taj način utjecati na propusnost oklopa, pa potencijalno i na unos drugih metala koji se stoga u uvjetima onečišćenja posljedično može povećati (Gagné i sur., 2005; Ternjej i sur., 2014).

4.5. Usporedba kukaša, rakušaca i probavila potočne pastrve iz rijeke Krke kao bioindikatora izloženosti metalima

Nastavno na prethodni dio istraživanja rakušaca, idući korak je bio detaljnije procijeniti potencijal kukaša kao pogodnih bioindikatora u procjeni onečišćenja okoliša metalima. Stoga su u istraživanju korišteni kukaši vrste *D. truttae*, njihovi međudomadari rakušci *G. balcanicus* te probavilo potočne pastrve *S. trutta* kako bismo uključili sve organizme uključene u životni ciklus ove vrste kukaša. Uzorci kukaša, rakušaca te komadić probavila riba su na jednak način razgrađeni te su pomoću HR ICP-MS izmjerene ukupne koncentracije 15 elemenata. S obzirom na nedostatak kukaša iz rijeke Ilove, prije svega zbog nedostatka rakušaca kao njihovih međudomadara na istim postajama na kojima su uzorkovane ribe, dio istraživanja na kukašima proveden je samo na uzorcima iz rijeke Krke te su ovi rezultati prezentirani u tablicama u Prilozima 3, 4 i 5.

4.5.1. Biološka i epidemiološka obilježja kukaša

Zastupljenost, odnosno invadiranost kukašima u pastrvama je bila veoma visoka na objema lokacijama i u objema istraživanim sezonama (92,3-100 %), dok je prosječni intenzitet invazije iznosio između 5,9 i 42,0 ovisno o postaji i sezoni, ali veće vrijednosti zabilježene su u jesen nego u proljeće na objema postajama. Isti trend zabilježen je i za ukupan broj kukaša na objema lokacijama te je minimalna vrijednost od 76 kukaša zabilježena u proljeće na onečišćenoj postaji kod grada Knina, a maksimalna od 669 jedinki kukaša u jesen na istoj postaji. Veća brojnost u jesenskom razdoblju vjerojatno je posljedica životnog ciklusa rakušaca kao njihovih međudomadara, koji se uglavnom razmnožavaju u kasno ljeto i jesen te tako utječu i na veću brojnost kukaša (Kennedy, 1985). Prosječnu zastupljenost od 73 % u potočnim pastrvama iz rijeke Krke su zabilježili i Vardić Smrzlić i sur. (2013) tijekom 11 uzorkovanja provedenih između 2005. i 2008. godine, pri čemu su najviše vrijednosti postignute upravo u jesenskim razdobljima. U skladu i sa svim ranije navedenim indikatorima, brojnost i zastupljenost kukaša također je ukazala na tek umjereno onečišćenje rijeke Krke, budući da u jako onečišćenim okolišima dolazi do pada brojnosti nametnika (Mackenzie, 1999). Međutim, kako na ove parametre utječu i sezona, brojnost međudomadara, spol domadara i biometrijske osobine, takve kompleksne odnose i sustave je teško dovesti u vezu isključivo s izvorom nekog onečišćenja.

Brojnost kukaša pozitivno je korelirala s ukupnom dužinom riba (TL), a negativno s FCI. Iako u literaturi postoje podaci o utjecaju spola ribe na brojnost kukaša, u ovom istraživanju to nije dokazano kao značajan čimbenik, iako su prosječan i ukupan broj kukaša ukazivali na nešto veću stopu invazije u mužjaka. Manju brojnost kukaša vrste *D. truttae* u ženkama potočne pastrve zabilježili su i Vardić Smrzlić i sur. (2013), što je suprotno većini istraživanja koja često izvještavaju o većoj invadiranosti ženki nego mužjaka (Valtonen i Crompton, 1990; Filipović Marijić i sur., 2013).

4.5.2. Akumulacija metala u kukašima, rakušcima i probavilu potočne pastrve

Razlike u koncentraciji ukupnih metala između referentne i onečišćene postaje rijeke Krke bile su vidljive u svim organizmima, ali najosjetljivijim indikatorima su se pokazali kukaši s najviše zabilježenih statistički značajnih razlika. Naime, statistički značajne razlike između postaja zabilježene su za Rb, Cd, Co, Sr, Ca i Mg u objema sezonama te za Fe, Pb, Mn, Zn i K u jednoj sezoni, s trendom povišenja na onečišćenoj postaji za sve navedene elemente, osim za Cd i Rb koji su bili povišeni na referentnoj postaji. Dodatno, kao i u većini tkiva i/ili frakcija, i Tl je bio povišen u kukašima s referentne postaje, iako ne značajno

(Mijošek i sur., 2020b). I srednje vrijednosti većine drugih elemenata bile su više na onečišćenoj postaji, ali zbog varijabilnosti koncentracija između jedinki kukaša, te razlike nisu dokazane kao značajne. Sezonske razlike u akumulaciji metala u kukašima uglavnom nisu bile značajne, ali neki su elementi ipak bili povišeni u proljeće u odnosu na jesen, dakle izvan reproduktivnog razdoblja potočne pastrve. Povišene koncentracije metala u kukašima vrste *P. laevis* izvan reproduktivnog razdoblja njihovog domadara, mrena, zabilježili su Nachev i Sures (2016), objasnivši da sezonalnost ovisi o transmisiji kukaša, stadiju razvoja u probavilu domadara, kao i promjenama u aktivnosti i fiziologiji ribe domadara. Padajući niz ukupnih koncentracija u kukašima iz našeg istraživanja bio je $K > Na > Ca > Mg > Fe \geq Zn > Cu > Mn > Sr > Rb > Tl > Se > Pb \geq Cd > Co > Cs$. Filipović Marijić i sur. (2014) su istraživali akumulaciju metala u vrstama kukaša *P. laevis* i *A. anguillae* iz rijeke Save te su dobiveni redosljedi koncentracija $Zn > Fe > Cu > Mn > Pb > Cd$ za obje vrste što potvrđuje istu učinkovitost akumulacije u više vrsta kukaša iz različitih područja, dok vrijednosti i razlike u ukupnim koncentracijama govore o različitim mehanizmima akumulacije i detoksikacije u različitim vrstama, a sigurno i o razlikama u koncentraciji otopljenih metala u vodi između rijeka Save i Krke (Dragun i sur., 2009; Filipović Marijić i sur., 2018; Sertić Perić i sur., 2018). Analiza korelacija ukazala je na negativan odnos ukupne dužine riba (TL) i koncentracije Mn, Fe, Zn, Ca, Co, Sr, Mg i K u kukašima, kao i negativan odnos mase kukaša i koncentracije Na, Mg, Ca, K, Sr i Co. Spol riba nije pokazao značajnu povezanost s akumulacijom metala u kukašima.

U rakušaca vrste *G. balcanicus*, pretpostavljeno međudomadara kukaša vrste *D. truttae*, prostorne razlike u koncentraciji ukupnih metala su se očitovale kao značajno više vrijednosti Mn, Fe, Co i Pb na onečišćenoj postaji nego referentnoj postaji u objema sezonama te Cs, Cu, K, Sr i Zn u jednoj sezoni, dok su Cd, Rb i Tl bili povišeni u organizmima s referentne u odnosu na onečišćenu postaju. Sličan obrazac bio je vidljiv i u ranije opisanim citosolskim koncentracijama iste vrste rakušaca. Sezonski trendovi nisu bili ujednačeni, no nekoliko je elemenata imalo višu akumulaciju u jesen nego u proljeće. Koncentracije metala u rakušcima su pratile padajući niz $Ca > K > Na > Mg > Fe \geq Sr > Zn > Cu > Mn > Rb > Se > Cd > Co \geq Pb > Tl > Cs$, slično kao i za citosolske koncentracije rakušaca, izuzev Fe koji je u citosolskoj frakciji prisutan u malom postotku. Rakušci su pokazali malen broj značajnih korelacija s masom uzorka rakušaca. Preciznije, masa uzorka negativno je korelirala samo s koncentracijom Cs, a pozitivno samo s koncentracijom Na, što upućuje na to da rakušci dobro prate i odražavaju okolišne uvjete, kao i na potencijal njihove primjene kao dobrih

bioindikatora stanja okoliša (Geffard i sur., 2007; Lebrun i sur., 2014; Filipović Marijić i sur., 2016a).

Najmanje značajnih razlika u koncentracijama ukupnih metala između postaja pokazalo je probavno tkivo potočne pastrve s višim vrijednostima Sr na onečišćenoj postaji u objema sezonama te Tl na referentnoj, dok je razlika u samo jednoj sezoni bila vidljiva za Rb, Cs, Mn, Se, Cd, Co i Na. Pri tome su Cd, Cs, Na i Rb bili viši u riba s referentne postaje, a ostali navedeni elementi na onečišćenoj postaji. Kao i kod kukaša i rakušaca, ukupne koncentracije u probavilu pokazale su mali broj sezonskih razlika, uglavnom samo na po jednoj istraživanoj postaji. Padajući niz koncentracija u probavilu riba bio je $K > Na > Mg > Ca > Zn > Fe > Rb > Cu \geq Se > Mn > Sr > Cd > Pb > Co \geq Tl > Cs$, što je potvrdilo već zabilježene trendove ukupnih koncentracija dobivenih razgradnjom homogenata i citosolskih koncentracija za jedinke ove vrste iz istih uzorkovanja. Spol ribe nije imao utjecaj na akumulaciju metala, dok je mali broj statistički značajnih korelacija zabilježen s dužinom riba, ali ne na objema lokacijama i/ili sezonama. I ovaj dio istraživanja potvrdio je da s obzirom na mali broj korelacija između koncentracija metala i spola, dužine ili mase riba, probavilo može biti korišteno kao pouzdan bioindikator izloženosti metalima i stvarnih okolišnih uvjeta.

Iako ne uvijek statistički značajno, kukaši, rakušci i probavilo potočnih pastrva održavali su okolišne uvjete na sličan način s povišenim koncentracijama Co, Cu, Fe, Mn, Se i Sr na onečišćenoj u odnosu na referentnu postaju rijeke Krke, dok su koncentracije Cd i Tl te uglavnom Rb bile povišene na referentnoj postaji u svim organizmima. Padajući nizovi koncentracija također su bili slični s najvišim koncentracijama esencijalnih elemenata, a najnižim koncentracijama Cd, Co, Pb, Tl i Cs. Utjecaj sezone nije bio jasno izražen niti potpuno ujednačen među ovim bioindikatorskim organizmima. Nadalje, najviše koncentracije Cd, Cu, Mn, Na i Pb zabilježene su u kukašima, Ca, Co, Cs, Fe, Mg, Se i Sr u rakušcima te K, Rb i Zn u probavilu potočnih pastrva.

Kako bismo dodatno opisali kompleksni odnos kukaša i njihovih domadara, napravili smo i analizu korelacije akumulacije metala u probavilu i pripadajućih kukaša te dobili pozitivan odnos za Cs, Rb i Sr. Nadalje, broj kukaša nađen u probavilu nije značajno utjecao na akumulaciju metala u riba, ali pozitivan odnos, odnosno povišene koncentracije u probavilu u jedinkama s većim brojem kukaša, zabilježen je za Cd i Na, te negativan za Co. Iako je više istraživanja potvrdilo i zaštitnu ulogu kukaša za njihove domadare u smislu smanjene akumulacije metala u riba invadiranih kukašima (Sures i Siddall, 1999; Oyoo-Okoth i sur., 2012; Filipović Marijić i sur., 2013; Paller i sur., 2016), u našem istraživanju s obzirom na nedostatak neinvadiranih riba nije bilo moguće testirati tu tezu. Suprotno zaštitnoj ulozi,

ukoliko su kukaši pričvršćeni duboko u epitelnoj sluznici probavila, mogu uzrokovati i histopatološka stanja koja dovode do fibroze. Može doći do perforacija, upale tkiva i sustavnih kliničkih promjena organizma (Shafaquat i sur., 2016). Dva su glavna čimbenika koja utječu na potencijalno negativno djelovanje kukaša, njihova gustoća te dubina prodiranja u tkivo (Taraschewski, 2000). Kako je istraživana vrsta *D. truttae* poznata kao vrsta koja prodire duboko u tkivo svojih domadara (Dezfuli i sur., 2011), u jednom dijelu istraživanja smo pratili histopatološke promjene stanica probavila potočnih pastrva pod utjecajem povišenih razina metala, ali i velike brojnosti kukaša, te se primjećena oštećenja crijevnih resica, smanjeni broj nabora sluzice kao i povećana brojnost eozinofila mogu dovesti u izravnu vezu s invazijom nametnika, dok su se promjene poput degeneracije resica, nepravilnog izgleda epitelnih stanica i narušavanja epitelne površine, smanjenog broja vrčastih stanica s ubrzanom proizvodnjom sluzi, nekroza te atrofija s infiltracijom leukocita mogle povezati s povišenim razinama metala na onečišćenoj, u odnosu na referentnu postaju (Barišić i sur., 2018).

Naši rezultati su potvrdili veću sposobnost akumulacije metala u kukašima, nego mekim tkivima riba (Sures i sur., 1999; Thielen i sur., 2004; Filipović Marijić i sur., 2014), što je vjerojatno posljedica njihove ovisnosti o nutrijentima domadara s obzirom na to da sami kukaši nemaju probavni sustav. Dodatno su izračunati biokoncentracijski faktori (BCF) u odnosu na probavilo riba kao kombinacija kratkoročne i dugoročne izloženosti metalima s obzirom na to da je životni vijek kukaša relativno kratak i traje 50-140 dana (Kennedy, 1985) u odnosu na životni vijek riba od 10-15 godina (Kottelat i Freyhof, 2007). Kao popratnu informaciju izračunali smo BCF i u odnosu na rakušce, iako u istraživanju nismo imali ličinački stadij cistakanta koji bi omogućio preciznu usporedbu s rakušcima, ali već je ranije dokazano da u tom stadiju kukaši imaju puno manju sposobnost akumulacije metala, nego kao odrasli oblici (Sures, 2001).

S obzirom na uglavnom veće koncentracije ukupnih metala u rakušcima nego u ribama, BCF su bili niži u odnosu na rakušce nego u odnosu na probavilo riba. Padajući niz $BCF > 1$ u odnosu na rakušce bio je $Tl > Pb \geq Cd > Cu \geq Zn > Mn \geq Rb \geq K > Na$, dok su vrijednosti BCF manje od 1 zabilježene za Co, Cs, Fe, Se, Sr, Ca i Mg što je značilo da su koncentracije tih elemenata bile više u rakušcima, nego u kukašima. Vrlo niske vrijednosti BCF očekivano su zabilježene za Ca i Sr s obzirom na njihove visoke razine u skeletu rakušaca (Greenway, 2008). Vrijednosti BCF u odnosu na rakušce su bile uglavnom više na onečišćenoj postaji, dodatno sugerirajući visoku osjetljivost na prisutnost metala i brzu reakciju kukaša na promjene u okolišu. Padajući niz $BCF > 1$ u odnosu na probavilo potočnih pastrva bio je $Cd \geq$

$Tl > Cu > Pb > Sr > Mn > Ca > Co \geq Fe \geq Na > Mg$, što je ukazalo na osobito učinkovitu akumulaciju potencijalno toksičnih metala poput Cd, Tl i Pb u kukaša u odnosu na ribe, kao i na njihovu eventualnu zaštitnu ulogu, što zahtijeva dodatna istraživanja. Cezij, K, Rb, Se i Zn su bili elementi koji su pokazali veću akumulaciju u probavilu potočne pastrve nego u kukašima. Sezonske su vrijednosti BCF (u odnosu na ribe) bile uglavnom više u proljeće nego u jesen, što je vjerojatno posljedica uglavnom nižih koncentracija metala u probavilu u tom razdoblju, jer se radi o sezoni izvan razdoblja mrijesta, a ne samog utjecaja izloženosti metalima (Filipović Marijić i sur., 2013). Vrijednosti BCF su, kao i u odnosu na rakušce, također bile više na onečišćenoj postaji. Visoke vrijednosti BCF (u odnosu na ribe) za Cd, Cu, Mn, Pb i Sr ukazale su na značajnu nedavnu izloženost ovim elementima u rijeci Krki, dok vrijednosti BCF malo iznad 1 za Co, Fe, Mg i Na upućuju na izloženost organizama tim elementima kroz duže vrijeme. Iako je isti trend bio vidljiv i na izvoru rijeke i na onečišćenoj postaji nizvodno od Knina, zabilježene više vrijednosti BCF mnogih elemenata na onečišćenoj, nego referentnoj postaji govore o izraženijem unosu metala na onečišćenoj postaji, vjerojatno kao posljedica antropogenih aktivnosti i otpadnih voda u tom dijelu toka rijeke.

U znanstvenoj literaturi nešto je češće opisivana akumulacija metala u kukašima rodova *Pomphorhynchus* te *Acanthocephalus*, odnosno najčešće vrsta *P. laevis* te *A. lucii* i *A. anguillae* (Sures i Taraschewski, 1995; Sures i sur., 1999; Schludermann i sur., 2003; Thielen i sur., 2004; Nachev i sur., 2010; Filipović Marijić i sur., 2013, 2014). Zajednička karakteristika svih tih istraživanja i vrsta, uključujući i naše, je da su uvijek zabilježene najviše vrijednosti BCF za Cd, Cu, Pb i Ag. To potvrđuje činjenicu o izuzetno velikoj sposobnosti akumulacije metala, osobito toksičnih, u različitim vrsta kukaša. U Hrvatskoj su od ranije poznati rezultati o akumulaciji metala u vrstama *P. laevis* te *A. anguillae* iz klenova iz rijeke Save (Filipović Marijić i sur., 2013, 2014). Od tih dviju vrsta, BCF u odnosu na probavilo klena su bili viši za vrstu *P. laevis*, slično kao što su dokazali i Sures i Taraschewski (1995), a u objema vrstama su pratili redoslijed $Ag > Pb > Cd > Cu > Mn$. Vrijednosti BCF u odnosu na probavilo klena u objema vrstama kukaša kretale su se u rasponu 13,2-57,6 za Ag, 19,1-48,1 za Pb, 6,6-10,8 za Cd, 3,5-6,4 za Cu te 1,9-3,5 za Mn (Filipović Marijić i sur., 2014). Dakle, izuzev Pb, vrijednosti BCF za *D. truttae* u našem istraživanju bile su više nego vrijednosti za *P. laevis* i *A. anguillae* u prethodno provedenim istraživanjima u Hrvatskoj. S obzirom da su koncentracije metala u vodi rijeke Krke niže nego u Savi (Dragun i sur., 2009; Sertić Perić i sur., 2018), ovi rezultati upućuju na to da je vrsta *D. truttae* dobar indikatorski organizam, osjetljiv čak i na niske okolišne koncentracije metala te da bi mogao imati i

prednost u odnosu na neke bolje istražene vrste kukaša poput vrste *P. laevis*. Time je izravno potvrđena hipoteza H5, kao i neposredno hipoteze H1 i H2.

4.6. Usporedba akumulacije metala u mekim i tvrdim tkivima riba te u kukašima u proljeće 2015. godine

Prethodno opisane analize metala u probavilu riba, kao i u svim mekim tkivima korisni su pokazatelji nedavne izloženosti onečišćenju, ali detoksikacija, metabolička transformacija i preraspodjela u tkivima mogu promijeniti biološke odgovore organizma te njihova upotreba zahtijeva kontinuirano uzorkovanje kako bi se dobila realna procjena stanja okoliša.

Iz tog razloga smo dodatno koristili ljuske i otolite potočne pastrve iz rijeke Krke, kako bismo procijenili njihovu primjenu u procjeni dugoročne akumulacije metala u riba, a ljuske još i kao neletalnu alternativu bioindikatorskog tkiva. Analiza upotrebe tvrdih tkiva riba u procjeni stanja okoliša i izloženosti metalima primjenjena na uzorcima iz uzorkovanja na rijeci Krki u travnju 2015. godine, koje je prethodilo ranije opisanim istraživanjima, kada su uz probavilo korištena tvrda tkiva (otoliti i ljuske), zatim jetra i mišić kao dodatna meka tkiva te nametnici u probavilu, kukaši, u svrhu sveobuhvatne procjene utjecaja onečišćenja u cjelini. Jetra je odabrana kao glavni metabolički i detoksikacijski organ (Linde i sur., 1998), a mišić zbog svoje važnosti u ljudskoj prehrani, zbog čega posljedično može utjecati i na zdravlje ljudi (Carvalho i sur., 2005). Kukaši su, kako je već navedeno, opisani kao organizmi s visokom sposobnošću akumulacije metala, koja je puno učinkovitija nego u inače korištenim indikatorskim organizmima (Sures, 2004; Sures i sur., 2017), ali za vrstu *D. truttae* nije bilo dostupnih podataka što daje važan doprinos okolišnoj parazitologiji. Suprotno od mekih tkiva, gdje utjecaj na akumulaciju imaju brojni čimbenici, kalcificirane strukture su neaktivne i metabolički inertne, što omogućava dobivanje trajnog zapisa o izloženosti pojedinim biološki dostupnim metalima (Campana, 1999; Tzadik i sur., 2017). Rezultati ovog dijela istraživanja, fokusiranog prvenstveno na kukaše i kalcificirane strukture kao bioindikatore izloženosti metalima, predstavljeni su u pripremljenom radu u Prilogu 6.

U istraživanju je odabrano osam elemenata na temelju rezultata dobivenih u uzorcima vode i tkiva, odnosno oni elementi koji su bili mjerljivi u (gotovo) svim tkivima, što je omogućilo usporedbu mekih i tvrdih tkiva (esencijalni Fe, Mg, Mn i Zn te neesencijalni Ba, Rb, Sr i Tl). U kukašima te jetri i mišiću potočne pastrve mjereni su svi navedeni elementi, dok u otolitima nisu mjerene koncentracije Fe, a u ljuskama su zbog nedostatka pouzdanih referentnih materijala izmjerene samo koncentracije Fe, Mg, Mn, Sr i Zn. Uzorci vode su pokazali da su ribe s lokacije blizu grada Knina u uzorkovanju u travnju 2015. godine bile

izložene značajno višim koncentracijama svih ovih elemenata, osim Mg i Tl koji su bili viši u vodi s referentne postaje na izvoru rijeke Krke.

Korištena kombinacija bioindikatora ukazala je da postoje razlike u stupnju akumulacije i koncentracijama metala u pojedinim tkivima, ali da unatoč tome sva tkiva odražavaju okolišne uvjete na sličan način. Unatoč malo statistički značajnih razlika, trendovi su ukazali na uglavnom više prosječne koncentracije Ba, Mn, Fe, Sr i Zn u svim korištenim bioindikatorima s onečišćene postaje, potvrđujući utjecaj tvornice vijaka i neadekvatno pročišćenih otpadnih voda. Vrijednosti Rb u jetri, mišićima i otolitima potočne pastrve te kukašima, Tl u mekim tkivima te otolitima riba te kukašima, kao i Mg u jetri, ljuskama i otolitima potočne pastrve bile su više u uzorcima s referentne postaje, kao i u ostalim uzorkovanjima, unatoč najčešće nižim koncentracijama Rb i Tl u vodi na toj postaji, što je potvrdilo da izvor tih elemenata nije primarno u vodi. Upravo za Tl su Clearwater (2000) te Lapointe i Couture (2009) pokazali da unos putem hrane predstavlja glavni put ulaska u ribe. Koncentracije metala u mekim tkivima i kukašima uglavnom su slijedile sličan redoslijed s najvišim koncentracijama Mg, Fe i Zn i najnižim Ba i Tl (Mg > Fe > Zn > Rb > Mn > Ba > Tl > Sr u jetri, Mg > Fe > Rb > Zn > Sr > Mn > Ba > Tl u mišiću te Mg > Zn > Fe > Mn > Sr > Rb > Tl > Ba u kukašima).

Redoslijed koncentracija metala u vrsti *D. truttae* je usporediv s našim prvim dijelom istraživanja na kada je niz analiziranih metala bio K > Na > Ca > Mg > Fe ≥ Zn > Cu > Mn > Sr > Rb > Tl > Se > Pb ≥ Cd > Co > Cs, dok drugih literaturnih podataka o ovoj vrsti nema. Ukupne koncentracije Cd, Mn i Rb pokazale su se sličnima u svim uzorkovanjima 2015. i 2016. godine, dok su koncentracije Fe, Sr, Tl i Zn bile nešto niže u jesen 2015. i proljeće 2016. u odnosu na proljeće 2015. godine.

Statistički značajne razlike između postaja zabilježene su za najveći broj metala kod kukaša, čime je još jednom potvrđena njihova izuzetna osjetljivost na promjene u okolišu kao i visoka učinkovitost akumulacije metala (Sures, 2004), što ih čini korisnim bioindikatorima i ranim pokazateljima izloženosti metalima i pri niskim okolišnim koncentracijama. Naime, statistički značajno više koncentracije Ba, Fe, Sr i Zn zabilježene su u kukaša s onečišćene postaje, kao i Rb u kukaša s referentne postaje. Međutim, u skladu s prethodnim istraživanjima drugih istraživača, i naši podaci potvrdili su veliku varijabilnost u koncentracijama u pojedinim jedinkama kukaša koja može nastati kao posljedica mobilnosti domadara, razlika u starosti te time i dužine izloženosti (Sures i sur., 1999; Filipović Marijić i sur., 2014). Uz to, potvrđeno je i da je akumulacija u kukaša učinkovitija nego u mekim tkivima riba, što su potvrdili i BCF koji opisuju odnos koncentracije metala u kukašima i

tkivima domadara (Sures i sur., 1999). S obzirom nisku metaboličku aktivnost mišića i očekivano niže koncentracije metala u mišiću, nego jetri (Jarić i sur., 2011; Nachev i Sures, 2016), BCF u odnosu na mišić bili su znatno viši, nego u odnosu na jetru. Najviše vrijednosti BCF u našem istraživanju su zabilježene za Mn, Sr, Tl i Zn što je ukazalo na nedavnu povećanu izloženost tim elementima, a same vrijednosti BCF uglavnom su bile više na onečišćenoj postaji, što je kao i u prethodnom dijelu istraživanju na kukašima, potvrdilo veću akumulaciju metala u kukašima koji su izloženi jačem antropogenom utjecaju, kao i mogućnost njihove primjene kao osjetljivih bioindikatora biološki dostupnih razina metala i brzih odgovora na promjene u okolišu. U odnosu na prethodni dio našeg istraživanja, gdje su BCF izračunati u odnosu na probavilo potočne pastrve, vrijednosti BCF u odnosu na jetru bile su usporedive s vrijednostima za probavilo za Fe, Mn i Rb, dok su vrijednosti BCF za Mg, Sr i Zn bile veće, a za Tl niže u jetri. Što se tiče mišića, BCF za Fe, Mn, Tl i Zn bili su viši u odnosu na BCF za probavilo, za Mg niži, a za Rb i Sr usporedivi s probavilom. Dok je Zn imao učinkovitiju akumulaciju u kukašima nego u jetri i mišiću, probavilo je i u ovom dijelu istraživanja potvrđeno kao njegova glavna zaliha u tijelu s puno višim koncentracijama nego u kukašima (Sun i Jeng, 1999), a Rb je u svim analiziranim slučajevima pokazao slabiju akumulaciju u kukašima u odnosu na indikatorske organe riba.

Iako bez statistički značajnih razlika između lokacija, koncentracije Fe, Mn, Sr i Zn u ljuskama, te Ba, Mn, Sr i Zn bile su više na onečišćenoj postaji kod Knina, dok su koncentracije Mg u ljuskama, te Mg, Rb i Tl u otolitima bile više na referentnoj postaji na izvoru rijeke Krke. Padajući redosljedi koncentracija u ljuskama bili su $Mg > Sr > Zn > Fe > Mn$ te $Sr > Zn > Mg > Rb > Mn > Ba > Tl$ u otolitima. Kalcificirane strukture su također pokazale međusobne razlike u akumulaciji metala, s višim vrijednostima akumulacije većine metala u ljuskama nego otolitima, što odgovara i ranijim literaturnim podacima (Wells i sur., 2000; Ramsay i sur., 2011; Kalantzi i sur., 2019), pri čemu je najveća razlika između dvaju tkiva zabilježena za Mg i Mn. S obzirom na kemijsku strukturu ljusaka čiju osnovu čini hidroksiapatit, više vrijednosti Mg u ljuskama su očekivane, jer Mg čini osnovu u izgradnji apatita (Bigi i sur., 1992). Nadalje, i sintetski i biogeni apatiti imaju visok afinitet za vezanje Mn (Wells i sur., 2000), što objašnjava i povišenu vrijednost Mn u ljuskama u odnosu na otolite. Suprotan trend je uočen za Sr čije vrijednosti su bile više u otolitima nego u ljuskama, vjerojatno kao posljedica kemijske sličnosti Sr i Ca, koji je glavni gradivni element otolita (Campana, 1999; Kalantzi i sur., 2019). Korištenje ljusaka u odnosu na otolite je korisno iz nekoliko razloga: koncentracije elemenata općenito su veće što smanjuje pogreške i povećava preciznost mjerenja osobito za elemente prisutne u vrlo niskim koncentracijama; lako ih je

prikupiti i mogu se koristiti kao neletalna alternativa, što je osobito korisno ako se istražuju neke rijetke i ugrožene vrste; njihova priprema za mjerenje na LA ICP-MS-u je lakša i brža, a zbog vidljivih zona rasta ljusaka nije potreban postupak poliranja, što smanjuje mogućnost onečišćenja. Međutim, problem je što još uvijek nema dostupnih relevantnih i u potpunosti pouzdanih referentnih materijala koji bi omogućili mjerenje širokog spektra elemenata u ljuskama te se koriste dostupni materijali koji sadrže ili mali broj elemenata ili po kemijskom sastavu ne odgovaraju najbolje sastavu ljusaka riba (Clarke i sur., 2007; Ramsay i sur., 2011). Uz to, budući da ribe mogu regenerirati ljuske, takve ljuske ne ukazuju na čitavo razdoblje života te ne daju potpun podatak o izloženosti, ali i kao takve također mogu dati informaciju o nedavnoj, kao i relativno dugotrajnoj izloženosti onečišćenju, bez utjecaja fizioloških promjena organizma (Hammond i Savage, 2009).

Usporedba svih korištenih bioindikatora ukazala je na uglavnom najviše koncentracije u kukašima, jetri i ljuskama, a najnižu u mišiću i otolitima. Naime, učinkovitost akumulacije bila je: kukaši > jetra > otoliti > mišić za Ba i Tl, jetra > kukaši > ljuske > mišić za Fe, ljuske > mišić > kukaši > jetra > otoliti za Mg, ljuske ≥ kukaši > jetra > otoliti ≥ mišić za Mn, jetra > mišić > kukaši > otoliti za Rb, otoliti > ljuske > kukaši > mišić > jetra za Sr i kukaši > ljuske ≥ jetra > otoliti > mišić za Zn. Učinkovitost akumulacije, odnosno visoke koncentracije nekih elemenata u ljuskama su također potvrdile njihov potencijal u procjeni stanja okoliša, dok otoliti zbog aragonitnog sastava imaju relativno nizak afinitet za metale, osobito u usporedbi s apatitnim strukturama poput ljusaka (Adey i sur., 2009; Goto i Sasaki, 2014).

Ovim dijelom istraživanja potvrdili smo hipoteze H2, H3 i H5, s osobitim naglaskom na prednosti korištenja kukaša i kalcificiranih struktura kao bioindikatora. Također, potvrđena je i H1 budući da su vrijednosti koncentracija metala bile više u organizmima s onečišćene postaje, iako su sva tkiva ukazala na još uvijek umjereno, ali kontinuirano onečišćenje rijeke Krke, potvrđeno sličnim trendovima mekih i tvrdih tkiva i BCF vrijednostima.

4.7. Biomarkerski odgovori u probavilima riba i rakušcima u procjeni stanja okoliša

Uz određivanje koncentracija metala u okolišu, kao i akumulacije u organizmima, neophodno je prikupiti informacije i o štetnim učincima zagađivala na biotu (Livingstone, 1993), što se često provodi analizom različitih biomarkera. Molekularni i stanični biomarkeri se definiraju kao najranije mjerljive promjene i biokemijski odgovori u organizmima izloženim onečišćenju (Phillips i Rainbow, 1993). Kako bi se istovremeno odredili različiti biološki odgovori koji ukazuju na promjene kakvoće okoliša najbolje je koristiti multibiomarkerski pristup koji omogućava jasniju i sveobuhvatnu procjenu stanja okoliša i

izloženosti određenim skupinama zagađivala, čak i pri niskim razinama (Broeg i Lehtonen, 2006; Monserrat i sur., 2007). Stoga je u našem istraživanju korišten set biomarkera koji je obuhvaćao promjene koncentracija ukupnih citosolskih proteina (TP) koji se koriste kao biomarkeri općeg stresa, odnosno očituju se bilo kao porast koncentracija uslijed povećane sinteze proteina ili pad uslijed oštećenja i raspada proteina. Strukturu ili funkciju proteina često narušavaju i povećane količine kisikovih radikala i oksidacijski stres u stanici (Carney Almroth i sur., 2008; Lushchak, 2011). Aktivnost acetilkolinesteraze (AChE) se koristi kao biomarker učinka na živčani sustav, najčešće izloženosti organofosfornim i karbamatnim spojevima (Lionetto i sur., 2011), ali se može javiti i kao rezultat izloženosti drugim zagađivalima, poput teških metala, policiličkih aromatskih ugljikovodika i deterdženata, koji najčešće uzrokuju inhibiciju (Elumalai i sur., 2007; Richetti i sur., 2011; de Lima i sur., 2012). Nadalje, kako su istraživanja u zadnjih nekoliko desetljeća pokazala da metali poput Fe, Cu, Cr, Cd, Hg, Ni, Pb i V značajno utječu na povećanu proizvodnju reaktivnih kisikovih spojeva (ROS) uzrokujući oštećenja tkiva i staničnih dijelova, odnosno oksidacijski stres (Winston i Di Giulio, 1991; Valko i sur., 2005), biomarkeri vezani uz ravnotežu oksidacijske i antioksidacijske aktivnosti imaju veliki značaj u akvatičkim studijama gdje se kao indikatorska tkiva najčešće koriste jetra, bubrezi i škrge riba (Valavanidis i sur., 2006; Fernandes i sur., 2008; Sevcikova i sur., 2011). Česta posljedica oksidacijskog stresa je peroksidacija lipida, kao pokazatelj oštećenja stanične membrane (Valavanidis i sur., 2006), a jedan od produkata peroksidacije lipida je malondialdehid (MDA), čija razina izravno odražava stupanj oksidacijskog oštećenja izazvanog zagađivalima (Banerjee i sur., 1999). U aktivnu obranu organizma ipak su uključeni različiti stanični antioksidansi od kojih su u ovom istraživanju mjereni ukupni glutation (GSH) te katalaza (CAT) koja štiti stanicu od djelovanja vodikovog peroksida te je jedan od prvih predloženih biomarkera oksidacijskog stresa, odnosno antioksidacijskog kapaciteta stanica (Livingstone i sur., 1993). Njihovo djelovanje na prisutnost oksidacijskog stresa najčešće se prvo očituje kao indukcija aktivnosti, no u uvjetima jačeg oksidacijskog stresa i onečišćenja dolazi do inhibicije. Na kraju, biomarker specifičan za procjenu izloženosti metalima je koncentracija metalotioneina (MT), niskomolekulskih proteina bogatih cisteinom čija pojačana sinteza predstavlja odgovor na povećanu razinu metala u okolišu (Amiard i sur., 2006) jer ima ključnu ulogu u održavanju homeostaze esencijalnih elemenata poput Cu i Zn te detoksikacije elemenata poput Cd i Hg (Vašak, 2005). S obzirom na nespecifičnost biomarkera TP i AChE, istraživanje je posebno usmjereno na biomarkere vezane uz oksidacijski stres (GSH, CAT, MDA) i izloženost

metalima (MT) u okolišu i organizmima. Rezultati dijela istraživanja vezanog uz biomarkere predstavljeni su u radovima 1, 2 i 3 na popisu radova te Prilozima 7 i 8.

4.7.1. Biomarkeri u probavilu riba

U oba istraživana ekosustava, dinaridskoj rijeci Krki i panonskoj rijeci Ilovi, biomarkerski odgovori su ukazali na umjereno onečišćenje okoliša i ne toliko izražen utjecaj na biološke odgovore organizama. Ukupni proteini (TP) kao pokazatelji općeg stresa ukazali su na neke prostorne i sezonske razlike, ali njihov porast ne može se dovesti u vezu isključivo s povišenim razinama metala budući da na metabolizam proteina u tkivima utječu i temperatura, razina kisika ili salinitet, a značajan doprinos daju i hranidbene navike, osobito u probavilu (Peragón i sur., 1994). Specifične razlike u koncentraciji TP u potočnim pastrvama ogledale su se kao značajno više vrijednosti na onečišćenoj nego referentnoj postaji u jesen (54,1 mg g⁻¹ m. m. (mokre mase) u odnosu na 47,8 mg g⁻¹ m. m., a u babuški kao značajno više vrijednosti na onečišćenoj nego referentnoj postaji u proljeće (80,8 u odnosu na 66,6 mg g⁻¹ m. m.). Dodatno, kod babuški je primjećena i sezonska razlika na referentnoj postaji kod sela Ilove s višom vrijednosti u jesen (82,4 u odnosu na 66,6 mg g⁻¹ m. m.). Takvi rezultati su u skladu sa studijama koje su dokazale povišene razine proteina u ribama s onečišćenih lokacija (Filipović Marijić i Raspor, 2012). Poznato je da organizmi prije štetnih učinaka zagađenja, prvo odgovaraju porastom koncentracija proteina kako bi zadržali homeostazu. Također, uočljivije razlike u razinama TP između postaja u ovome istraživanju zabilježene su u reproduktivnom razdoblju svake vrste te je moguće da su započele i specifične fiziološke promjene u riba koje uzrokuju porast citosolskih koncentracija proteina (Filipović Marijić i Raspor, 2010). Uz to, Stegman i sur. (1992) su zabilježili da se porast koncentracije TP dovodi u vezu i s različitim organskim zagađivačima koja su, s obzirom na ispušte komunalnih i industrijskih otpadnih voda, kao i poljoprivredne aktivnosti, svakako prisutna na onečišćenim postajama obiju rijeka. Nadalje, porast TP je moguć i zbog veće dostupnosti hrane na onečišćenim postajama, što je u skladu i s vrijednostima FCI, kao i masama riba koje su bile veće na onečišćenim postajama kod grada Knina i sela Trebeža nego na referentnim postajama kod izvora Krke i sela Ilove. Dakle, iako porast TP može upućivati i na izlaganje stresnom utjecaju koji može rezultirati i indukcijom stres proteina te porastom njihove koncentracije, a time i porastom koncentracije ukupnih citosolskih proteina, razine TP značajno ovise i o fiziologiji riba, a ne samo o vanjskim utjecajima.

Do inhibicije aktivnosti AChE dolazi uslijed izloženosti organofosfatima, karbamatima, ali i metalima, no u našem istraživanju nisu zabilježene statistički značajne razlike niti između

postaja, niti između sezona. Ipak, nešto manje prosječne vrijednosti su zabilježene na onečišćenim postajama, nego na referentnim postajama i u rijeci Krki i Ilovi, vjerojatno kao posljedica povišenih razina metala u vodi i probavilu riba s onečišćenih postaja, kao i korištenja pesticida koji u ovim regijama imaju stalnu primjenu zbog povećanja prinosa u poljoprivredi, a poznato je da u blizini grada Knina i sela Trebeža ima puno obradivih površina. S obzirom na to da smo proveli prva mjerenja AChE u probavilu potočnih pastrva i babuški na ovom području, ne možemo pouzdano zaključiti predstavljaju li dobivene vrijednosti aktivnosti AChE (koje su kretale od 7,5 do 9,4 nmol min⁻¹ mg⁻¹ prot. u potočnih pastrva te 7,8 do 8,8 nmol min⁻¹ mg⁻¹ prot. u babuški) bazalne vrijednosti ovih vrsta ili se radi o eventualnim promjenama aktivnosti. Svakako, vrijednosti AChE su bile usporedive između dviju vrsta riba, odnosno dvaju slatkovodnih dinaridskih i panonskih ekosustava. Szabó i sur. (1991) su istraživali aktivnost AChE u mišiću, srcu, mozgu i probavilu 12 vrsta riba, među kojima su bile i kalifornijska pasrtva, šaran te bijeli tolstolobik, kao vrste najsirodnije potočnoj pastrvi, odnosno babuški. Među njima, vrijednosti AChE u probavilu kalifornijske pastrve bile su najniže u usporedbi s preostalim istraživanim vrstama riba, dok je probavilo u ovoj vrsti pokazalo najnižu aktivnost AChE od svih testiranih indikatorskih tkiva. Vrijednosti u probavilu su bile oko 10 nmol min⁻¹ mg⁻¹ prot. što je nešto više nego u potočnoj pastrvi iz našeg istraživanja. Što se tiče šarana i bijelog tolstolobika, u probavilu su također zabilježene najniže aktivnosti AChE u usporedbi s drugim tkivima, ali prosječne vrijednosti bile su 19, odnosno 35 nmol min⁻¹ mg⁻¹ prot., što je značajno više nego što je zabilježeno u babuškama iz našeg istraživanja (Szabó i sur., 1991).

Kako bismo dobili uvid u ravnotežu oksidansa i antioksidansa u potočnim pastrvama i babuškama, izmjerene su razine GSH te aktivnosti CAT kao pokazatelja učinkovitosti antioksidacijskog sustava organizama, koji su ukazali na nešto nepovoljnije uvjete i veću izloženost oksidacijskom stresu na onečišćenim postajama. Naime, u rijeci Krki zabilježena je statistički značajno viša vrijednost GSH na onečišćenoj nego na referentnoj postaji u jesen (1642,3 nmol g⁻¹ m.m. u odnosu na 1102,1 nmol g⁻¹ m.m.), a isti trend bio je vidljiv i u proljeće no nije bio statistički značajan. Sličan odgovor zabilježen je i za aktivnost CAT, ali bez značajnih razlika. U rijeci Ilovi razlike između postaja očitovale su se kao značajno više vrijednosti CAT na onečišćenoj postaji u proljeće, kada su uglavnom zabilježene i više koncentracije metala, dok GSH nije pokazao jedinstven obrazac. Atli i sur. (2006) su mjerili aktivnost CAT u probavilu slatkovodne vrste Nilske tilapije nakon 96 h izlaganja različitim koncentracijama (0,1, 0,5, 1,0 i 1,5 mg L⁻¹) Ag, Cd, Cr, Cu i Zn pri čemu su svi elementi osim Cu uzorkovali inhibiciju ovog enzima, a dobivene vrijednosti kretale su se između 25 i 225

$\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ prot. ovisno o metalu i koncentraciji. Ipak, može se pretpostaviti da prvotno pojačano djelovanje antioksidansa ukazuje na njihovu indukciju kisikovim radikalima, kao mehanizma obrane i zaštite od oksidacijskog stresa (Winston i Di Giulio, 1991). S obzirom na nedostatak podataka za probavilo kao indikatorsko tkivo, kao i za istraživane vrste, ne može se reći radi li se o nekoj značajnoj inhibiciji ili aktivaciji CAT, ali očekivana reakcija je da u uvjetima povišenih koncentracija metala, odnosno povećane količine ROS, dođe do porasta aktivnosti enzima uslijed njihove indukcije, nakon čega slijedi pad aktivnosti uslijed kataboličke aktivnosti i/ili inhibicije enzima djelovanjem zagađivala (Viarengo i sur., 2007). U literaturi su generalno zabilježeni različiti odgovori antioksidansa na prisutno onečišćenje metalima i u laboratorijskim i okolišnim uvjetima ovisno o dozi i trajanju izlaganja, elementu, vrsti organizama i načinu izlaganja (Liu i sur., 2005; Atli i sur., 2006; Tsangaris i sur., 2011; Greani i sur., 2017). Primjerice, Liu i sur. (2005) su zabilježili porast koncentracija GSH u jetri s produženjem izlaganja zlatnih ribica Cu, dok Berntssen i sur. (2001) u svojem izlaganju nisu zabilježili promjene u vrijednostima GSH u jetri i probavilu lososa s povišenjem koncentracija Cd, a Greani i sur. (2017) su objavili rezultate o povišenoj razini MDA i aktivnosti CAT u jetri, gonadama i bubrezima u pastrvama kao odgovor na kroničnu izloženost As u okolišu. S obzirom da su u našim istraživanjima mnogi elementi bili povišeni na onečišćenim postajama, to ukazuje na njihovu moguću ulogu u nastanku oksidacijskog stresa i posljedične aktivacije ili inhibicije odgovora staničnih antioksidansa.

Povišene koncentracije MDA u organizmima odražavaju oksidacijski stres kao posljedicu oštećenja lipida (Banerjee i sur., 1999). Prosječne vrijednosti MDA u potočnim pastrvama kretale su se između 147,1 i 166,1 $\text{nmol g}^{-1} \text{ m.m.}$, a u babuškama između 39,9 i 104,7 $\text{nmol g}^{-1} \text{ m.m.}$, ovisno o lokaciji i sezoni. Značajno povišene razine MDA u našem istraživanju zabilježene su samo u babuškama iz rijeke Ilove s višim vrijednostima na onečišćenoj postaji kod sela Trebeža u objema sezonama što ukazuje i na možebitni nastanak oksidacijskih oštećenja uslijed prisutnog oksidacijskog stresa. Razine MDA u jesen bile su dvostruko, a u proljeće čak trostruko više na onečišćenoj nego na referentnoj postaji rijeke Ilove. U potočnih pastrva iz rijeke Krke nisu bile zabilježene statistički značajne razlike između postaja. Usporedba s razinama MDA u prirodnim populacijama riba bila je moguća samo za probavilo vardarskog klana iz triju makedonskih rijeka pod utjecajem rudnika i poljoprivrednih aktivnosti. Iako su sve tri rijeke, Zletovska, Kriva i Bregalnica više onečišćene metalima, nego rijeka Krka (Ramani i sur., 2014; Filipović Marijić i sur., 2018), prosječne vrijednosti MDA u klenu su se kretale u rasponu od 3,3 do 155,8 $\text{nmol g}^{-1} \text{ m.m}$

(Dragun i sur., 2017). Značajno povišenje MDA nije nađeno u rijekama jako onečišćenim metalima (Zletovska i Kriva), nego u rijeci Bregalnici koja je jako onečišćena pesticidima, što je ukazalo na veći utjecaj organskog zagađenja, nego metala na nastanak oksidacijskog stresa (Dragun i sur., 2017). Stoga je u nedostatku drugih podataka o vrijednosti MDA u probavilu slatkovodnih vrsta, napose vrsta istraživanih u ovome radu, teško zaključiti radi li se u našem istraživanju o pojačanoj sintezi MDA i pojavi oksidacijskog stresa na objema postajama rijeke Krke. U slučaju babuški, uzimajući u obzir jasno vidljiv porast MDA na onečišćenoj postaji, vjerojatno je u pitanju pojava izvjesne povišene razine oksidacijskog stresa u blizini sela Trebeža, no nije moguće utvrditi je li uzrok povišeno onečišćenje vode metalima ili organskim zagađivalima prisutnim u tom ekosustavu. Okolišni uvjeti poput temperature, koncentracije kisika i dostupnosti hrane također mogu djelovati na oksidacijski stres i odgovore organizama uslijed promjena u metabolizmu ili reprodukciji (Sheehan i Power, 1999). Ipak, nastanak kisikovih radikala i oštećenja tkiva, DNK, lipida ili proteina često se dovodi u vezu upravo s metalima poput As, Cu i Fe (Berntssen i sur., 2000; Carriquirriborde i sur., 2004; Bhattacharya i Bhattacharya, 2007; Greani i sur., 2017), kao i s organskim zagađivalima (Dragun i sur., 2017). S obzirom da su i u našem istraživanju potvrđene povišene koncentracije nekih od tih elemenata, a vjerojatna je i prisutnost organskih zagađivala iz okolnih poljoprivrednih područja u riječnoj vodi te je pozitivna korelacija zabilježena za razine As i MDA kod sela Trebeža, vrlo je vjerojatna prisutnost oksidacijskog stresa u babušcima iz rijeke Ilove. Za probavilo potočne pastrve potrebno je dodatno istražiti bazalne razine MDA, kako bismo mogli sa sigurnošću utvrditi jesu li dobivene visoke vrijednosti MDA na objema postajama posljedica oksidacijskog stresa ili normalnog fiziološkog stanja ove vrste.

Metalotioneini, kao specifičniji biomarkeri izloženosti metalima, sudjeluju u pohrani i detoksikaciji metala poput Cd, Cu i Zn, kao i obrani organizama od oksidacijskog stresa (Viarengo i sur., 1999, 2007; Fernandes i sur., 2008). Njihova antioksidacijska uloga vjerojatno počiva na visokom udjelu tiolnih skupina u molekuli MT i specifičnoj dinamici vezanja i otpuštanja metala (Atif i sur., 2006; Viarengo i sur., 2007), ali utjecaj zagađenja na odnos između vezanja metala i antioksidacijske funkcije MT nije dobro razjašnjen (Chesman i sur., 2007). Iako se indukcija MT koristi kao učinkovit pokazatelj izloženosti metalima u mnogim organizmima (Langston i sur., 2002; Ivanković i sur., 2005; Mosleh i sur., 2006; Dragun i sur., 2007; Calisi i sur., 2013), u ovom istraživanju njihova značajna indukcija nije zabilježena niti u jednoj od istraživanih vrsta, neovisno o lokaciji i sezoni. Srednje vrijednosti razine MT u objema vrstama riba ipak su bile više na onečišćenim postajama rijeke Krke,

odnosno Ilove, u odnosu na referentne postaje, ali to nije dokazano kao statistički značajno. Nadalje, vrijednosti su uglavnom bile blago povišene u reproduktivnim razdobljima riba, moguće kao posljedica povećanih metaboličkih aktivnosti i ishrane u tom razdoblju (Filipović Marijić i Raspor, 2010), ali ni to nije bilo jasno izraženo. Srednje vrijednosti MT u potočnim pastrvama kretale su se u rasponu 0,85-1,5 mg g⁻¹ m.m., a u babuškama od 2,0-2,5 mg g⁻¹ m.m. Iako je indukcija MT u probavilu različitih vrsta riba potvrđena nakon izloženosti Cu (Berntssen i sur., 1999; Handy i sur., 1999), Ni (Ptashynski i Klaverkamp, 2002) ili Cd (Berntssen i sur., 2001; Roesijadi i sur., 2009), usporedba dobivenih vrijednosti za probavilo bila je moguća samo s klenovima iz rijeke Save budući da vrijednosti MT jako variraju ovisno o metodi određivanja (Isani i sur., 2000; Zorita i sur., 2005). Vrijednosti u probavilu klena iz rijeke Save bile su 2,9-3,1 mg g⁻¹ m.m., što je ukazalo na sličnije rezultate s babuškom koja također pripada porodici Cyprinidae, nego s potočnom pastrvom (Filipović Marijić i Raspor, 2010). U istraživanju klenova, uočeno je da su vrijednosti MT u probavilu bile više nego u škragama (oko 2 mg g⁻¹ m.m.) i jetri (oko 1,5 mg g⁻¹ m.m.) istih jedinki (Dragun i sur., 2009; Podrug i Raspor, 2009). Budući da su i škrge i probavilo uključeni u unos, detoksikaciju, kao i izlučivanje tvari i metala (Van Cleef i sur., 2000), više koncentracije MT u tim tkivima su vjerojatno povezane s važnom funkcijom ovih proteina u unosu metala, kao i zaštitnoj ulozi od prekomjernog unosa, održavanju homeostaze i detoksikaciji. Iako se zna da su Cd, Cu i Zn među glavnim elementima koji induciraju sintezu MT, taj odgovor u našem istraživanju također nije uvijek bio jednoznačan. Naime, u potočnim pastrvama uglavnom nije bilo značajnih razlika u citosolskim koncentracijama ovih elemenata između dviju postaja, izuzev Cd, ali zabilježena je značajna pozitivna korelacija između razina MT i citosolskih koncentracija Cd i Cu u pastrva s referentne postaje u proljeće, te s Cu u pastrva s onečišćene postaje u jesen. Značajne korelacije nisu zabilježene s citosolskim koncentracijama Zn, no jasna korelacija ponekad izostaje kad je raspon analiziranih vrijednosti uzak. S druge strane, razine Cd i Cu u babuškama iz rijeke Ilove bile su značajno više u ribama ulovljenim na onečišćenoj postaji u odnosu na referentnu, ali to nije uzrokovalo značajnu indukciju niti je zabilježena značajna korelacija koncentracija ovih elemenata s razinom MT u probavilu. Ipak, u babuškama je zabilježena pozitivna korelacija razine MT s citosolskim koncentracijama Zn na referentnoj postaji u jesen te onečišćenoj postaji u proljeće. Ukupne koncentracije metala nisu pokazale značajne korelacije s koncentracijama MT, vjerojatno stoga što se samo citosolski metali mogu vezati na biomolekule te utjecati na njihove koncentracije, aktivnosti ili strukturu (Caron i sur., 2018). Ipak, sama raspodjela Zn među citosolskim biomolekulama pokazala je da ne dolazi do značajnog vezanja Zn u vremenu eluiranja standarda MT niti u

jednoj istraživanoj vrsti riba, dok je pokazano da se Cd i Cu u najvećim količinama vežu za MT. Iako su MT specifičniji biomarkeri u odnosu na TP ili MDA te se koriste kao biomarkeri izloženosti metalima, zapravo svi čimbenici koji utječu na metabolizam proteina utječu izravno i na MT, dok mnogi drugi čimbenici koji utječu i na akumulaciju metala, poput spola, reproduktivnog razdoblja ili veličine organizma neizravno utječu i na MT (Hylland i sur., 1998; Amiard i sur., 2006). Također, same razine izloženosti metalima u oba ekosustava potencijalno nisu bile dovoljne da izazovu značajnu indukciju sinteze MT jer je raspoloživa količina u stanicama bila dovoljna za vezanje unesenih metala. Poremećaji u fizikalno-kemijskim čimbenicima poput temperature, vodljivosti, količine kisika i količine organskih tvari također mogu djelovati i na razinu sinteze MT. S obzirom na saznanje da na sintezu MT utječu brojni biotički i abiotički čimbenici, te da su organizmi u prirodnom okolišu izloženi različitim zagađivačima, nemoguće je povećanu sintezu MT povezati samo sa specifičnim elementom te je ovaj biomarker također potrebno pratiti kao dio sveobuhvatne analize.

4.7.2. Biomarkeri u rakušaca

S obzirom da je za elektrokemijsku analizu MT bila dovoljna mala masa uzorka, razine MT su određene i u rakušcima iz rijeke Krke, ulovljenim na istim postajama kao i potočne pastrve. Vrsta *G. balcanicus* pokazala je značajne razlike u koncentraciji MT između dviju postaja sa značajnom višom vrijednosti MT na onečišćenoj nego na referentnoj postaji u proljeće, dok su u jesen koncentracije bile usporedive. Sezonske razlike su se očitovale kao više vrijednosti MT u jesen nego u proljeće na objema lokacijama. Prosječne vrijednosti MT u vrsti *G. balcanicus* bile su između 2,43 i 3,30 mg g⁻¹ m. m. Slične vrijednosti zabilježene su i u vrsti *E. acarinatus* (2,94 mg g⁻¹ m. m. u jesen te 2,53 mg g⁻¹ m. m. u proljeće), koju je bilo moguće uzorkovati samo na izvoru rijeke Krke, a značajne sezonske razlike u ovoj vrsti nisu zabilježene. Iako su metali poput Cu i Zn imali povišene citosolske koncentracije u rakušcima s onečišćene postaje, Cd je bio značajno povišen u organizama s referentne postaje, te je značajna korelacija MT dokazana samo s citosolskim koncentracijama Zn na onečišćenoj postaji u jesen za vrstu *G. balcanicus*, dok nije bilo značajnih korelacija razine MT i metala u vrsti *E. acarinatus*. Indukcija MT u rakušaca kao i u drugih organizama može biti uzrokovana i sezonom, temperaturom, spolom ili reproduktivnom fazom (Rainbow i Moore, 1986; Geffard i sur., 2007; Filipović Marijić i sur., 2016a) te više vrijednosti postignute u jesen mogu biti posljedica utjecaja reproduktivnog razdoblja ovih vrsta. Dobivene vrijednosti MT u ovom istraživanju su usporedive s rezultatima dvaju dostupnih istraživanja na rakušcima istog roda; vrsti *G. pulex* iz rijeke La Bourbre u Francuskoj, okarakteriziranom kao rijekom s

niskim koncentracijama metala u kojoj su koncentracije MT bile u rasponu od 1,25 do 3,25 mg g⁻¹ m. m. (Geffard i sur., 2007), te vrsti *G. fossarum* iz rijeke Sutle, koja se pokazala kao jače onečišćenom metalima nego rijeka Krka, u kojoj su koncentracije MT bile u rasponu od 1,55 do 3,65 mg g⁻¹ m. m. (Filipović Marijić i sur., 2016a).

Iako i biomarkerski odgovori potvrđuju hipoteze H1 i H2, slično kao i obrasci akumulacije metala te njihove raspodjele među citosolskim biomolekulama, oni također potvrđuju umjereno onečišćenje obaju istraživanih sustava te zasad samo blage negativne utjecaje i učinke na organizme. Naime, iako markeri upućuju na više razine oksidacijskog stresa ili nešto jaču indukciju MT na onečišćenim postajama, odgovori antioksidansa ukazuju na djelotvoran rad na objema lokacijama bez značajnog pada aktivnosti, koji je često vidljiv u jako onečišćenim okolišima. To pokazuje da u bioti nije došlo do značajnog ili trajnog oštećenja pod utjecajem ispuštanja otpadnih voda i ostalih antropogenih aktivnosti. Ipak, dobivene razlike između postaja ukazuju na potencijalne opasnosti ukoliko se isto djelovanje i trendovi nastave i u budućnosti bez prikladnog i učinkovitog sustava pročišćavanja te da u tom slučaju tijekom vremena može doći i do izraženijih i trajnih poremećaja, oštećenja i negativnih učinaka na same organizme, kao i zaštićena područja Nacionalnog parka Krka i Parka prirode Lonjsko polje.

5. ZAKLJUČCI

U znanstvenoj literaturi malobrojna su istraživanja o unosu metala putem hrane, odnosno probavilom riba kao izabranim bioindikatorskim organom. Naše istraživanje o akumulaciji ukupnih i citosolskih metala, raspodjeli elemenata među citosolskim biomolekulama te multibiomarkerskim odgovorima u probavnom tkivu obuhvatilo je dvije vrste riba različitih bioloških i ekoloških karakteristika (potočnu pastrvu i babušku), koje su ulovljene na dvjema postajama (referentnoj i onečišćenju) i u dvjema sezonama (izvan i tijekom mrijesta, odnosno u jesen i proljeće) u dinaridskoj rijeci Krki te panonskoj rijeci Ilovi pod antropogenim utjecajem komunalnih i industrijskih otpadnih voda. Dobiveni rezultati predstavljaju prve podatke za koncentracije metala u citosolu i biomarkerske odgovore u probavilu navedenih vrsta riba u znanstvenoj zajednici. Analiza kalcificiranih struktura (ljuske, otoliti) potočne pastrve također je donijela nove podatke za ovu dobro istraženu vrstu i ukazala na prednosti korištenja ljusaka u monitoring studijama. Nadalje, novi podaci o akumulaciji metala u kukašu vrste *D. truttae*, prvi ovakve vrste u svijetu, daju doprinos polju okolišne parazitologije, a napravljena je i usporedba akumulacije metala u nekoliko vrsta rakušaca roda *Gammarus* što je omogućilo sveobuhvatnu analizu stanja okoliša i ekosustava rijeka Krke i Ilove. Specifični zaključci istraživanja su:

I. Analiza akumulacije metala, raspodjele elemenata među citosolskim biomolekulama te biomarkerskih odgovora u probavilu dviju vrsta riba predstavlja važan dio istraživanja unutarstaničnog ponašanja metala u ribama i pruža sveobuhvatnu informaciju o stanju okoliša i organizama te pruža mogućnost buduće primjene i usporedbe u monitoring programima.

II. Fiziološke promjene tijekom mrijesta i reproduktivnog razdoblja utječu na vrijednosti nekih bioloških pokazatelja, npr. povišene vrijednosti gonadosomatskih indeksa, kao i snižene vrijednosti Fultonovog kondicijskog indeksa i hepatosomatskog indeksa te na vrijednosti biokemijskih pokazatelja, npr. akumulacija metala, osobito esencijalnih, često je povećana u reproduktivnom razdoblju te je za praćenje unosa metala i izloženosti metalima pogodnije uzorkovati ribe u razdoblju izvan razdoblja mrijesta.

III. Ukupne koncentracije metala u probavilu riba, koje samo djelomično odražavaju okolišne uvjete i ne pružaju informaciju o potencijalno toksičnim i štetnim učincima na organizme, ukazale su na uglavnom više vrijednosti u babuškama nego u potočnim pastrvama, bilo uslijed veće izloženosti metalima u rijeci Ilovi, ili zbog fizioloških i bioloških specifičnosti svake vrste.

IV. Redoslijed koncentracija ukupnih i citosolskih metala u tragovima bio je sličan u probavilima objiju vrsta riba s najvišim koncentracijama Zn, Fe, Mn i Rb, a najnižim Cs i V.

V. S obzirom na to da koncentracije metala u citosolu odražavaju biološki, odnosno metabolički dostupne koncentracije, preporuča se određivanje citosolskih koncentracija metala u probavilu, a ne samo ukupnih.

VI. Analiza raspodjele metala/metaloida/nemetala između topljive i netopljive citosolske frakcije probavila potočnih pastrva i babuški ukazala je na visoku zastupljenost (> 70 %) u citosolu za Na, K, Se, Cs, Cd, Mo i Rb, ukazujući na njihovu veliku metaboličku raspoloživost te u nekim slučajevima i mogući toksični potencijal, dok su najmanju zastupljenost imali Mn, Ni i V, što upućuje na njihovu uspješnu detoksikaciju u objema vrstama riba u oba riječna ekosustava.

VII. Ukupne i citosolske koncentracije mnogih elemenata bile su više u ribama s onečišćenih nego referentnih postaja, a također su koncentracije više elemenata često bile povišene u reproduktivnom razdoblju svake od istraživanih vrsta, u jesen za potočnu pastrvu i proljeće za babušku. Ipak, koncentracije Cd, Cs i Tl bile su uglavnom povišene i u ribama, rakušcima i kukašima s referentne postaje rijeke Krke, kao i Mn i V u babuškama s referentne postaje rijeke Ilove, što se nije moglo objasniti samo unosom metala vodom te upućuje na važnost unosa putem hrane i sedimenta kao dodatnih izvora metala za organizme.

VIII. Primjenom SEC-HPLC-a i HR ICP-MS-a određene su raspodjele esencijalnih elemenata Co, Cu, Fe, Mo, Se i Zn te neesencijalnog Cd među citosolskim biomolekulama različitih molekulskih masa u probavilu potočnih pastrva iz rijeke Krke te babuški iz rijeke Ilove te se može istaknuti sljedeće:

- dominantna raspodjela Cd i Cu u području biomolekula niskih molekulskih masa, čija molekulska masa i vrijeme eluiranja odgovaraju MT u probavilu objiju vrsta riba
- dominantna raspodjela Co u području biomolekula visokih molekulskih masa (85-235 kDa) u potočnih pastrva te u području biomolekula jako niskih molekulskih masa (0,7-18 kDa) u babuškama
- raspodjela Fe uglavnom u područjima visokih (VMM; ~180-1100 kDa) i srednjih molekulskih masa (SMM; ~20-110 kDa), pri čemu je SMM područje dominantno kod potočnih pastrva i sugerira vezanje za različite proteine poput hemoglobina,

transferina, feroportina ili podjedinice katalaze, a VMM kod babuški i obuhvaća molekulska masu feritina, proteina koji služi za skladištenje Fe

- dominantna raspodjela Mo u području biomolekula jako niskih molekulskih masa (2-8 kDa) te manji dio u području visokih molekulskih masa (100-400 kDa) u objema vrstama
- dominantna raspodjela Se u području jako niskih molekulskih masa (0,4-5,1 kDa) u potočnih pastrva, koje obuhvaćaju molekulske mase poznatih selenospojeva poput selenoneina i selenometionina, te ravnomjerna raspodjela u području jako niskih (0,4-11 kDa) i visokih molekulskih masa (30-300 kDa) u babuški, što je uključivalo i molekulske mase glutation peroksidaze i tioredoksin reduktaze
- dominantna raspodjela Zn u području visokih molekulskih masa (30-1100 kDa) u objema vrstama, uz dodatak eluiranja u području jako niskih molekulskih masa (0,7-14 kDa) u babuškama, što je ukazalo i na mogućnost vezanja za MT, ali nije bilo jasno vidljivo u probavilu.

IX. Meka (jetra i mišić) i tvrda tkiva (ljuske i otoliti) unatoč razlikama u razinama akumulacije odabranih elemenata odražavaju okolišne uvjete na sličan način, pri čemu je akumulacija najizraženija u jetri i ljuskama, a najniža u mišićima i otolitima.

X. Ljuske su potencijalna neletalna alternativa uobičajenim bioindikatorskim tkivima u procjeni stanja okoliša jer omogućavaju izbjegavanje usmrćivanja riba, ali i zbog uglavnom viših koncentracija akumuliranih metala, lakog sakupljanja te jednostavne pripreme za lasersku ablaciju, zbog čega ih se može preporučiti za korištenje u istraživanjima endemskih i/ili ugroženih vrsta.

XI. Akumulacija metala u citosolima bila je usporediva u svim vrstama rakušaca rodova *Gammarus* i *Echinogammarus* koji su pokazali slične trendove i razlike kao i probavilo riba u oba riječna ekosustava.

XII. Crijevni nametnici kukaši akumuliraju više razine većine metala u odnosu na probavno tkivo potočne pastrve, pri čemu je najveći porast u kukašima zabilježen za Cd, Cu, Mn, Pb i Sr. Visoka osjetljivost na promjene u okolišu pokazuje da je vrsta *D. truttae* prikladan i obećavajući bioindikator u okolišnim istraživanjima, u usporedbi s bolje istraženim vrstama

kukaša. Ipak, zbog velike varijabilnosti između pojedinih jedinki iste vrste, njihova primjena zahtijeva dodatna istraživanja.

XIII. Primjenjeni multibiomarkerski pristup daje sveobuhvatnu informaciju o stanju okoliša i istraživane biote te, unatoč nešto povišenim razinama oksidacijskog stresa (GSH, CAT) u ribama s onečišćenih postaja obiju rijeka ili oksidacijskog oštećenja (MDA) u ribama s onečišćene postaje rijeke Ilove, ukazuje na još uvijek djelotvoran antioksidacijski sustav na svim lokacijama, što ukazuje da u bioti nije došlo do značajnog ili trajnog oštećenja pod utjecajem ispuštanja komunalnih i industrijskih otpadnih voda, kao i ostalih antropogenih utjecaja na rijeke Krku i Ilovu.

XIV. S obzirom na dobivene rezultate, mjerljivost metala i svih biomarkera u probavilu riba, visoku ponovljivost i osjetljivost mjerenja te slabo izraženu povezanost razina metala u probavilu s drugim čimbenicima poput dužine, mase ili spola riba, probavilo se pokazalo kao važno i korisno bioindikatorsko tkivo u procjeni unosa metala prehranom i izloženosti metalima te ga je potrebno koristiti u okolišnim studijama kako se nebi zanemario značajan udio unosa metala hranom.

XV. Cjelokupno istraživanje upućuje na zasad umjeren utjecaj otpadnih voda i antropogenih aktivnosti na istraživana područja i vrste dinaridske rijeke Krke i panonske rijeke Ilove, ali postojeće razlike u koncentracijama metala, od kojih mnogi imaju porast na onečišćenim postajama, kao i veće razine oksidacijskog stresa i/ili oksidacijskog oštećenja, upućuju na prisutnu opasnost i ukazuju na potrebu kontinuirane kontrole i monitoringa ovih područja, strateški osobito bitnih zbog blizine zaštićenih područja Nacionalnog parka Krka te Parka prirode Lonjsko polje.

6. POPIS LITERATURE

Able KW, Lamonaca JC (2006). Scale formation in selected western North Atlantic flatfishes. *Journal of Fish Biology* 68: 1679 - 1692.

Adams SM, McLean RB (1985) Estimation of Largemouth Bass, *Micropterus salmoides* Lacèpede, growth using liver somatic index and physiological variables. *Journal of Fish Biology* 26: 111–126.

Adey EA, Black KD, Sawyer T, Shimmield TM, Trueman CN (2009) Scale microchemistry as a tool to investigate the origin of wild and farmed *Salmo salar*. *Marine Ecology Progress Series* 390: 225–235.

Alaya A, Muñoz MF, Argüelos S (2014) Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity* 2014: 1-31.

Alfonso-Prieto M, Biarnes X, Vidossich P, Rovira C (2009) The molecular mechanism of the catalase reaction. *Journal of the American Chemical Society* 131: 11751-11761.

Almirall JR, Trejos T (2016) Applications of LA-ICP-MS to forensic science. *Elements* 12 (5): 335-340.

Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS (2006) Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology* 76 (2): 160–202.

Amin OM (2013) Classification of Acanthocephala. *Folia Parasitologica* 60: 273-305.

Andreji J, Stránai I, Massányi P, Valent M (2006) Accumulation of some metals in muscles of five fish species from lower Nitra River. *Journal of Environmental Science and Health, Part A* 41 (11): 2607–2622.

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.

Asmamaw B (2016) Transferrin in fishes: A review article. *Journal of Coastal Life Medicine* 4: 176-180.

- Atif F, Kaur M, Yousuf S, Raisuddin S (2006) In vitro free radical scavenging activity of hepatic metallothionein induced in an Indian freshwater fish, *Channa punctata* Bloch. *Chemico-biological Interactions* 162(2):172-180.
- Atli G, Alptekin I, Tukul S, Canli M (2006) Response of catalase activity to Ag^+ , Cd^{2+} , Cr^{6+} , Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus*. *Comparative Biochemistry and Physiology* 143: 218–224.
- Banerjee BD, Seth V, Bhattacharya A (1999) Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicology Letters* 107: 33–47.
- Banerjee R, Ragsdale, SW (2003) The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. *Annual Review of Biochemistry* 72: 209-247.
- Barak NAE, Mason CF (1990) Mercury, cadmium and lead concentrations in five species of freshwater fish from Eastern England. *Science of the Total Environment* 92: 257–263.
- Barišić J, Filipović Marijić V, Mijošek T, Čož-Rakovac R, Dragun Z, Krasnići N, Ivanković D, Kružlicová D, Erk M (2018) Evaluation of architectural and histopathological biomarkers in the intestine of brown trout (*Salmo trutta* Linnaeus, 1758) challenged with environmental pollution. *Science of the Total Environment* 642: 656–664.
- Barst BD, Rosabal M, Campbell 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.
- Barton BA, Morgan JD, Vijayan MM (2002) Physiological condition-related indicators of environmental stress in fish. U: Adams SM (urednik) *Biological indicators of stress in fish* American Fisheries Society, 111–148.
- Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. *Geochimica et Cosmochimica Acta* 64: 1705–1714.
- Beard JL, Dawson H, Pifiero DJ (1996) Iron metabolism: a comprehensive review. *Nutrition Reviews* 54: 295–317.

- Benejam L, Benito J, Ordóñez J, Armengol J, García-Berthou E (2008) Short-term effects of a partial drawdown on fish condition in a eutrophic reservoir . *Water Air and Soil Pollution* 190: 3-11.
- Berntssen MHG, Hylland K, Wendelaar Bonga SE, Maage A (1999) Toxic levels of dietary copper in Atlantic salmon (*Salmo salar* L.) parr. *Aquatic Toxicology* 46: 87–99.
- Berntssen MHG, Lundebye A, Hamre K (2000) Tissue lipid peroxidative responses in Atlantic salmon (*Salmo salar* L.) parr fed high levels of dietary copper and cadmium. *Fish Physiology and Biochemistry* 23: 35–48.
- Berntssen MHG, Aspholm OØ, Hylland K, Wendelaar Bonga SE, Lundebye A-K (2001) Tissue metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo salar* L.) parr fed elevated dietary cadmium. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 128 (3): 299–310.
- Bhattacharya A, Bhattacharya S (2007) Induction of oxidative stress by arsenic in *Clarias batrachus*: involvement of peroxisomes. *Ecotoxicology and Environmental Safety* 66 (2): 178–187.
- Bigi A, Foresti E, Gregorini R, Ripamonti A, Roveri N, Shah JS (1992) The role of magnesium on the structure of biological apatites. *Calcified Tissue International* 50: 439–444.
- Bisswanger H (2014) Enzyme assays. *Perspectives in Science* 1: 41-55.
- Blindauer CA, Leszczyszyn O (2010) Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more. *Natural Product Reports* 27(5): 720-741.
- Blust R (2012) Cobalt. U: Wood CM, Farrell AP, Brauner CJ (urednici) *Fish Physiology: homeostasis and toxicology of essential metals*. Vol. 31A. Academic, London, 291–326.
- Boeker C, Geist J (2015). Effects of invasive and indigenous amphipods on physico-chemical and microbial properties in freshwater substrates. *Aquatic Ecology* 49(4): 467–480.
- Bojko J, Burgess AL, Baker AG, Orr CH (2020) Invasive Non-Native Crustacean Symbionts: Diversity and Impact. *Journal of invertebrate pathology*, u objavi, 107482.
- Bonnail E, Sarmiento AM, DelValls TA, Nieto JM, Riba I (2016) Assessment of metal contamination, bioavailability, toxicity and bioaccumulation in extreme metallic

environments (Iberian Pyrite Belt) using *Corbicula fluminea*. Science of the Total Environment 544: 1031–1044.

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 (4): 319–334.

Britton JR, Gozlan RE, Copp GH (2011) Managing non-native fish in the environment. Fish and Fisheries 12: 256–274.

Brkić Ž, Kuhta M, Larva O, Gottstein S (2019) Groundwater and connected ecosystems: an overview of groundwater body status assessment in Croatia. Environmental Sciences Europe 31: 75.

Broeg K, Lehtonen K (2006) Indices for the assessment of environmental pollution of the Baltic Sea coasts: Integrated assessment of a multi-biomarker approach. Marine Pollution Bulletin 53: 508-522.

Brothers EB, Mathews CP, Lasker R (1976) Daily growth increments in otoliths from larval and adult fishes. U.S. National Marine Fisheries Service Fishery Bulletin 74:1-8.

Burgess RR (2018) A brief practical review of size exclusion chromatography: Rules of thumb, limitations, and troubleshooting. Protein Expression and Purification 150: 81-85.

Bury NR, Walker PA, Glover CN (2003). Nutritive metal uptake in teleost fish. Journal of Experimental Biology 206: 11-23.

Bury NR, Boyle D, Cooper CA (2012) Iron. U: Wood CM, Farrell AP, Brauner CJ (urednici) Fish physiology: Homeostasis and toxicology of essential metals, Vol. 31A. Academic, London, 201–251.

Calisi A, Zaccarelli N, Lionetto MG, Schettino T (2013) Integrated biomarker analysis in the earthworm *Lumbricus terrestris*: application to the monitoring of soil heavy metal pollution. Chemosphere 90: 2637–2644.

Campana SE, Neilson, JD (1982) Daily Growth Increments in Otoliths of Starry Flounder (*Platichthys stellatus*) and the Influence of Some Environmental Variables in Their Production. Canadian Journal of Fisheries and Aquatic Sciences 39: 937-942.

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. *Marine Ecology Progress Series* 188: 263–297.

Campana SE, Chouinard GA, Hanson JM, Frechet A, Bratney J (2000) Otolith elemental fingerprints as biological tracers of fish stocks. *Fisheries Research* 46: 343–357.

Campbell PGC, Giguère A, Bonneris E, Hare L (2005) Cadmium-handling strategies in two chronically exposed indigenous freshwater organisms - the yellow perch (*Perca flavescens*) and the floater mollusc (*Pyganodon grandis*). *Aquatic Toxicology* 72: 83-97.

Campbell PGC, Kraemer LD, Giguère A, Hare L, Hontela A (2008) Subcellular distribution of cadmium and nickel in chronically exposed wild fish: inferences regarding metal detoxification strategies and implications for setting water quality guidelines for dissolved metals. *Human and Ecological Risk Assessment* 14: 290–316.

Can E, Yabanli M, Kehayias G, Aksu Ö, Kocabaş M, Demir V, Kayim M, Kutluyer F, Şeker S (2012) Determination of bioaccumulation of heavy metals and selenium in tissues of brown trout *Salmo trutta macrostigma* (Duméril, 1858) from Munzur Stream, Tunceli, Turkey. *Bulletin of Environmental Contamination and Toxicology* 89: 1186–1189.

Canesi L, Viarengo A, Leonzio C, Filipelli M, Gallo G (1999) Heavy metals and glutathione metabolism in mussel tissue. *Aquatic Toxicology* 46: 67–76.

Carignan V, Villard M-A (2002) Selecting indicator species to monitor ecological integrity: a review. *Environmental Monitoring and Assessment* 78: 45–61.

Carney Almroth B, Albertsson E, Sturve J, Förlin L (2008) Oxidative stress, evident in antioxidant defences and damage products, in rainbow trout caged outside a sewage treatment plant. *Ecotoxicology and Environmental Safety* 70(3):370-378.

Caron A, Rosabal M, Drevet O, Couture P, Campbell PGC (2018) Binding of trace elements (Ag, Cd, Co, Cu, Ni, and Tl) to cytosolic biomolecules in livers of juvenile yellow perch (*Perca flavescens*) collected from lakes representing metal contamination gradients. *Environmental Toxicology and Chemistry* 37: 576-586.

Carriquiriborde P, Handy RD, Davies SJ (2004) Physiological modulation of iron metabolism in rainbow trout (*Oncorhynchus mykiss*) fed low and high iron diets. *Journal of Experimental Biology* 207: 75–86.

Carvalho ML, Santiago S, Nunes ML (2005) Assessment of the essential element and heavy metal content of edible fish muscle. *Analytical and Bioanalytical Chemistry* 382: 426-432.

Chesman B, O'hara S, Burt GR, Langston WJ (2007) Hepatic metallothionein and total oxyradical scavenging capacity in Atlantic cod. *Aquatic Toxicology* 84: 310-320.

Chovanec A, Hofer R, Schiemer F (2003) Fish as bioindicators. U: Markert BA, Breure AM, Zechmeister HG (urednici) *Bioindicators & Biomonitoring: principles, concepts and applications*. Elsevier, 639-676.

Cindrić M, Marković A, Horvatić A (2009) Sprengnute tehnike tekućinski kromatograf–spektrometar masa: osnove metodologije i primjene. *Medicina* 45: 218-232.

Cindrić AM, Garnier C, Oursel B, Pižeta I, Omanović D (2015) Evidencing the natural and anthropogenic processes controlling trace metals dynamic in a highly stratified estuary: The Krka River estuary (Adriatic, Croatia). *Marine Pollution Bulletin* 94: 199-216.

Clarke AD, Telmer KH, Shrimpton JM (2007) Elemental analysis of otoliths, fin rays and scales: a comparison of bony structures to provide population and life-history information for the Arctic grayling (*Thymallus arcticus*). *Ecology of Freshwater Fish* 16: 354-361.

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.

Coleman JE (1992) Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. *Annual Review of Biochemistry* 61: 897–946.

Collins SM, Kohler TJ, Thomas SA, Fetzer WW, Flecker AS (2016) The importance of terrestrial subsidies in stream food webs varies along a stream size gradient. *Oikos* 125: 674–685.

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.

Cravo A, Lopes B, Serafim A, Company R, Barreira L, Gomes T, Bebianno MJ (2009) A multibiomarker approach in *Mytilus galloprovincialis* to assess environmental quality. *Journal of Environmental Monitoring* 11: 1673–1686.

Crompton DWT, Nickol BB (1985) *Biology of Acanthocephala*. Cambridge Univ Press, Cambridge.

Cukrov N, Barišić D (2006) Spatial Distribution of ^{40}K and ^{232}Th in Recent Sediments of the Krka River Estuary. *Croatica Chemica Acta* 79: 115-118.

Cukrov N, Frančišković-Bilinski S, Mikac N, Roje V (2008a) Natural and anthropogenic influences recorded in sediments from the Krka River estuary (Eastern Adriatic Coast), evaluated by statistical methods. *Fresenius Environmental Bulletin* 17: 855-863.

Cukrov N, Cmur P, Mlakar M, Omanović D (2008b) Spatial distribution of trace metals in the Krka River, Croatia. An example of the self-purification. *Chemosphere* 72: 1559–1566.

Cukrov N, Tepić N, Omanović D, Lojen S, Bura-Nakić E, Vojvodić V, Pižeta I (2012) Qualitative interpretation of physico-chemical and isotopic parameters in the Krka River (Croatia) assessed by multivariate statistical analysis. *International Journal of Environmental Analytical Chemistry* 92: 1187–1199.

Cukrov N, Cuculić V, Barišić D, Lojen S, Lovrenčić Mikelić I, Oreščanin V, Vdović N, Fiket Ž, Čermelj B, Mlakar M (2013) Elemental and isotopic records in recent fluvio lacustrine sediments in karstic river Krka, Croatia. *Journal of Geochemical Exploration* 134: 51-60.

Culioli J-L, Calendini S, Mori C, Orsini A (2009) Arsenic accumulation in a freshwater fish living in a contaminated river of Corsica, France. *Ecotoxicology and Environmental Safety* 72: 1440-1445.

Cummins K (1973) Trophic relations of aquatic insects. *Annual Review of Entomology* 18: 183-206.

Čanković M, Delić S, Kiškarolj M, Rukavina J (1968) Parazito-fauna slatkovodnih riba Bosne i Hercegovine (Trematoda, Cestoda, Nematoda, Acanthocephala). *Akademija Nauka i Umjetnosti Bosne i Hercegovine. Odijeljenje Prirodnih i Matematičkih Nauka*, Sarajevo.

Čolović MB, Krstić DZ, Lazarević-Pašti TD, Bondžić AM, Vasić VM (2013) Acetylcholinesterase inhibitors: pharmacology and toxicology. *Current Neuropharmacology* 11(3): 315-335.

Dallinger, R., Kautzky, H., 1985. Contaminated The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdneri*): a field study. *Oecologia* 67: 82-89.

Daniels G (2007) Functions of red cell surface proteins. *Vox Sanguinis* 93: 331-334.

Darafsh F, Mashinchian A, Fatemi M, Jamili S (2008) Study of the application of fish scale as bioindicator of heavy metal pollution (Pb, Zn) in the *Cyprinus carpio* of the Caspian Sea. *Research Journal of Environmental Sciences* 2(6): 438– 444.

Dautović J (2006) Determination of metals in natural waters using high resolution inductively coupled plasma mass spectrometry, završni rad.

De Boeck G, Meeus W, Coen WD, Blust R (2004) Tissue-specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). *Aquatic Toxicology* 70 (3): 179–188.

De Giosa M, Czerniejewski P, Rybczyk A (2014) Seasonal changes in condition factor and weight-length relationship of invasive *Carassius gibelio* (Bloch, 1782) from Leszczynskie Lakeland, Poland. *Journal of Advanced Zoology* 2014: 1–7.

de la Calle Guntiñas MB, Bordin G, Rodriguez AR (2002) Identification, characterization and determination of metal binding proteins by liquid chromatography. A review. *Analytical and Bioanalytical Chemistry* 374: 369–378.

de Lima D, Roque GM, de Almeida EA (2012) In vitro and in vivo inhibition of acetylcholinesterase and carboxylesterase by metals in zebrafish (*Danio rerio*). *Marine Environmental Research* 91: 45-51.

Deb SC, Fukushima T (1999) Metals in aquatic ecosystems: mechanisms of uptake, accumulation and release-ecotoxicological perspectives. *International Journal of Environmental Studies* 56: 385-417.

Del Rio D, Stewart AJ, Pellegrini N (2005) A review of fecent studeis on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism & Cardiovascular Diseases*, 15(4): 316-328.

Delić A (1989) Ihtiofauna rijeke Ilove u području gornjeg Poilovlja (središnja Hrvatska). *Ribarstvo Jugoslavije* 44: 26-28.

Delić A (1991) Kvalitativni i kvantitativni sastav makrozoobentosa rijeke Ilove. *Ribarstvo Jugoslavije* 46: 10-13.

Dezfuli BS, Lui A, Giari L, Boldrini P, Giovinazzo G (2008) Ultra-structural study on the body surface of the acanthocephalan parasite *Dentitruncus truttae* in brown trout. *Microscopy Research and Technique* 71: 230–235.

Dezfuli BS, Lui A, Giovinazzo G, Boldrini P, Giari L (2009) Intestinal inflammatory response of powan *Coregonus lavaretus* (Pisces) to the presence of acanthocephalan infections. *Parasitology* 136: 929–937.

Dezfuli BS, Giari L, Squerzanti S, Lui A, Lorenzoni M, Sakalli S, Shinn AP (2011) Histological damage and inflammatory response elicited by *Monobothrium wagneri* (Cestoda) in the intestine of *Tinca tinca* (Cyprinidae). *Parasites & Vectors* 4:225.

Dezfuli BS, Lui A, Squerzanti S, Lorenzoni M, Shinn AP (2012) Confirmation of the hosts involved in the life cycle of an acanthocephalan parasite of *Anguilla anguilla* (L.) from Lake Piediluco and its effect on the reproductive potential of its amphipod intermediate host. *Parasitology Research* 110: 2137–2143.

Dossi C, Ciceri E, Giussani B, Pozzi A, Galgaro A, Viero A, Vigano A (2007) Water and snow chemistry of main ions and trace elements in the karst system of Monte Pelmo massif (Dolomites, Eastern Alps, Italy). *Marine Freshwater Research* 58: 649–656.

Dove SG, Kingsford MJ (1998) Use of otoliths and eye lenses for measuring trace-metal incorporation in fishes: A biogeographic study. *Marine Biology* 130: 377–387.

Dragun Z, Raspor B, Podrug M (2007) The influence of the season and the biotic factors on the cytosolic metal concentrations in the gills of the European chub (*Leuciscus cephalus* L.). *Chemosphere* 69: 911-919.

Dragun Z, Podrug M, Raspor B (2009) Combined use of bioindicators and passive samplers for the assessment of river water contamination with metals. *Archives of Environmental Contamination and Toxicology* 57: 211-220.

Dragun Z, Kapetanović D, Raspor B, Teskeredžić E (2011) Water quality of medium size watercourse under baseflow conditions: the case study of river Sutla in Croatia. *Ambio* 40(4): 391–407.

Dragun Z, Filipović Marijić V, Krasnići N, Ramani S, Valić D, Rebok K, Kostov V, Jordanova M, Erk M (2017) Malondialdehyde concentrations in the intestine and gills of Vardar chub (*Squalius vardarensis* Karaman) as indicator of lipid peroxidation. *Environmental Science and Pollution Research* 24(20): 16917-16926.

Dragun Z, Filipović Marijić V, Krasnići N, Ivanković D, Valić D, Žunić J, Kapetanović D, Vardić Smrzlić I, Redžović Z, Grgić I, Erk M (2018a) 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.

Dragun Z, Krasnići N, Kolar N, Filipović Marijić V, Ivanković D, Erk M (2018b) Cytosolic distributions of highly toxic metals Cd and Tl and several essential elements in the liver of brown trout (*Salmo trutta* L.) analyzed by size exclusion chromatography and inductively coupled plasma mass spectrometry. *Chemosphere* 207: 62-173.

Dragun Z, Tepić N, Ramani S, Krasnići N, Filipović Marijić V, Valić D, Kapetanović D, Erk M, Rebok K, Kostov V, Jordanova M (2019) Mining waste as a cause of increased bioaccumulation of highly toxic metals in liver and gills of Vardar chub (*Squalius vardarensis* Karaman, 1928). *Environmental Pollution* 247: 564–576.

Dragun Z, Krasnići N, Ivanković D, Filipović Marijić V, Mijošek T, Redžović Z, Erk M (2020) Comparison of intracellular trace element distributions in the liver and gills of the invasive freshwater fish species, Prussian carp (*Carassius gibelio* Bloch, 1782). *Science of the Total Environment* 730: 138923

Durgo K, Oreščanin V, Lulić S, Kopjar N, Želježić D, Franekić Čolić J (2009) The assessment of genotoxic effects of wastewater from a fertilizer factory. *Journal of Applied Toxicology* 29: 42–51.

Durmaz H, Sevgiler Y, Üner N (2006) Tissue-specific antioxidative and neurotoxic responses to diazinon in *Oreochromis niloticus*. *Pesticide Biochemistry and Physiology* 84: 215–226.

Đikanović V, Skorić S, Gačić Z (2016) Concentrations of metals and trace elements in different tissues of nine fish species from the Međuvršje Reservoir (West Morava River Basin, Serbia). *Archives of Biological Sciences* 68(4): 811–819.

Dorđević VB (2004) Free radicals in cell biology. *International Review of Cytology* 237: 57-89.

Elumalai E, Antunes C, Guilhermino L (2007) Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NM Portugal) exposed to zinc and mercury. *Chemosphere* 66 (7): 1249–1255.

Ergüden SA (2015) Age and growth properties of Prussian carp, *Carassius gibelio* (Bloch, 1782) living in the middle basin of Seyhan River in Adana, Turkey. *Pakistan Journal of Zoology* 47: 1365–1371.

Erk M, Ivanković D, Raspor B, Pavičić J (2002) Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins. *Talanta* 57 (6): 1211–1218.

Fairweather Tait SJ (1983) The availability of minerals in food, with particular reference to iron. *Journal of the Royal Society of Health* 103: 74-77.

Falfushynska HI, Gnatyshyna LL, Stoliar OB, NamYK (2011) Various responses to copper and manganese exposure of *Carassius auratus gibelio* from two populations. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* 154 (3): 242–253.

Farkas A, Salanki J, Specziar A (2003) Age- and size-specific patterns of heavy metals in the organs of freshwater fish *Abramis brama* L. populating a low-contaminated site. *Water Research* 37 (5): 959–964.

Farombi EO, Adelowo OA, Ajimoko YR (2007) Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African catfish (*Clarias gariepinus*) from Nigeria Ogun River. *International Journal of Environmental Research and Public Health* 4(2): 158-165.

Fatima M, Ahmad I, Sayeed I, Athar M, Raisuddin S (2000) Pollutant induced over-activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. *Aquatic Toxicology* 49:243–250.

Fernandes C, Fontainhas-Fernandes A, Ferreira M, Salgado MA (2008) Oxidative stress response in gill and liver of *Liza saliens*, from the Esmoriz-Paramos coastal lagoon, Portugal. *Archives of Environmental Contamination and Toxicology* 55: 262-269.

Fijan N (2006) *Zaštita zdravlja riba*. Faculty of Agriculture Osijek

Filipović Marijić V, Raspor B (2003) Metallothionein and metal levels in cytosol of liver, kidney and brain in relation to growth parameters of *Mullus surmuletus* and *Liza aurata* from the Eastern Adriatic Sea. *Water Research* 37: 3253-3262.

Filipović Marijić V, Raspor B (2005) Biološka raspoloživost metala u morskom ekosustavu. *Kemija u Industriji* 54 (3): 143–148.

Filipović Marijić V, Raspor B (2007) Metal exposure assessment in native fish, *Mullus barbatus* L., from the Eastern Adriatic Sea. *Toxicology Letters* 168: 292-301.

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 Part C* 708: 133–138.

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 and Soil Pollution* 223: 4769–4783.

Filipović Marijić V, Vardić Smrzlić I, Raspor B (2013) Effect of acanthocephalan infection on 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.

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.

Filipović Marijić V, Dragun Z, Sertić Perić M, Matoničkin Kepčija R, Gulin V, Velki M., Ečimović S, Hackenberger BK, Erk, M (2016a) Investigation of the soluble metals in tissue as biological response pattern to environmental pollutants (*Gammarus fossarum* example). *Chemosphere* 154: 300–309.

Filipović Marijić V, Sertić Perić M, Matoničkin Kepčija R, Dragun Z, Kovarik I, Gulin V, Erk M (2016b) Assessment of metal exposure, ecological status and required water quality monitoring strategies in small- to medium-size temperate rivers. *Journal of Environmental Science and Health, Part A* 51(4): 309–317.

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.

Florence TM, Batley GE (1977) Determination of the chemical forms of trace metals in natural waters, with special reference to copper, lead, cadmium and zinc. *Talanta* 24: 151-158.

Fogarasi E, Croitoru M, Fülöp I, Muntean D (2016) Is the Oxidative Stress Really a Disease? *Acta Marisiensis - Seria Medica* 62(1): 112-120.

Forman HJ, Zhang H, Rinna A (2009) Glutathione: overview of its protective roles, measurement and biosynthesis. *Molecular Aspects of Medicine* 30: 1-12.

Forstner U, Wittmann GTW (1981) *Metal Pollution in the Aquatic Environment*. 2 izd., Springer-Verlag, Berlin Heidelberg.

Frasco M, Fournier D, Carvalho F, Guilhermino L (2005) Do metals inhibit cholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. *Biomarkers* 10: 360-375.

Friedrich LA, Halden NM (2008) Alkali element uptake in otoliths: a link between the environment and Otolith microchemistry. *Environmental Science and Technology* 42: 3514-3518.

Glazier DS (2009) Amphipoda. U: Likens GE (urednik) *Encyclopedia of Inland Waters*. Oxford, Elsevier, vol. 2, 89-115.

Goenaga Infante H, Van Campenhout K, Schaumlöffel D, Blust R, Adams FC (2003) Multi-element speciation of metalloproteins in fish tissue using size-exclusion chromatography coupled “on-line” with ICP-isotope dilution-time-of-flight-mass spectrometry. *Analyst* 128, 651–657.

Gaetke LM, Chow-Johnson HS, Chow CK (2014) Copper: toxicological relevance and mechanisms. *Archives of Toxicology* 88: 1929–1938.

Gagné F, Blaise C, Pellerin J (2005) Altered exoskeleton composition and vitellogenesis in the crustacean *Gammarus* sp. collected at polluted sites in the Saguenay Fjord, Quebec, Canada. *Environmental Research* 98: 89-99.

Gaillardet J, Viers J, Dupré B (2004) Trace elements in river waters. U: Holland HD, Turekian KK (urednici) *Treatise on geochemistry: Surface and ground water, weathering, and soils*, Vol. 5, Elsevier, Amsterdam, 225-272.

Garcia JS, Schmidt de Magalhaes C, Zezzi Arruda MA (2006) Trends in metal-binding and metalloprotein analysis. *Talanta* 69: 1–15.

García-Varela M, Pérez-Ponce de León G (2015) Advances in the classification of acanthocephalans: Evolutionary history and evolution of the parasitism. U: Morand S, Krasnov B, Littlewood D (urednici) *Parasite Diversity and Diversification: Evolutionary Ecology Meets Phylogenetics*. Cambridge: Cambridge University Press, 182-201.

Gazi M, Sultana T, Min GS, Park YC, García-Varela M, Nadler SA, Park JK (2012) The complete mitochondrial genome sequences of *Oncicola luehei* (Acanthocephala: Archiacanthocephala) and its phylogenetic position within Syndermata. *Parasitology International* 61: 307–316.

Geffard A, Quéau H, Dedourge O, Biagianti-Risboug S, Geffard O (2007) Influence of biotic and abiotic factors on metallothionein level in *Gammarus pulex*. *Comparative Biochemistry and Physiology C* 145: 632–640.

Gerhardt A (2002) Bioindicator species and their use in biomonitoring. *Environmental monitoring I. Encyclopedia of life support systems*. UNESCO ed. Oxford (UK): Eolss Publisher.

Gerhardt A, Bloor M, Mills CL (2011) Gammarus: important taxon in freshwater and marine changing environments. *International Journal of Zoology* 2011: 524276.

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.

Giguère A, Campbell PGC, Hare L, Couture P (2006) Subcellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient. Aquatic Toxicology 77: 178–189.

Gillanders BM, Kingsford MJ (2000) Elemental fingerprints of otoliths of fish may distinguish estuarine 'nursery' habitats. Marine Ecology Progress Series 201: 273–286.

Giller PS, Malmqvist B (1998) The Biology of Streams and Rivers, 1. izd., Oxford University Press, Oxford.

Goto T, Sasaki K (2014) Effects of trace elements in fish bones on crystal characteristics of hydroxyapatite obtained by calcination. Ceramics International 40(7): 10777–10785.

Gottstein S, Žganec K, Maguire I, Kerovec M, Jalžić B (2007) Viši rakovi slatkih i bočatih voda porječja rijeke Krke. U: Marguš D (urednik), Zbornik radova sa simpozija Rijeka Krka i Nacionalni park Krka, Šibenik, 421–431.

Gray AL (1985) Solid sample introduction by laser ablation for inductively coupled plasma source mass spectrometry. Analyst 110: 551–556.

Greani S, Lourkisti R, Berti L, Marchand B, Giannettini J, Santini J, Quilichini Y (2017) Effect of chronic arsenic exposure under environmental conditions on bioaccumulation, oxidative stress, and antioxidant enzymatic defenses in wild trout *Salmo trutta* (Pisces, Teleostei). Ecotoxicology 26 (7): 930–941.

Greenaway P (2008) Calcium balance and moulting in the Crustacea. Biological reviews of the Cambridge Philosophical Society 60 (3): 425–454.

Gutteridge JM (2015) Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clinical Chemistry 41: 1819–1828.

Habdija I, Prime Habdija B, Radanović I, Špoljar M, Matoničkin Kepčija R, Vujčić Karlo S, Miliša M, Ostojić A, Sertić Perić M (2011) Protista – Protozoa, Metazoa – Invertebrata, strukture i funkcije. Alfa, Zagreb, Hrvatska.

- Hamer PA, Jenkins GP (2007) Comparison of spatial variation in otolith chemistry of two fish species and relationships with water chemistry and otolith growth. *Journal of Fish Biology* 71: 1035–1055.
- Hamer B, Jakšić Ž, Pavičić-Hamer D, Perić L, Medaković D, Ivanković D, Pavičić J, Zilberberg C, Schröder HC, Müller WEG, Smodlaka N, Batel R (2008) Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels *Mytilus galloprovincialis* on biological parameters used for pollution assessment. *Aquatic Toxicology* 89: 137-151.
- Hammond MP, Savage C (2009) Use of regenerated scales and scale marginal increments as indicators of recent dietary history in fish. *Estuaries and Coasts* 32(2): 340-349.
- Hamza-Chaffai A, Amiard JC, Pellerin J, Joux L, Berthet B (2000) The potential use of metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal exposure. *Comparative Biochemistry and Physiology Part C* 127: 185-197.
- Handy RD (1996) Dietary exposure to toxic metals in fish. U: Taylor EW (urednik) *Toxicology of aquatic pollution: physiological, molecular, and cellular approaches*. Society of Experimental Biology Seminar Series 57, Cambridge University Press, Cambridge, 29–60.
- Handy RD, Sims DW, Giles A, Campbell HA, Musonda MM (1999) Metabolic tradeoff between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquatic Toxicology* 47: 23–41.
- Handy RD, Galloway TS, Depledge MH (2003) A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotoxicology* 12: 331- 343.
- Harris ED (2000) Cellular copper transport and metabolism. *Annual Review of Nutrition* 20: 291-310.
- Hauser-Davis RA, de Campos RC, Ziolli RL (2012) Fish metalloproteins as biomarkers of environmental contamination. *Reviews of Environmental Contamination and Toxicology* 218: 101-123.
- Hernández-Moreno D, de la Casa-Resino I, Maria Flores J, González-Gómez MJ, María Neila C, Soler F, Pérez-López M (2014) Different enzymatic activities in carp (*Cyprinus carpio* L.)

as potential biomarkers of exposure to the pesticide methomyl. *Arhiv za higijenu rada i toksikologiju* 65: 311-318.

Holá M, Kalvoda J, Bábek O, Brzobohatý R, Holoubek I, Kanický V, Škoda R (2009) LA-ICP-MS heavy metal analyses of fish scales from sediments of the Oxbow Lake Certak of the Morava River (Czech Republic). *Environmental Geology* 58: 141–151.

Holm R, Kennepohl P, Solomon EI (1996) Structural and functional aspects of metal sites in biology. *Chemistry Reviews* 96: 2239–2314.

Holt EA, Miller SW (2010) Bioindicators: using organisms to measure environmental impacts. *Nature Education Knowledge* 3(10): 8

Hou Z, Sket B (2016) A review of Gammaridae (Crustacea: Amphipoda): the family extent, its evolutionary history, and taxonomic redefinition of genera. *Zoological Journal of the Linnean Society* 176: 323–348.

Huggett RJ, Kimerle RA, Mehrle PM Jr, Bergman HL (1992) Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. Boca Raton, FL: Lewis Publishers.

Illies J (1978) *Limnofauna Europaea*. A checklist of the animals inhabiting European inland waters, with an account of their distribution and ecology, 2. izd., Gustav Fischer Verlag, Stuttgart.

IPCS (1999) Manganese and its compounds. World Health Organization, international Programme on chemical safety (concise international chemical assessment document 12), Geneva.

Isani G, Andreani G, Kindt M, Carpenè E (2000) Metallothioneins (MTs) in marine molluscs. *Cell and Molecular Biology* 46 (2): 311–330.

Isani G, Carpenè E (2014) Metallothioneins, unconventional proteins from unconventional animals: a long journey from nematodes to mammals. *Biomolecules* 4: 435-457.

Ivanković D, Pavičić J, Erk M, Filipović Marijić V, Raspor B (2005) Evaluation of *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term field study: seasonal and spatial variability. *Marine Pollution Bulletin* 50: 1303-1313.

Izvadak iz Registra vodnih tijela prema Planu upravljanja vodnim područjima 2016. - 2021. Klasifikacijska oznaka: 008-02/21-02/74, urudžbeni broj: 314-21-1, veljača 2021, Hrvatske vode.

Izvješće o provedbi planova upravljanja vodnim područjima iz Okvirne direktive o vodama; Država članica: HRVATSKA, 2015, Bruxelles

Izvješće o stanju površinskih voda u 2019. godini, Hrvatske vode, Zagreb, Hrvatska

Janz DM (2012) Selenium. U: Wood CM, Farrell AP, Brauner CJ (urednici) Fish physiology: Homeostasis and toxicology of essential metals, Vol 31A. Academic, London, 327-374.

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.

Javna ustanova NP Krka, <http://np-krka.hr/>, ustupljeno: 15.04.2021.

Jebali, J., Banni, M., Almeida, E. A., Bannaoui, A. & Boussetta, H., (2006). Effects of malathion and cadmium on acetylcholinesterase activity and metallothionein levels in the fish *Seriola dumerilli*. Fish Physiology and Biochemistry, 32, 93-98.

Jelić D, Žutinić P, Jelić M (2009) Značenje i karakteristike ihtiofaune rijeke Ilove (središnja Hrvatska). Ribarstvo 67: 53-61.

Jenkins JA (2004) Fish bioindicators of ecosystem condition at the Calcasieu Estuary, Louisiana. National wetlands research center USGS, Lafayette.

Jensen JK, Holm PE, Nejrup J, Larsen MB, Borggaard OK (2009) The potential of willow for remediation of heavy metal polluted calcareous urban soils. Environmental Pollution 157: 931–937.

Johnson JL, Rajagopalan KV (1976) Purification and properties of sulphite oxidase from human liver. Journal of Clinical Investigation 58: 543–550.

Jonas DA, Antignac E, Antoine JM, Classen HG, Huggett A, Knudsen I, Mahler J, Ockhuizen T, Smith M, Teuber M, Walker R, De Vogel P (1996) The safety assessment of novel foods.

Guidelines prepared by ILSI Europe Novel Food Task Force. Food and Chemical Toxicology 34: 931–940.

Kalantzi I, Mylona K, Pergantis SA, Coli A, Panopoulos S, Tsapakis M (2019) Elemental distribution in the different tissues of brood stock from Greek hatcheries. Aquaculture 503: 175-185.

Kamunde C, MacPhail R (2008) Bioaccumulation and hepatic speciation of copper in rainbow trout (*Oncorhynchus mykiss*) during chronic waterborne copper exposure. Archives of Environmental Contamination and Toxicology 54: 493–503.

Kamunde C (2009) Early subcellular partitioning of cadmium in gill and liver of rainbow trout (*Oncorhynchus mykiss*) following low-to-near-lethal waterborne cadmium exposure. Aquatic Toxicology 91: 291–301.

Karaman G, Pinkster S (1987) Freshwater *Gammarus* species from Europe, North Africa and adjacent regions of Asia (Crustacea-Amphipoda) Part III. *Gammarus balcanicus* group and related species. Bijdragen tot de Dierkunde 57: 207–260.

Kashani A, Mostaghimi J (2010) Aerosol characterization of concentric pneumatic nebulizer used in inductively coupled plasma-mass spectrometry (ICP-MS). Atomization and sprays 20: 415-433.

Kelly DW, Dick JTA, Montgomery WI (2002) The functional role of *Gammarus* (Crustacea, Amphipoda): shredders, predators, or both? Hydrobiologia 485: 199-203.

Kennedy CR (1985) Regulation and dynamics of acanthocephalan populations. U: Crompton, DWT, Nickol BB (urednici) Biology of the Acanthocephala. Cambridge University Press, Cambridge, 385-416.

Kennedy CR (2006) Ecology of the Acanthocephala. Cambridge University Press, Cambridge.

Kirschbaum J (1981) Cyanocobalamin. U: Florey K (urednik) Analytical profiles of drug substances, Vol. 10. Academic, New York, 183–288.

Klemetsen A, Amundsen P-A, Dempson JB, Jonsson B, Jonsson N, O'Connell MF, Mortensen E (2003) Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic

charr *Salvelinus alpinus* (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish* 12: 1-59.

Koç HT, Erdoğan Z, Tinkci M, Treer T (2007) Age, growth and reproductive characteristics of chub, *Leuciscus cephalus* (L., 1758) in the İkizcetepeler Dam Lake (Balıkesir), Turkey. *Journal of Applied Ichthyology* 23: 19-24

Kohen R, Nyska A (2002) Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic Pathology* 30: 620-650.

Kolda A, Petrić, I, Mucko M, Gottstein S, Žutinić, P, Goreta G, Ternjej I, Rubinić R, Radišić, M, Gligora Udovič M (2019). How environment selects: Resilience and survival of microbial mat community within intermittent karst spring Krčić (Croatia). *Ecohydrology* 12(2): e2063.

Korfali SI, Davies BE (2004) Speciation of metals in sediment and water in a river underlain by limestone: role of carbonate species for purification capacity of river. *Advances in Environmental Research* 8: 599-612.

Kottelat M, Freyhof J (2007) *Handbook of European freshwater fishes*. Kottelat and Freyhof, Cornot, Berlin.

Kraemer LD, Campbell PGC, Hare L (2006) Seasonal variations in hepatic Cd and Cu concentrations and in the sub-cellular distribution of these metals in juvenile yellow perch (*Perca flavescens*). *Environmental Pollution* 142: 313–325.

Krasnići N, Dragun Z, Erk M, Raspor B (2013) Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se and Zn) and nonessential (Cd, Pb) trace elements among protein fractions from hepatic cytosol of European chub (*Squalius cephalus* L.). *Environmental Science and Pollution Research* 20: 2340–2351.

Krasnići N, Dragun Z, Erk M, Raspor B (2014) Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (*Squalius cephalus* L.). *Environmental Science and Pollution Research* 21: 13512–13521.

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 northeastern Macedonia. *Science of the Total Environment* 613-614:1055–1068.

Krasnići N, Dragun Z, Kazazić S, Muharemović H, Erk M, Jordanova M, Rebok K, Kostov V (2019) Characterization and identification of selected metal-binding biomolecules from hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928) using various techniques of liquid chromatography and mass spectrometry. *Metallomics* 11: 1060–1078.

Kuhn DE, O'Brien KM, Crockett EL (2016) Expansion of capacities for iron transport and sequestration reflects plasma volumes and heart mass among white-blooded notothenioid fishes. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 311: 649–657.

Kunz PY, Kinle C, Gerhardt A (2010) *Gammarus* spp. in aquatic ecotoxicology and water quality assessment: toward integrated multilevel tests. *Reviews of Environmental Contamination and Toxicology* 205: 1-76.

Lacorn M, Lahrssen A, Rotzoll N, Simat T J, Steinhart H (2001) Quantification of metallothionein isoforms in fish liver and its implications for biomonitoring. *Environmental Toxicology and Chemistry* 20: 140-145.

Laflamme JS, Couillard Y, Campbell PGC, Hontela A (2000) Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. *Canadian Journal of Fisheries and Aquatic Sciences* 57: 1692–1700.

Lagrue C, Poulin R (2015) Measuring fish body condition with or without parasites: Does it matter? *Journal of Fish Biology* 87(4): 836-847.

Lam PKS (2009) Use of biomarkers in environmental monitoring. *Ocean & Coastal Management* 52(7): 348-354.

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.

Landis WG, Yu M-H (1995) *Introduction to environmental toxicology : impacts of chemicals upon ecological systems*. Lewis Publishers, Boca Raton Fla.

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

Langston WJ, Chesman BS, Burt GR, Pope ND, McEvoy J (2002) Metallothionein in liver of eels *Anguilla anguilla* from the Thames Estuary: an indicator of environmental quality? *Marine Environmental Research* 53: 263–293.

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.

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 & Technology* 43: 8665–8670.

Lavradas RT, Chávez Rocha RC, Dillenburg Saint' Pierre T, Godoy JM, Hauser-Davis RA (2016) Investigation of thermostable metalloproteins in *Perna perna* mussels from differentially contaminated areas in Southeastern Brazil by bioanalytical techniques. *Journal of Trace Elements in Medicine and Biology* 34: 70–78.

Lebrun JD, Uher E, Tusseau-Vuillemin M-H, Gourlay-Francé C (2014) Essential metal contents in indigenous gammarids related to exposure levels at the river basin scale: metal-dependent models of bioaccumulation and geochemical correlations. *Science of the Total Environment* 466: 100–108.

Lecerf A, Dobson M, Dang CK, Chauvet E (2005) Riparian plant species loss alters trophic dynamics in detritus-based stream ecosystems. *Oecologia* 146: 432–442.

Linde AR, Sánchez-Galán S, Izquierdo JI, Arribas P, Marañón E, García-Vázquez E (1998) Brown trout as biomonitor of heavy metal pollution: effect of age on the reliability of the assessment. *Ecotoxicology and Environmental Safety* 40: 120–125.

Leonardos ID, Tsikliras AC, Eleftheriou V, Cladas Y, Kagalou I, Chortatou R, Papigiotti O (2008) Life history characteristics of an invasive cyprinid fish (*Carassius gibelio*) in Chimaditis Lake (northern Greece). *Journal of Applied Ichthyology* 24 (7): 213-217.

Lionetto MG, Caricato R, Giordano ME, Pascariello MF, Marinosci L, Schettino T (2003) Integrated use of biomarkers (acetylcholinesterase and antioxidant enzyme activities) in

Mytilus galloprovincialis and *Mullus barbatus* in an Italian coastal marine area. Marine Pollution Bulletin 46: 324-330.

Lionetto MG, Caricato R, Calisi A, Schettino T (2011) Acetylcholinesterase inhibition as a relevant biomarker in environmental biomonitoring: new insights and perspectives. U: Visser JE (urednik), Ecotoxicology Around the Globe. Nova Science Publishers, Hauppauge (USA), 87–115.

Liu H, Zhang JF, Shen H, Wang XR, Wang WM (2005) Impact of copper and its EDTA complex on the glutathione-dependent antioxidant system in freshwater fish (*Carassius auratus*). Bulletin of Environmental Contamination and Toxicology 74: 1111–1117.

Liu Y, Hu Z, Li M, Gao S (2013) Applications of LA-ICP-MS in the elemental analyses of geological samples. Chinese Science Bulletin 58: 3863–3878.

Livingstone DR (1993) Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. Journal of Chemical Technology and Biotechnology 57: 195-211.

Lobinski R, Marczenko Z (1997) Spectrochemical trace analysis for metals and metalloids. U: Weber SG (urednik) Wilson and Wilsons Comprehensive Analytical Chemistry, Vol. 30, 1 izd., Elsevier, Amsterdam.

Lončar V (2015) Nastajanje i izlučivanje slobodnih radikala kod životinja pri stresnim uvjetima. Završni rad. Poljoprivredni fakultet, Osijek

Lopez Heras I, Palomo M, Madrid Y (2011) Selenoproteins: the key factor in selenium essentiality. State of the art analytical techniques for selenoprotein studies. Analytical and Bioanalytical Chemistry 400: 1717–1727.

Lundova K, Matousek J, Prokesova M, Vanina T, Sebesta R, Urban J, Stejskal V (2018) The effects of a prolonged photoperiod and light source on growth, sexual maturation, fin condition, and vulnerability to fungal disease in brook trout *Salvelinus fontinalis*. Aquaculture Research 50: 256-267.

Luoma SN (1989) Can we determine the biological availability of sediment-bound trace elements? Hydrobiologia 176/177: 379-401.

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101: 13-30.

Luperchio S, Tamir S, Tannenbaum SR (1996) NO-Induced oxidative stress and glutathione metabolism in rodent and human cells. *Free Radical Biology and Medicine* 21: 513-519.

Mackenzie K (1999) Parasites as pollution indicators in marine ecosystems: a proposed early warning system. *Marine Pollution Bulletin* 38: 955– 959.

MacNeil C, Dick JTA, Elwood R (1997) The trophic ecology of freshwater *Gammarus* (Crustacea: Amphipoda); problems and perspectives concerning the Functional Feeding Group concept. *Biological Reviews* 72 (3): 349–364.

MacNeil C, Dick JTA, Elwood RW (2000) Differential physico-chemical tolerances of amphipod species revealed by field transplantations. *Oecologia* 124:1-7.

MacNeil C, Prenter J (2000) Differential microdistributions and interspecific interactions in coexisting native and introduced *Gammarus* spp. (Crustacea: Amphipoda). *Journal of Zoology, London* 251: 377-384.

Marcogliese DJ (2004). Parasites: Small players with crucial roles in the ecological theater. *EcoHealth* 1: 151–164.

Maddock 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.

Marcinek S, Santinelli C, Cindrić AM, Evangelista V, Gonnelli M, Layglon N, Mounier S, Lenoble V, Omanović D (2020) Dissolved organic matter dynamics in the pristine Krka River estuary (Croatia). *Marine Chemistry* 225: 103848.

Martin-Antonio B, Jimenez-Cantizano RM, Salas-Leiton E, Infante C, Manchado M (2009) Genomic characterization and gene expression analysis of four hepcidin genes in the red banded seabream (*Pagrus auriga*). *Fish and Shellfish Immunology* 26: 483–491.

Martínez-Álvarez RM, Morales AE, Sanz A (2005) Antioxidant Defenses in Fish: Biotic and Abiotic Factors. *Reviews in fish biology and fisheries* 15: 75–88.

Masella R, Di Benedetto R, Vari R, Filesi C, Giovannini C (2005) Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *The Journal of Nutritional Biochemistry* 16(10): 577-586.

Mason AZ, Jenkins KD (1995) Metal detoxification in aquatic organisms. U: Tessier, A., Turner, D.R. (urednici) Metal speciation and bioavailability in aquatic systems. IUPAC, Wiley, New York, 479–608.

Mason C (2002) Biology of freshwater pollution. 4. izd., Pearson Education Limited, Harlow, England.

Mathews T, Fisher NS (2008) Trophic transfer of seven trace metals in a four-step marine food chain. *Marine Ecology Progress Series* 367: 23–33.

McGeer JC, Niyogi S, Smith DS (2012) Cadmium. U: Wood CM, Farrell AP, Brauner CJ (urednici) Fish physiology: Homeostasis and toxicology of non-essential metals, Vol. 31B. Elsevier Academic, London, 125–184.

Mertz W (1987) Trace elements in human and animal nutrition, 5. izd., vol. 1. Press Inc., London.

Michalke B, Nischwitz V (2010) Review on metal speciation analysis in cerebrospinal fluid-current methods and results: A review. *Analytica Chimica Acta* 682: 23–36.

Mihaljević Z, Ternjej I, Stanković I, Ivković M, Zelježić D, Mladinić M, Kopjar N (2011) Assessment of genotoxic potency of sulfate-rich surface waters on medicinal leech and human leukocytes using different versions of the Comet assay. *Ecotoxicology and Environmental Safety* 74(5):1416-1426.

Mijošek T, Filipović Marijić V, Dragun Z, Ivanković D, Krasnići N, Redžović Z, Sertić Perić M, Vdović N, Bačić N, Dautović J, Erk M (2020a) The assessment of metal contamination in water and sediments of the lowland Ilova River (Croatia) impacted by anthropogenic activities. *Environmental Science and Pollution Research* 27: 25374-25389.

Mijošek T, Filipović Marijić V, Dragun Z, Ivanković D, Krasnići N, Redžović Z, Veseli M, Gottstein S, Lajtner J, Sertić Perić M, Matoničkin Kepčija R, Erk M (2020b) Thallium accumulation in different organisms from karst and lowland rivers of Croatia under wastewater impact. *Environmental Chemistry* 17(2): 201–212.

Milton D, Halliday I, Sellin M, Marsh R, Staunton-Smith J, Woodhead J (2008) The effect of habitat and environmental history on otolith chemistry of barramundi *Lates calcarifer* in

estuarine populations of a regulated tropical river. *Estuarine, Coastal and Shelf Science* 78: 301–315.

Miramand P, Lafaurie M, Fowler SW, Lemaire P, Guary JC, Bentley D (1991) Reproductive cycle and heavy metals in the organs of red mullet, *Mullus barbatus* (L), from the northwestern Mediterranean. *Science of the Total Environment* 103: 47–56.

Monna F, Camizuli E, Revelli P, Biville C, Thomas C, Losno R, Scheifler R, Bruguier O, Baron S, Chateau C, Ploquin A, Alibert P (2011) Wild brown trout affected by historical mining in the Cévennes National Park, France. *Environmental Science and Technology* 45: 6823–6830.

Monserrat JM, Martínez PE, Geracitano L, Amado LL, Gaspar Martins CM, Leães Pinho GL, Chaves IS, Ferreira-Cravo M, Ventura-Lima J, Bianchini A (2007) Pollution biomarkers in estuarine animals: critical review and new perspectives. *Comparative Biochemistry and Physiology - Part C* 146: 221–234.

Montaser A (1998) *Inductively Coupled Plasma Mass Spectrometry*, WILEY-VCH.

Montes-Bayón M, DeNicola K, Caruso JA (2003) Liquid chromatography-inductively coupled plasma mass spectrometry. *The Journal of Chromatography A* 1000: 457–476.

Moravec F (2004) *Metazoan parasites of salmonid fishes of Europe*. Academia, Prague.

Mosleh YY, Paris-Palacios S, Biagianti-Risbourg S (2006) Metallothioneins induction and antioxidative response in aquatic worms *Tubifex tubifex* (Oligochaeta, Tubificidae) exposed to copper. *Chemosphere* 64: 121–128.

Mounicou S, Szpunar J, Lobinski R (2009) Metallomics: the concept and methodology. *Chemical Society Reviews* 38: 1119–1138.

Mrakovčić M, Brigić A, Buj I, Čaleta M, Mustafić P, Zanella D (2006) *Crvena knjiga slatkovodnih riba Hrvatske*. Ministarstvo kulture, Državni zavod za zaštitu prirode, Republika Hrvatska

Muhlfeld CC, Marotz B (2005) Seasonal movement and habitat use by subadult bull trout in the upper Flathead River system, Montana. *North American Journal of Fisheries Management* 25: 797–810.

- Mulligan TJ, Lapi L, Kieser R, Yamada SB, Duewer DL (1983) Salmon stock identification based on elemental composition of vertebrae. *Canadian Journal of Fisheries and Aquatic Sciences* 40: 215–229.
- Mussali-Galante P, Tovar-Sánchez E, Valverde M, Rojas del Castillo E (2013) Biomarkers of exposure for assessing environmental metal pollution: From molecules to ecosystems. *Revista Internacional de Contaminacion Ambiental* 29: 117-140.
- Nachev M, Zimmermann S, Rigaud T, Sures B (2010) Is metal accumulation in *Pomphorhynchus laevis* dependent on parasite sex or infrapopulation size? *Parasitology* 137: 1239–1248.
- 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.
- Nunes JR, Ramos-Miras J, Lopez-Piñeiro A, Loures L, Gil C, Coelho J, Loures A (2014) Concentrations of available heavy metals in Mediterranean agricultural soils and their relation with some soil selected properties: a case study in typical Mediterranean soils. *Sustainability* 6: 9124–9138.
- Ohira Y, Shimizu M, Ura K, Takagi Y (2007) Scale regeneration and calcification in goldfish *Carassius auratus*: quantitative and morphological processes. *Fisheries Science* 73: 46–54.
- Ojo AA, Wood CM (2008) In vitro characterization of cadmium and zinc uptake via the gastro-intestinal tract of the rainbow trout (*Oncorhynchus mykiss*): Interactive effects and the influence of calcium. *Aquatic Toxicology* 89: 55–64.
- Olsson PE, Kling P, Hogstrand C (1998) Mechanisms of heavy metal accumulation of toxicity in fish. U: Langston WJ, Bebianno MJ (urednici) Metal metabolism in aquatic environments. London, UK: Chapman and Hall.
- Olsvik PA, Gundersen P, Andersen RA, Zachariassen KE (2000) Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a run-off episode. *Aquatic Toxicology* 50: 301-316.

Orešić D, Čanjevac I, Plantak M (2018) Promjena protoka i protočnih režima na rijeci Ilovi. *Acta Geographica Croatica* 43/44: 1-20.

Oyoo-Okoth E, Admiraal W, Osano O, Kraak MHS, Gichuki J, Ogwai C (2012) Parasites modify subcellular partitioning of metals in the gut of fish. *Aquatic Toxicology* 106/107: 76–84.

Ovaskainen O, Weigel B, Potyutko O, Buyvolov Y (2019) Long-term shifts in water quality show scale-dependent bioindicator responses across Russia – Insights from 40 year-long bioindicator monitoring program. *Ecological Indicators* 98: 476-482.

Paller VG, Resurreccion DJ, de la Cruz CP, Bandal MZ Jr (2016) Acanthocephalan parasites (*Acanthogyrus* sp.) of Nile Tilapia (*Oreochromis niloticus*) as biosink of lead (Pb) contamination in a Philippine freshwater lake. *Bulletin of Environmental Contamination and Toxicology* 96(6): 810-815.

Paustenbach D, Tvermoes B, Unice K, Finley B, Kerger B (2013) A review of the health hazards posed by cobalt: potential importance of free divalent cobalt ion equilibrium in understanding systemic toxicity in humans. *Critical Reviews in Toxicology* 43: 316–362.

Peragón J, Barroso JB, Garcia-Salguero L, de la Higuera M, Lupiañez JA (1994) Dietary protein effects on growth and fractional protein synthesis and degradation rates in liver and white muscle of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 124: 35–46.

Petlevski R, Juretić D, Hadžija M, Slijepčević M, Lukač Bajalo J (2006) Koncentracija malondialdehida u NOD miševa tretiranih akarbozom. *Biochemia Medica* 16(1): 43-49.

Phillips DJH, Rainbow PS (1993) *Biomonitoring of trace aquatic contaminants*. Essex. Elsevier Science Publishers Ltd.

Pinder III JE, Hinton TG, Taylor BE, Whicker FW (2011) Cesium accumulation by aquatic organisms at different trophic levels following an experimental release into a small reservoir. *Journal of Environmental Radioactivity* 102 (3): 283–293.

Pinkster S (1993) A revision of the genus *Echinogammarus* Stebbing, 1899 with some notes on related genera (Crustacea, Amphipoda). *Memorie del Museo Civico di Storia Naturale (serie 2a) Sezione Scienze della Vita, Verona* 10: 1–185.

Pinto E, Sigaud-Kutner TCS, Leitão MAS, Okamoto OK, Morse D, Colepicolo P (2003) Heavy metal-induced oxidative stress in algae. *Journal of Phycology* 39:1008–1018.

Piscator M (1964) On cadmium in normal human kidneys with a report on the isolation of metallothioneine from cadmium-exposed rabbit livers. *Nordisk hygienisk tidskrift* 45: 76-82.

Plantak M, Čanjevac I, Vidaković I (2016) Morphological state of rivers in the Ilova River catchment. *Hrvatski geografski glasnik* 78(1): 5–24.

Podrug M, Raspor B (2009) Seasonal variation of the metal (Zn, Fe, Mn) and metallothionein concentrations in the liver cytosol of the European chub (*Squalius cephalus* L.). *Environmental Monitoring and Assessment* 157: 1-10.

Pozebon D, Scheffler GL, Dressler VL, Nunes MAG (2014) Review of the applications of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) to the analysis of biological samples. *Journal of Analytical Atomic Spectrometry* 29: 2204-2228.

Pozebon D, Scheffler GL, Dressler VL (2017) Recent applications of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) for biological sample analysis: a follow-up review. *Journal of Analytical Atomic Spectrometry* 32: 890-919.

Pröfrock D, Prange A (2012) Inductively coupled plasma-mass spectrometry (ICP-MS) for quantitative analysis in environmental and life sciences: a review of challenges, solutions, and trends. *Applied Spectroscopy* 66(8): 843-868.

Prohaska T, Irrgeher J, Zitek A (2016) Simultaneous multi-element and isotope ratio imaging of fish otoliths by laser ablation split stream ICP-MS/MC ICP-MS. *Journal of Analytical Atomic Spectrometry* 3: 1612-1621.

Ptashynski MD, Klaverkamp JF (2002) Accumulation and distribution of dietary nickel in lake whitefish (*Coregonus clupeaformis*). *Aquatic Toxicology* 58: 249–264.

Rada B, Leto T (2008) Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contributions to Microbiology* 15: 164–187.

Radhakrishnan KV, Liu M, He W, Murphy BR, Xie S (2010) Otolith retrieval from faeces and reconstruction of prey-fish size for Great Cormorant (*Phalacrocorax carbo*) wintering at the East Dongting Lake National Nature Reserve, China. *Environmental Biology of Fishes* 89: 505–512.

- 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.
- Radovanović TB, Borković-Mitić SS, Perendija BR, Despotović SG, Pavlović SZ, Cakić PD, Saičić ZS (2010) Superoxide dismutase and catalase activities in the liver and muscle of barbel (*Barbus barbus*) and its intestinal parasite (*Pomphorynchus laevis*) from the Danube river, Serbia. *Archives of Biological Sciences* 62: 97-105.
- Rahman I, MacNee W (1999) Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *American Journal of Physiology* 277: 1067-1088.
- Rainbow PS, Moore PG (1986) Comparative metal analyses in amphipod crustaceans. *Hydrobiologia* 141: 273–289.
- Ramani S, Dragun Z, Kapetanović D, Kostov V, Jordanova H, Erk M, Hajrulai- Musliu Z (2014) Surface water characterization of three rivers in the lead/zinc mining region of Northeastern Macedonia. *Archives of Environmental Contamination and Toxicology* 66 (4): 514–528.
- Ramsay AL, Milner NJ, Hughes RN, McCarthy ID (2011) Comparison of the performance of scale and otolith microchemistry as fisheries research tools in a small upland catchment. *Canadian Journal of Fisheries and Aquatic Sciences* 68: 823–833.
- Ranaldi MM, Gagnon MM (2008) Trace metal incorporation in otoliths of black bream (*Acanthopagrus butcheri* Munro), an indicator of exposure to metal contamination. *Water, Air, & Soil Pollution* 194: 31-43.
- Rashed MN (2001) Monitoring of environmental heavy metals in fish from Nasser lake. *Environment International* 27: 27–33.
- Reule AG (1976) Errors in spectrophotometry and calibration procedures to avoid them. *Journal of Research of the National Bureau of Standards. Section A, Physics and Chemistry* 80A (4): 609-624.
- Ribera D, Narbonne JF, Arnaud C, Saint-Denis M (2001) Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. *Soil Biology and Biochemistry* 33: 1123-1130.

Richetti SK, Rosemberg DB, Ventura-Lima J, Monserrat JM, Bogo MR, Bonan CD (2011) Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. *NeuroToxicology* 32: 116–122.

Riley JP, Chester R (1971) *Introduction to marine chemistry*, Academic Press, London and NY

Rinderhagen M, Ritterhoff J, Zauke G-P (2000) Crustaceans as bioindicators. U: Gerhardt A (urednik) *Biomonitoring of polluted water – reviews on actual topics*. TransTech Publications – Scitech Publications, *Environmental Research Forum* 9:161-194.

Ritterhoff J, Zauke GP, Dallinger R (1996) Calibration of the estuarine amphipods, *Gammarus zaddachi* Sexton (1912), as biomonitors: toxicokinetics of cadmium and possible role of inducible metal-binding proteins in Cd detoxification. *Aquatic Toxicology* 34(4): 351-369.

Robinson PK (2015) *Enzymes: principles and biotechnological applications*. *Essays in biochemistry* 59: 1–41.

Roesijadi G (1992) Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology* 22: 81 – 114.

Roesijadi G, Robinson WE (1994) Metal regulation in aquatic animals: mechanisms of uptake, accumulation and release. U: Malins DC, Ostrander GK (urednici), *Aquatic toxicology: molecular, biochemical and cellular perspectives*. Lewis Publishers, Boca Raton, 387–420.

Roesijadi G, Rezvankhah S, Perez-Matus A, Mitelberg A, Torruellas K, Van Veld PA (2009) Dietary cadmium and benzo(a)pyrene increased intestinal metallothionein expression in the fish *Fundulus heteroclitus*. *Marine Environmental Research* 67 (1): 25–30.

Romani R, Antognelli C, Baldacchini F, De Santis A, Isani G, Giovannini E, Rosi G (2003) Increased acetylcholinesterase activities in specimens of *Sparus auratus* exposed to sublethal copper concentrations. *Chemico-Biological Interactions* 145: 321-329.

Roosa S, Prygiel E, Lesven L, Wattiez R, Gillan D, Ferrari BJ, Criquet J, Billon G (2016) On the bioavailability of trace metals in surface sediments: a combined geochemical and biological approach. *Environmental Science and Pollution Research* 23(11): 10679–10692.

- 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.
- Rowan DJ, Rasmussen JB (1994) Bioaccumulation of radiocesium by fish: the influence of physiochemical factors and trophic structure. *Canadian Journal of Fisheries and Aquatic Sciences* 51: 2388–2410.
- Russo RE, Mao X, Mao SS (2002) The physics of laser ablation in microchemical analysis. *Analytical Chemistry* 74 (3): 70-77.
- Sabrah MM, Mohamedein LI, El-Sawy MA, Abou El-Naga EH (2016) Biological characteristics in approaching to biochemical and heavy metals of edible fish *Terapon puta*, Cuvier, 1829 from different fishing sites along the Suez Canal, Egypt. *Journal of Fisheries and Aquatic Science* 11: 147-162.
- Sadiq M (1992) Toxic metal chemistry in marine environments. Marcel Dekker, Inc., NY.
- Saint-Denis M, Labrot F, Narbonne JF, Ribera D (1998) Glutathione, glutathione-related enzymes, and catalase activities in the earthworm *Eisenia fetida andrei*. *Archives of Environmental Contamination and Toxicology* 35: 602-614.
- Sanchez W, Palluel O, Meunier L, Coquery M, Porcher J-M, Aît-Aïssa S (2005) Copper-induced oxidative stress in the three-spined stickleback: relationship with hepatic metal levels. *Environmental Toxicology and Pharmacology* 19: 177–183.
- Saquet M, Halden NM, Babaluk J, Campbell JL, Nejedly Z (2002) Micro- PIXE analysis of trace element variation in otoliths from fish collected near acid mine tailings: Potential for monitoring contaminant dispersal. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms* 189: 196–201.
- Şaşı 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.
- Schludermann C, Konecny R, Laimgruber S, Lewis JW, Schiemer F, Chovanec A, Sures B (2003) Fish macroparasites as indicators of heavy metal pollution. *Parasitology* 126: 61–69.

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.

Sevcikova M, Modra H, Slaninova Z, Svobodova Z (2011) Metals as a cause of oxidative stress in fish: a review. *Veterinarni Medicina* 56, 537–546.

Shafaquat AS, Syed T, Showket AG, Shazia A, Uzma N, Iram A (2016) Acanthocephalan infestation in fishes – a review. *Journal of Zoology* 2 (6): 32–37.

Sharma P, Bhushan A, Shanker Dubey R, Pessaraki M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Hindawi Publishing Corporation, *Journal of Botany* 2012, 217037

Sheehan D, Power A (1999) Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 123(3): 193-199.

Shulgin KK, Popova TN, Rakhmanova TI (2008) Isolation and purification of glutathione peroxidase. *Applied Biochemistry and Microbiology* 44: 247–250.

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.

Simkiss K (1998) Mechanisms of metal uptake. U: Langston WJ, Bebianno MJ (urednici) *Metal Metabolism in Aquatic Environments*. Springer, Boston, MA.

Sindayigaya E, Vancauwenbergh R, Robberecht H, Deelstra H (1994) Copper, zinc, manganese, iron, lead, cadmium, mercury and arsenic in fish from Lake Tanganyika, Burundi. *Science of the Total Environment* 144: 103–115.

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

Steele BB, Bayn RL JR, Grant CV (1984) Environmental monitoring using population of birds and small mammals: analyses of sampling effort. *Biological Conservation* 30: 157–172.

Steinauer ML, Nickol BB, Broughton R, Ortí G (2005) First sequenced mitochondrial genome from the phylum Acanthocephala (*Leptorhynchoides thecatus*) and its phylogenetic position within Metazoa. *Journal of Molecular Evolution* 60: 706–715.

Strižak Ž, Ivanković D, Pröfrock D, Helmholz H, Cindrić A-M, Erk M, Prange A (2014) Characterization of the cytosolic distribution of priority pollutant metals and metalloids in the digestive gland cytosol of marine mussels: seasonal and spatial variability. *Science of the Total Environment* 470(471): 159–170.

Sturrock AM, Trueman CN, Darnaude AM, Hunter E (2012) Can otolith elemental chemistry retrospectively track migrations in fully marine fishes? *Journal of Fish Biology* 81: 766–795.

Sultana T, Siddique A, Sultana S, Mahboob S, Al-Ghanim K, Ahmed Z (2017) Fish scales as a non-lethal tool of the toxicity of wastewater from the River Chenab. *Environmental science and pollution research international* 24(3): 2464-2475.

Sun L-T, Jeng S-S (1998) Comparative zinc concentrations in tissues of common carp and other aquatic organisms. *Zoological Studies* 37: 184-190.

Suren AM (1991) Bryophytes as invertebrate habitat in two New Zealand alpine streams. *Freshwater Biology* 26: 399–418.

Sures B, Taraschewski H (1995) Cadmium concentrations in two adult acanthocephalans, *Pomphorhynchus laevis* and *Acanthocephalus lucii*, as compared with their fish hosts and cadmium and lead levels in larvae of *A. lucii* s compared with their crustacean host. *Parasitology Research* 81: 494-497.

Sures B, Taraschewski H, Rydlo M (1997) Intestinal fish parasites as heavy metal bioindicators: a comparison between *Acanthocephalus lucii* (Palaeacanthocephala) and the zebra mussel, *Dreissena polymorpha*. *Bulletin of Environmental Contamination and Toxicology* 59, 14-21.

Sures B, Siddall R (1999) *Pomphorhynchus laevis*: the intestinal acanthocephalan as a lead sink for its fish host, chub (*Leuciscus cephalus*). *Experimental Parasitology* 93: 66–72.

Sures B, Siddall R, Taraschewski H (1999) Parasites as accumulation indicators of heavy metal pollution. *Parasitology Today* 15: 16–21.

Sures B (2001) The use of fish parasites as bioindicators of heavy metals in aquatic ecosystems: a review. *Aquatic Ecology* 35: 245-255.

Sures B (2004) Fish acanthocephalans of the genus *Pomphorhynchus* sp. as globally applicable bioindicators for metal pollution in the aquatic environment? *Wiener klinische Wochenschrift* 116(4): 19-23.

Sures B (2006) How parasitism and pollution affect the physiological homeostasis of aquatic hosts. *Journal of Helminthology* 80(2):151-157.

Sures B, Nachev M, Selbach C, Marcogliese DJ (2017) Parasite responses to pollution: what we know and where we go in 'Environmental Parasitology'. *Parasites & Vectors* 10: 65-83.

Syrovátka V, Zhai M, Bojková J, Šorfová V, Horsák M (2020) Native *Gammarus fossarum* affects species composition of macroinvertebrate communities: evidence from laboratory, field enclosures, and natural habitat. *Aquatic Ecology* 54: 505–518.

Szabó A, Nemcsók J, Kása P, Budai D (1991) Comparative study of acetylcholine synthesis in organs of freshwater teleosts. *Fish Physiology and Biochemistry* 9: 93–99.

Szpunar J, Lobiński R (1999) Species-selective analysis for metal biomacromolecular complexes using hyphenated techniques. *Pure and Applied Chemistry* 71: 899–918.

Szpunar J (2005) Advances in analytical methodology for bioinorganic speciation analysis: metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics. *Analyst* 130: 442-465.

Šinžar D (1956) Prilog poznavanju entoparazita pastrmke *Salmo trutta* L. U: Zbornik radova Poljoprivrednog fakulteta. IV 2, Univerzitet Beograd: 165–170.

Štambuk-Giljanović N, Smolčić V (1982) Pokazatelji kvalitete voda u Dalmaciji. *Voda i sanitarna tehnika* 12: 39-57.

Štefan L, Tepšić T, Zavidčić T, Urukalo M, Tota D, Domitrović R (2007) Lipidna peroksidacija- uzroci i posljedice. *Medicina* 43: 84-93.

Tabouret H, Bareille G, Claverie F, Pécheyran C, Prouzet P, Donard OFX (2010) Simultaneous use of strontium:calcium and barium:calcium ratios in otoliths as markers of

habitat: application to the European eel (*Anguilla anguilla*) in the Adour basin, South West France. *Marine Environmental Research* 70: 35–45.

Tadić L, Šperac M, Kareluša B, Rubinić J (2020) Water Quality Status of Croatian Surface Water Resources. U: Negm AM, Romanescu G, Zelenakova M (urednici) *Water Resources Management in Balkan Countries*, Springer Water, Švicarska.

Tanner S, Reis-Santos P, Cabral H (2016). Otolith chemistry in stock delineation: a brief overview, current challenges and future prospects. *Fisheries Research* 173: 206–213.

Taraschewski H (2000) Host-parasite interactions in Acanthocephala: a morphological approach. *Advances in Parasitology* 46: 1–179.

Tepić N, Olujić G, Ahel M (2007) Raspodjela ugljikohidrata u estuariju rijeke Krke. U: Marguš, D. (Urednik) *Zbornik radova - Simpozij Rijeka Krka i Nacionalni park Krka 2005*, Javna ustanova “Nacionalni park Krka”, 839-847.

Ternjej I, Gaurina Srček V, Mihaljević Z, Kopjar N (2013) Cytotoxic and genotoxic effects of water and sediment samples from gypsum mining area in channel catfish ovary (CCO) cells. *Ecotoxicology and Environmental Safety* 98: 119-127.

Ternjej I, Mihaljević Z, Ivković M, Previšić A, Stanković I, Maldini K, Želježić D, Kopjar N (2014) The impact of gypsum mine water: a case study on morphology and DNA integrity in the freshwater invertebrate, *Gammarus balcanicus*. *Environmental Pollution* 189: 229–238.

Thiele DJ, Gitlin JD (2008) Assembling the pieces. *Nature chemical biology* 4: 145-147.

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.

Timi JT, Poulin R (2020) Why ignoring parasites in fish ecology is a mistake. *International Journal for Parasitology* 50: 755-761.

Topić-Popović N, Strunjak-Perović I, Fonns A, Vilsgaard-Espersen T, Teskeredžić E (1999) Report of *Pseudorhadinorhynchus salmothymi* isolation from brown trout in Krka river (Croatia). *Periodicum Biologorum* 101: 273–275.

Topić Popović N, Strunjak-Perović I, Barišić J, Kepec S, Jadan M, Beer-Ljubić B, Matijatko V, Palić D, Klobučar G, Babić S, Gajdoš Kljusurić J, Čož-Rakovac R (2016) Native Prussian carp (*Carassius gibelio*) health status, biochemical and histological responses to treated wastewaters. *Environmental Pollution* 218: 689–701.

Truglio JJ, Theis K, Leimkühler S, Rappa R, Rajagopalan KV, Kisker C (2002) Crystal structures of the active and alloxanthine inhibited forms of xanthine dehydrogenase from *Rhodobacter capsulatus*. *Structure* 10: 115–125.

Trumbo TA, Schultz E, Borland MG, Pugh ME (2013) Applied spectrophotometry: analysis of a biochemical mixture. *Biochemistry and Molecular Biology Education : a Bimonthly Publication of the International Union of Biochemistry and Molecular Biology* 41(4): 242-250.

Tsangaris C, Vergolyas M, Fountoulaki E, Goncharuk VV (2011) Genotoxicity and oxidative stress biomarkers in *Carassius gibelio* as endpoints for toxicity testing of Ukrainian polluted river waters. *Ecotoxicology and Environmental Safety* 74 (8): 2240–2244.

Tušar, B (2004) Ispuštanje i pročišćavanje otpadne vode. CroatiaKnjiga, Zagreb.

Tzadik OE, Curtis JS, Granneman JE, Kurth BN, Pusack TJ, Wallace AA, Hollander DJ, Peebles EB, Stallings CD (2017) Chemical archives in fishes beyond otoliths: a review on the use of other body parts as chronological recorders of microchemical constituents for expanding interpretations of environmental, ecological and life-history changes. *Limnology and Oceanography: Methods* 15: 238–263.

Uchida H, Kondo D, Yamashita A, Nagaosa Y, Sakurai T, Fujii Y, Fujishiro K, Aisaka K, Uwajima T (2003) Purification and characterization of an aldehyde oxidase from *Pseudomonas* sp. KY 4690. *FEMS Microbiology Letters* 229: 31–36.

Urien N, Jacob S, Couture P, Campbell PGC (2018) Cytosolic distribution of metals (Cd, Cu) and metalloids (As, Se) in livers and gonads of field-collected fish exposed to an environmental contamination gradient: an SEC-ICP-MS analysis. *Environments* 5, 102.

Ünlü E, Akba O, Sevim S, Gümgüm B (1996) Heavy metal levels in Mullet, *Liza abu* (Heckel, 1843) (Mugilidae) from the Tigris River, Turkey. *Fresenius Environmental Bulletin* 5: 107-112.

Valko M, Morris H, Cronin M (2005) Metals, toxicity and oxidative stress. *Current Medicinal Chemistry* 12: 1161 – 1208.

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullou M (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety* 64: 178-189.

Valtonen ET, Crompton DWT (1990) Acanthocephala in fish from the Bothnian Bay, Finland. *Journal of Zoology, London* 220: 619–639.

Van Campenhout K, Infante HG, Goemans G, Belpaire C, Adams F, Blust R, Bervoets L (2008) A field survey of metal binding to metallothionein and other cytosolic ligands in liver of eels using an on-line isotope dilution method in combination with size exclusion (SE) high pressure liquid chromatography (HPLC) coupled to Inductively Coupled Plasma time-of-flight Mass spectrometry (ICP-TOFMS). *Science of the Total Environment* 394: 379-389.

Van Campenhout K, Goenaga Infante H, Hoff PT, Moens L, Goemans G, Belpaire C, Adams F, Blust R, Bervoets L (2010) Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (*Carassius auratus gibelio*) from metal-impacted habitats. *Ecotoxicology and Environmental Safety* 73 (3): 296–305.

van Cleef KA, Kaplan LAE, Crivello JF (2000) The relationship between reproductive status and metallothionein mRNA expression in the common killifish, *Fundulus heteroclitus*. *Environmental Biology of Fishes* 57: 97-105.

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13: 57–149.

Vannote RL, Minshall GW, Cummins KW, Sedell JR, Cushing CE (1980) The river continuum concept. *Canadian journal of fisheries and aquatic sciences* 37 : 130 - 137.

Vardić Smrzlić I, Valić D, Kapetanović D, Dragun Z, Gjurčević E, Četković 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.

Vašak M (2005) Advances in metallothionein structure and functions. *Journal of Trace Elements in Medicine and Biology* 19: 13–27.

Verma R, Dwivedi P (2013) Heavy metal water pollution- A case study. *Recent Research in Science and Technology* 5: 98-99.

Viarengo A (1985) Biochemical effects of trace metals. *Marine Pollution Bulletin* 16: 153-158.

Viarengo A, Ponzano E, Dondero F, Fabbri R (1997) A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic molluscs. *Marine Environmental Research* 44: 69 – 84.

Viarengo A, Burlando B, Dondero F, Marro A, Fabbri R (1999) Metallothionein as a tool in biomonitoring programmes. *Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals* 4: 455 – 466.

Viarengo A, Lowe D, Bolognesi C, Fabbri E, Koehler A (2007) The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 146(3): 281-300.

Vidal SM, Malo D, Vogan K, Skamene E, Gros P (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* 73: 469–485.

Vijever MG, Van Gestel CAM, Lanno RP, Van Straalen NM, Peijnenburg WJGM (2004) Internal metal sequestration and its ecotoxicological relevance: a review. *Environmental Science and Technology* 38: 4705-4712.

Villizi, L (2018) Age determination in common carp *Cyprinus carpio*: history, relative utility of ageing structures, precision and accuracy. *Reviews in Fish Biology and Fisheries* 28: 461-484.

Vítek T, Spurný P, Mareš J, Ziková A (2007) Heavy metal contamination of the Loučka River water ecosystem. *Acta Veterinaria Brno* 76: 149-154.

Vlada Republike Hrvatske (2008) Pravilnik o graničnim vrijednostima opasnih i drugih tvari u otpadnim vodama. *Narodne novine* 94/08, Zagreb, Hrvatska.

Vlada Republike Hrvatske (2016) Plan upravljanja vodnim područjima 2016.-2021. Narodne novine 66/16, Zagreb, Hrvatska.

Vlada Republike Hrvatske (2017) Plan gospodarenja otpadom Republike Hrvatske za razdoblje 2017.-2022. Narodne novine 3/17, Zagreb, Hrvatska.

Vlada Republike Hrvatske (2019) Uredba o standardu kakvoće voda. Narodne novine 96/19, Zagreb, Hrvatska.

Vlada Republike Hrvatske (2019) Zakon o vodama. Narodne novine 66/19, Zagreb, Hrvatska.

Vojvodić V, Bura-Nakić E, Čosović B (2007) Organske tvari u Krki: određivanje i karakterizacija. U: Marguš, D. (urednik) Zbornik radova - Simpozij Rijeka Krka i Nacionalni park Krka 2005, Javna ustanova "Nacionalni park Krka", 849-862.

Vutukuru SS, Chintada S, Madhavi KR, Rao JV, Anjaneyulu Y (2006) Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. *Fish Physiology and Biochemistry* 32: 221–229.

Walker RL, Fromm PO (1976) Metabolism of iron by normal and iron deficient rainbow trout. *Comparative Biochemistry and Physiology - Part A* 55: 311–318.

Walkner C, Gratzner R, Meisel T, Hussain Bokhari SN (2017) Multi-element analysis of crude oils using ICP-QQQ-MS. *Organic Geochemistry* 103: 22-30.

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.

Wallace WG, Luoma SN (2003) Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM). *Marine Ecology Progress Series* 257: 125–137.

Wallace JB, Webster JR (1996) The role of macroinvertebrates in stream ecosystem function. *Annual Review of Entomology* 41: 115–139.

Wang WX, Rainbow PS (2006) Subcellular partitioning and the prediction of cadmium toxicity to aquatic organisms. *Environmental Chemistry* 3: 395–399.

- Wang WX (2013) Prediction of metal toxicity in aquatic organisms. *Chinese Science Bulletin* 58: 194-202.
- Watanabe T, Kiron V, Satoh S (1997) Trace minerals in fish nutrition. *Aquaculture* 151:185-207.
- Wells BK, Thorrold SR, Jones CM (2000) Geographic variation in trace element composition of juvenile Weakfish scales. *Transactions of the American Fisheries Society* 129(4): 889-900.
- Wells BK, Thorrold SR, Jones CM (2003) Stability of elemental signatures in the scales of spawning weakfish, *Cynoscion regalis*. *Canadian Journal of Fisheries and Aquatic Sciences* 60: 361–369.
- White EP, Morgan Ernest SK, Kerkhoff AJ, Enquist BJ (2007) Relationships between body size and abundance in ecology. *Trends in Ecology & Evolution* 22: 323–330.
- Winston GW, Di Giulio RT (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology* 19:137–161.
- Wolff BA, Johnson BM, Landress CM (2013) Classification of hatchery and wild fish using natural geochemical signatures in otoliths, fin rays, and scales of an endangered catostomid. *Canadian Journal of Fisheries and Aquatic Sciences* 70: 1775–1784.
- Wood CM, Farrell AP, Brauner CJ (2012) *Fish physiology: Homeostasis and toxicology of non-essential metals*. Vol. 31B, Academic Press, San Diego.
- Xiong Y, Uys JD, Tew KD, Townsend DM (2011). S-glutathionylation: from molecular mechanisms to health outcomes. *Antioxidants & redox signaling* 15(1): 233–270.
- Yabanli M, Yozukmaz A, Sel F (2014) Bioaccumulation of heavy metals in tissues of the gibel carp *Carassius gibelio*: example of Marmara Lake, Turkey. *Russian Journal of Biological Invasions* 5 (3): 217–224.
- Yamashita Y, Yamashita M (2010) Identification of a novel selenium containing compound, selenoneine, as the predominant chemical form of organic selenium in the blood of a bluefin tuna. *The Journal of Biological Chemistry* 285: 18134–18138.

- Yadav A, Gopesh A, Pandey RS, Rai DK, Sharma B (2009) Acetylcholinesterase: a potential biochemical indicator for biomonitoring of fertilizer industry effluent toxicity in freshwater teleost, *Channa striatus*. *Ecotoxicology* 18(3): 325-333.
- Yarsan E, Yipel M (2013) The Important Terms of Marine Pollution “Biomarkers and Biomonitoring, Bioaccumulation, Bioconcentration, Biomagnification”. *Journal of Molecular Biomarkers & Diagnosis* s1: 003.
- 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(3): 1613–1621.
- Young IS, Woodside JV (2001) Antioxidants in health and disease. *Journal of Clinical Pathology* 54: 176-186.
- Zhelev ZhM, Mollova D, Boyadziev P (2016) Morphological and hematological parameters of *Carassius gibelio* (Pisces: Cyprinidae) in conditions of anthropogenic pollution in Southern Bulgaria. Use hematological parameters as biomarkers. *Trakia Journal of Sciences* 14 (1): 1–15.
- 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 Zoologica Bulgarica* 70: 547–556.
- Zhou Q, Zhang J, Fu J, Shi J, Jiang G (2008) Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. *Analytica Chimica Acta* 606: 135 – 150.
- Zitek A, Sturm M, Waidbacher H, Prohaska T (2010) Discrimination of wild and hatchery trout by natural chronological patterns of elements and isotopes in otoliths using LA-ICP-MS. *Fisheries Management and Ecology* 17: 435–445.
- Zorita I, Strogyloudi E, Buxens A, Mazon LI, Papathanassiou E, Soto M, Cajaraville MP (2005) Application of two SH-based methods for metallothionein determination in mussels and intercalibration of the spectrophotometric method: laboratory and field studies in the Mediterranean Sea. *Biomarkers* 10 (5): 342–359.

Žganec K (2009) Rasprostranjenost i ekologije nadzemnih rakušaca (Amphipoda: Gammaroidea) slatkih i bočatih voda Hrvatske, doktorska disertacija.

Žganec K, Gottstein S, Đurić P (2010) Distribution of native and alien gammarids (Crustacea: Amphipoda) along the course of the Una River. *Natura Croatica* 19:141-150.

Žganec K, Lunko P, Stroj A, Mamos T, Grabowski M (2016) Distribution, ecology and conservation status of two endemic amphipods, *Echinogammarus acarinatus* and *Fontogammarus dalmatinus*, from the Dinaric karst rivers, Balkan Peninsula. *Annales de Limnologie* 52: 13–26.

7. PRILOZI

Prilog 1. First insight in trace element distribution in the intestinal cytosol of two freshwater fish species challenged with moderate environmental contamination

Tatjana Mijošek^{1, *}, Vlatka Filipović Marijić¹, Zrinka Dragun¹, Nesrete Krasnići^{1, 2}, Dušica Ivanković¹, Zuzana Redžović^{1, 3}, Marijana Erk^{1, 3}

¹Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička cesta 54, 10000 Zagreb, Croatia

²Present address: University of Vienna, Department of Structural and Computational Biology, Campus-Vienna-Biocenter 5, 1030 Vienna, Austria

³Present address: Ruđer Bošković Institute, Division of Molecular Medicine, Laboratory for Bioanalytics, Bijenička cesta 54, 10000 Zagreb, Croatia

E-mail addresses: tmijosek@irb.hr, vfilip@irb.hr, zdragun@irb.hr, nkrasnic@irb.hr, djuric@irb.hr, zredzov@irb.hr, erk@irb.hr

*Corresponding author:

Tatjana Mijošek, tmijosek@irb.hr

Laboratory for Biological Effects of Metals, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia.

Abstract

Cytosolic distribution of six essential elements and nonessential Cd among biomolecules of different molecular masses was investigated in the intestine of brown trout (*Salmo trutta*) from the karst Krka River and Prussian carps (*Carassius gibelio*) from the lowland Ilova River. Fish were sampled at two locations (reference and contaminated) and in two seasons (autumn and spring). Analyses were conducted by size exclusion high performance liquid chromatography and high resolution inductively coupled plasma mass spectrometry. Although studied salmonid and cyprinid fish have different biological characteristics, obtained profiles often showed mostly similar patterns in both species. Specifically, Cd and Cu were dominantly bound to metallothioneins in both species, but the same association was not observed for Zn, whereas Mo distribution was similar in the intestine of both fish species with two well shaped and clear peaks in HMM (100-400 kDa) and VLMM (2-8 kDa) range. In brown trout, Se was mostly associated with biomolecules of very low molecular masses (VLMM, <10 kDa), whereas significant additional elution in HMM region (30-303 kDa) was observed only in Prussian carp. Iron binding to VLMM biomolecules (1.8–14 kDa) was observed only in brown trouts, and of Zn in Prussian carps. Cobalt was mostly bound to HMM biomolecules (85-235 kDa) in brown trout and to VLMM biomolecules (0.7-18 kDa) in Prussian carp. Comparison of intestinal profiles with previously published data on liver and gills revealed some similarities in distribution, but also organ-specific differences due to the different function and composition of each organ. Obtained results represent the novel findings, as there is no published data on intestinal trace metal distribution, and moreover the key point for the exact identification of specific metal-binding biomolecules which could eventually be used as biomarkers of metal exposure.

Keywords: SEC-HPLC, brown trout, Prussian carp, HR ICP-MS, metal detoxification, wastewaters

1. Introduction

Although essential metals have a significant role in variety of physiologically important processes, often as cofactors of a number of metalloproteins and enzymes (Holm et al., 1996), they can also be the cause of toxic effects if present in high concentrations. Elements as Cd or Pb, considered as non-essential, do not have any known biological role in organisms and can be toxic even in very low concentrations. However, commonly applied procedure of measuring only total concentrations of trace elements in bioindicator organisms and their tissues does not provide complete and reliable information on bioavailability, biological effects and toxicity of metals in aquatic environments as their real impact is mostly connected with binding to essential molecules such as enzymes or transporter proteins and their possible inactivation in the cytosol (Mason and Jenkins, 1995). Cytosolic metal fraction, soluble and metabolically available, consists of heat denaturable proteins (HDP, such as enzymes) and microsomes (biologically available and partially toxic metal fraction), and heat-stable proteins (HSP, such as metallothioneins) (detoxified metal fraction) (Wallace et al., 2003). In cytosol, metal toxic effects include blocking of functional groups of biomolecules, substitution of essential elements, and formation of reactive oxygen species (ROS) which have an important role in oxidative stress. However, they can also be detoxified either through sequestration in forms of granules or by binding to molecules such as metallothioneins (MTs) or metallothionein-like proteins in cytosols (Wallace et al., 2003; Vijver et al., 2004).

Therefore, to get the insight into biomolecules targeted by the metals and affected metabolic and physiological pathways, new approach, called metallomics, was developed (Szpunar, 2004). Combination of size-exclusion liquid chromatography (SEC-HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) is one of recognized approaches for screening cytosolic metal distribution among biomolecules of different molecular sizes. This methodology has already been used for determination of the cytosolic metal distribution in different tissues of aquatic organisms including bivalves *Mytilus galloprovincialis* (Strižak et al., 2014) and *Perna perna* (Lavradas et al., 2016), or fish such as European eel (*Anguilla anguilla*; Goenaga Infante et al., 2003), yellow perch (*Perca flavescens*; Caron et al., 2018), white sucker (*Catostomus commersonii*; Urien et al., 2018), European and Vardar chub (*Squalius cephalus* and *Squalius vardarensis*; Krasnići et al., 2013, 2014, 2018, 2019), brown trout (*Salmo trutta*; Dragun et al., 2018b) and Prussian carp (*Carassius gibelio*; Dragun et al., 2020).

As a continuation of comprehensive study of anthropogenic impact on Croatian rivers, Krka and Ilova, we have, for the first time, chosen the intestine of brown trout and Prussian carp, for the analysis of molecular masses (MM) of cytosolic biomolecules that bind specific trace elements. Both of the investigated rivers are under moderate influence of industrial and municipal wastewaters (Filipović Marijić et al., 2018; Sertić Perić et al., 2018; Mijošek et al., 2020). So far, we have investigated seasonal and spatial variability of total and cytosolic metal levels, as well as biomarker responses in the intestine of these two fish species (Mijošek et al., 2019a, 2019b, 2021). But, to our knowledge there is no available data on distribution of any element among cytosolic molecules in the intestine of these or any other fish species. Despite its great importance in fish digestion and dietborne metal uptake (Clearwater et al., 2000), intestine is still rarely used as a bioindicator tissue.

Thus, we applied SEC-HPLC combined with offline metal measurement using high resolution ICP-MS (HR ICP-MS) to separate, for the first time, intestinal cytosols of two fish species into fractions with the main aim to define the distribution among biomolecules of different MM for seven selected elements, including nonessential Cd and six essential elements (Co, Cu, Fe, Mo, Se, and Zn). The additional aim was to establish the differences/similarities between two studied species, as well as the differences/similarities between intestinal metal/nonmetal distributions and distributions reported for the other organs of the same fish species (Dragun et al., 2018b; Dragun et al., 2020). Moreover, the goals of the study were also to detect possible differences in metal-handling strategies of fish dwelling in differently polluted areas and of fish caught during the spawning (autumn) and post-spawning period (spring).

2. Materials and methods

2.1. Study areas

Two areas of differing ecological characteristics were selected, the karst Krka River and lowland Ilova River. At both watercourses, samplings involved two sampling sites of different pollution impact (reference and contaminated) and two sampling campaigns covering different physiological conditions of fish, specifically spawning (autumn) and post-spawning period (spring).

At Krka River, sampling campaigns were conducted in October 2015 and May 2016. As a reference site, river source was selected, whereas contaminated site was located downstream

of the Town of Knin, due to the known pollution sources (industrial wastewaters of screw factory and untreated municipal wastewaters of the Town of Knin). The information on sampling sites and water quality were already published (Filipović Marijić et al., 2018; Sertić Perić et al., 2018), as well as on metal accumulation in fish intestine (Mijošek et al., 2019a, 2019b). Iron and Mo were considerably higher in water from the contaminated site (Town of Knin), while Cu and Zn were below LOD in all sites and seasons. At Ilova River, the samplings were conducted in October 2017 and May 2018. The reference site was located upstream of contamination sources near the Ilova village, while contaminated site, affected by municipal (the Town of Kutina) and industrial (fertilizer factory) wastewaters, was located near the Trebež village. The information on sampling sites and water and sediment quality were already published (Mijošek et al., 2020), as well as on metal accumulation in the intestine of Prussian carp (Mijošek et al., 2021). In the Ilova River, Cd, Mo and Se in both seasons, and Co and Fe in one season were elevated in the water samples from contaminated site (Trebež village). All elements except Zn were higher in the Ilova than Krka River so fish from the Ilova River were exposed to higher water metal concentrations indicating that some specific differences in metal handling strategies and defense mechanisms of the two fish species might be presumed.

2.2. Fish sampling and tissue dissection and preparation

The selected bioindicator organisms were representative native fish from the Krka and Ilova rivers, brown trout (*Salmo trutta* Linnaeus, 1758) and Prussian carp (*Carassius gibelio* Bloch, 1782), respectively. Sampling campaigns were performed by electro-fishing, following the Croatian standard HRN EN 14011. Fish were kept alive in an opaque plastic tank filled with aerated river-water. Among the sampled fish, we selected twelve specimens from each ecosystem for cytosolic metal distribution analyses, three per each site in each season. Basic biometric characteristics of used fish are presented in Table 1. In the laboratory, fish were anesthetized using tricaine methane sulphonate (MS 222, Sigma Aldrich) in accordance with the Ordinance on the protection of animals used for scientific purposes (NN 55, 2013). Fish total body mass and total lengths were recorded and sex determined. The posterior part of the intestinal tissue was dissected, weighed and stored at $-80\text{ }^{\circ}\text{C}$ until further analyses.

Table 1. Biometric characteristics and cytosolic trace element concentrations in the intestine of 12 specimens of brown trout (*Salmo trutta* Linnaeus, 1758) from the Krka River and 12 specimens of Prussian carp (*Carassius gibelio* Bloch, 1782) from the Ilova River used for analyses of intestinal trace element distributions.

Site	Sample ID	Total length/cm	Total mass/g	Sex	Cd $\mu\text{g kg}^{-1}$	Co $\mu\text{g kg}^{-1}$	Cu mg kg^{-1}	Fe mg kg^{-1}	Mo $\mu\text{g kg}^{-1}$	Se mg kg^{-1}	Zn mg kg^{-1}
Krka River	K2	27.0	201.7	M	15.1	28.1	0.432	11.96	49.7	0.712	55.6
	K5	27.8	194.2	F	107.1	42.2	0.336	11.55	31.0	0.802	39.0
	K11	27.5	175.0	F	6.15	8.76	0.155	8.59	17.3	0.362	52.7
	K53	22.1	107.2	M	11.13	5.31	0.210	2.00	22.0	0.691	26.2
	K67	17.0	50.6	F	271.3	17.1	0.493	5.88	32.6	0.705	37.4
	K68	16.5	46.7	F	24.3	11.1	0.390	5.14	33.5	0.655	40.2
	K20	23.9	146.0	F	47.6	121.3	1.348	8.54	43.2	1.784	46.5
	K21	25.5	193.0	M	21.6	45.5	0.348	12.20	27.0	1.302	36.4
	K26	16.2	53.7	M	8.76	48.4	0.884	2.49	40.0	1.587	27.9
	K42	22.6	122.2	M	2.25	38.4	0.588	8.70	26.4	0.996	50.5
	K46	20.5	102.4	M	4.77	23.4	0.272	4.52	19.7	1.064	53.4
	K47	19.1	84.9	M	6.93	81.0	0.253	5.62	21.5	1.017	61.5
Ilova River	IL57	17.9	99.3	F	259.1	25.6	0.480	10.05	71.2	0.538	111.8
	IL65	17.1	83.4	F	387.2	16.1	1.043	9.37	58.5	0.377	84.3
	IL68	19.9	136.2	F	133.0	17.7	0.782	9.45	62.5	0.403	102.3
	IL116	18.2	71.2	M	30.9	33.8	0.816	17.58	64.3	/	126.4
	IL118	18.7	82.1	M	41.8	28.1	0.358	8.27	49.5	0.389	105.3
	IL121	17.7	68.1	M	25.1	33.8	0.427	8.51	58.2	0.474	147.2
	IL72	18.5	112.3	F	564.4	11.3	0.812	14.92	68.2	0.470	75.9
	IL76	23.7	239.3	F	683.8	15.1	1.060	22.03	48.1	0.414	76.2
	IL81	20.5	139.6	M	753.0	33.1	1.361	/	81.8	0.560	96.7
	IL94	17.1	96.2	F	129.2	39.9	0.818	12.47	56.7	0.499	139.6
	IL96	26.6	316.7	F	100.5	29.0	0.908	14.39	70.7	0.447	123.4
	IL100	27.2	339.2	F	220.5	18.8	1.332	12.42	51.9	0.445	108.0

2.4. Homogenization procedure and preparation of intestinal cytosolic fractions

Homogenization procedure of the fish intestinal tissue has been described in detail by Mijošek et al. (2019a). Homogenization buffer contained 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at 4 °C), 1 mM DTT (Sigma, USA) as a reducing agent, 0.5 mM PMSF (Sigma, USA) and 0.006 mM leupeptin (Sigma) as protease inhibitors. Intestinal tissue was homogenised on ice in five volumes of buffer at 6000 rpm by Potter-Elvehjem homogenizer (Glas-Col, USA). Obtained homogenates were afterwards centrifuged by Avanti J-E centrifuge (Beckman Coulter, USA) at 50,000×g for 2 h at 4 °C, and the resulting supernatant corresponded to total soluble cytosolic fraction, which was stored at -80 °C.

2.5. SEC-HPLC fractionation of intestinal cytosols of brown trout and Prussian carp

The distributions of elements among biomolecules of different MM in the cytosols of the intestine of brown trout and Prussian carp were determined using HPLC system (Perkin Elmer, 200 series, USA). Tricorn Superdex TM 200 10/300 GL column with a separation range of 10–600 kDa (GE Healthcare Biosciences, USA) was used as described by Krasnići et al. (2013, 2014, 2018, 2019) and Dragun et al. (2018b, 2020). 20 mM Tris-HCl/Base buffer solution (Sigma–Aldrich, pH 8.1 at 22 °C) was used as the mobile phase at a flow rate of 0.5 mL min⁻¹ (isocratic mode). The supernatant (cytosol) samples were injected directly into the HPLC system. One-minute fractions were collected starting at 13th minute and ending at 52nd using a fraction collector (Gilson FC 203B) after two consecutive injections (100 µL of supernatant sample each) and two chromatographic runs. For column calibration, seven protein standards (thyroglobulin, apoferritin, b-amylase, alcohol dehydrogenase, bovine albumin, and carbonic anhydrase, Sigma, USA) were run through the column under the same conditions as the samples and obtained equation of the calibration straight line is given in Table 2. Calibration straight line was created based on known MM of protein standards and their respective elution times (t_e ; Table 2). Metallothionein (MT) standards (Enzo Metallothionein-1, Enzo Metallothionein-2, Enzo Life Sciences, Switzerland) were also run through the column, whereas the void volume (V_o) of the column was determined by use of blue dextran.

Table 2. Elution times (t_e) and molecular masses (MM) of eight proteins used as standards for calibration of Superdex 200 10/300 GL size exclusion column, as well as of rabbit metallothionein standard (Enzo Metallothionein-1, Enzo Metallothionein-2). Equation of calibration straight line was: $K_{av} = -0.277 \times \log MM + 1.627$.

	Protein	t_e	MM	Concentration
		/	/ kDa	/ mg mL⁻¹
Superdex 200 10/300 GL	Thyroglobulin	16.12	669	8
	Apo-ferritin	17.88	443	10
	β -amylase	20.55	200	4
	Alcohol	21.8	150	5
	Bovine albumin	23.06	66	10
	Superoxide dismutase	27.71	32	1.25
	Carbonic anhydrase	29.60	29	3
	Metallothionein - 2	31.22	6.1	1
	Metallothionein - 1	32.32	6.1	1
	Vitamin B12	36.14	1.35	3

2.6. Measurement of trace element concentrations in the SEC-HPLC fractions of intestinal cytosols

Cytosolic trace element concentrations in the intestinal tissue of brown trout and Prussian carp were previously measured and reported by Mijošek et al. (2019b, 2021), and are now given in Table 1 for seven elements analyzed in this study. In present study, we have measured concentrations in one-minute fractions obtained by SEC-HPLC separation of cytosols. Fractions collected after SEC-HPLC separation were only acidified with HNO₃ (Suprapur, Merck, Germany, final acid concentration in the samples: 0.16%) prior to measurements. Indium (Fluka, Germany) was added to all samples as an internal standard (1 $\mu\text{g L}^{-1}$). 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 the measurements. Measurements of ⁸²Se, ⁹⁸Mo and ¹¹¹Cd we performed in low resolution mode, whereas ⁵⁶Fe, ⁵⁹Co, ⁶³Cu, and ⁶⁶Zn in medium resolution mode. External calibration was performed using diluted multielement standard solution for trace elements (Analitika, Czech Republic), prepared in 1.3% HNO₃ (Suprapur;

Merck, Germany), supplemented with In ($1 \mu\text{g L}^{-1}$; Fluka, Germany). Limits of detection (LODs) were reported by Krasnići et al. (2018) and Dragun et al. (2018b, 2020). The accuracy of measurements was checked by analysis of quality control sample (QC for trace metals, catalog no. 8072, lot no. 146142-146143, Burlington, Canada) and the following recoveries (%) were obtained based on 13 measurements: Cd 96.2 ± 1.8 ; Co 96.8 ± 1.7 ; Cu 97.3 ± 1.3 ; Fe 96.9 ± 4.4 ; Mo 97.4 ± 2.4 ; Se 96.7 ± 5.8 ; and Zn 106.6 ± 13.7 .

2.7. Data processing and statistics

Microsoft Excel 2007 and SigmaPlot 11.0 for Windows were used for data processing and creation of graphs. Column calibration (Table 2) enabled association of elution times of specific peaks to corresponding MM, with the aim to define MM of biomolecules that bind each element (Table 3). Based on t_r of protein standards and the chromatographic profiles of metals and previous studies (Krasnići et al., 2013, 2018), biomolecules were categorized in four classes: a high molecular mass range (HMM; >100 kDa), a medium molecular mass range (MMM; 30-100 kDa), a low molecular mass range (LMM; 10-30 kDa) and a very low molecular mass range (VLMM; <10 kDa).

3. Results and discussion

3.1. Fish biometry and cytosolic metal concentrations

The information on cytosolic intestinal metal levels in the same fish as analyzed in this study was previously published as a part of comprehensive research of metal exposure and bioaccumulation in brown trout and Prussian carp (Mijošek et al., 2019b; 2021). In the presented research, element distribution among biomolecules was assessed at different bioaccumulation rates by choosing fish individuals with variable cytosolic metal concentrations.

Biometric characteristics of twelve selected specimens of each species, as well as their cytosolic metal levels are listed in Table 1. Brown trouts used in this research were 16.2-27.8 cm long and had masses of 46.7-201.7 g. Altogether, we have analyzed five females and seven males (Table 1). Prussian carps varied in total length from 17.1 to 27.2 cm and weighed from 83.36 to 339.19 g. Female predominance was evident, as already noted by few authors for Prussian carp (Erdogan et al., 2014; Dragun et al., 2020), with eight females and four males analyzed (Table 1). However, comparison of obtained element distribution profiles did not indicate any considerable variations regarding fish sex or size. Differences, mainly

referring to peak heights, were mostly the consequence of variable intestinal accumulation in investigated specimens.

3.2. Distribution of trace elements in the intestinal cytosols of brown trout and Prussian carp

Distribution profiles of seven analyzed elements among cytosolic biomolecules of different MM in the intestine of brown trout and Prussian carp are presented in Figs. 1-4 for each river and location, while their elution times and MM of corresponding biomolecules are given in Table 3. Sampling season is also indicated in figures to consider the differences that might occur due to the physiological variability and reproductive status of fish species. In general, specific seasonal and spatial trends were mostly not observed in presented research, but rather connection with different levels of bioaccumulation. Each investigated element will further be independently presented and discussed.

Table 3. Elution times (t_e) and molecular masses (MM) of cytosolic proteins from intestine of brown trout (*Salmo trutta* Linnaeus, 1758) from the Krka River and of Prussian carp (*Carassius gibelio* Bloch, 1782) from the Ilova River contained within the fractions (obtained by separation of cytosols using SEC-HPLC with Superdex 200 10/300 GL column) in which respective elements were eluted. Presented data refer to maxima of trace element peaks (i.e. to fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks.

Element	Location	^a HMM peak 1		^a HMM peak 2		^b MMM peak		^c LMM peak		^d VLM peak 1		^d VLM peak 2	
		t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa
Cd	Krka River							30,31 (28-38)	18,14 (30-24)				
	Ilova River							32 (29-37)	11 (24-3)				
Co	Krka River			22 (20-24)	141 (235-85)	25 (24-28)	65 (85-30)			33,35 (30-38)	8,51 (18-24)	41 (39-44)	1,1 (1,80-5)
	Ilova River			22,23 (19-24)	141,109 (303-85)	26 (24-28)	51 (85-30)			36,37 (30-38)	3,9,3 (18-24)	41 (39-43)	1,1 (1,80-7)
Cu	Krka River					26,27 (24-28)	51,39 (85-30)	30,31 (28-36)	18,14 (30-39)				
	Ilova River					28 (26-30)	30 (51-18)	32 (30-37)	11 (18-3)				
Fe	Krka River	17,18 (14-21)	506,392 (1088-182)			25,26 (23-28)	65,51 (109-30)	33 (31-37)	8 (14-3)				
	Ilova River	18,19 (16-22)	392,303 (653-141)			27 (26-30)	39 (51-18)	33 (31-35)	8 (14-5,1)				
Mo	Krka River			19,20 (18-22)	303,235 (392-141)					35 (33-39)	5,1 (8-1,8)		
	Ilova River			20 (18-23)	235 (392-109)					35 (33-39)	5,1 (8-1,8)		
Se	Krka River									36 (35-39)	3,9 (5,1-1,8)	41,42 (40-45)	1,1,08 (1,40-4)
	Ilova River			23 (19-28)	109 (303-30)					36,37 (32-39)	3,9,3 (11-1,8)	42 (40-45)	0,8 (1,40-4)
Zn	Krka River	15,16 (14-18)	843,653 (1088-392)	23,24 (20-28)	109,85 (235-30)								
	Ilova River	15 (14-18)	843 (1088-392)	23 (20-28)	109 (235-30)					36,37 (31-39)	3,9,3 (14-1,8)	40 (39-43)	1,4 (1,80-7)

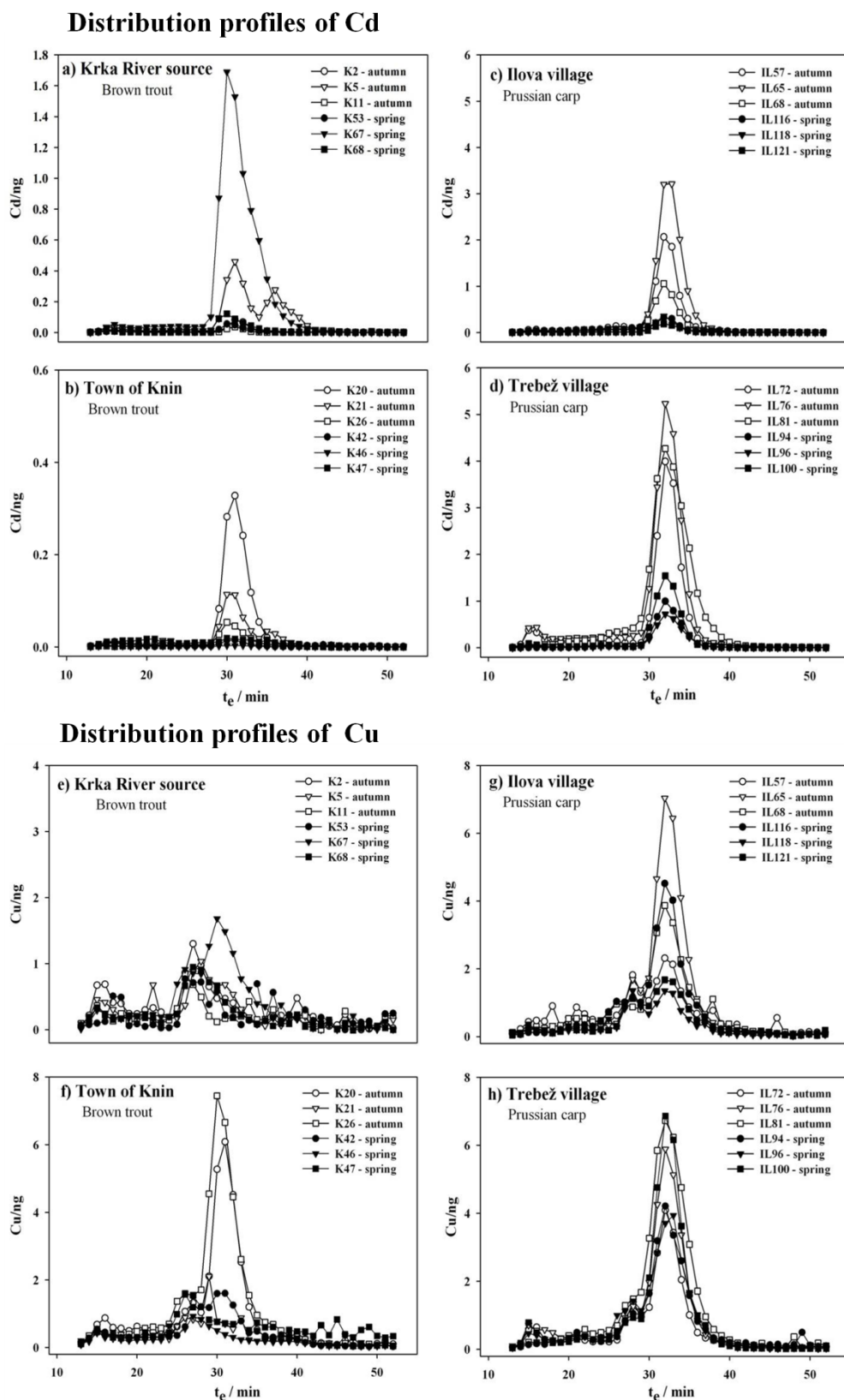
3.2.1. Cadmium

Cd is considered as nonessential and toxic element, and it was distributed within a single peak in LMM biomolecule region (28th-38th minute) in the majority of specimens of both investigated fish species (Fig. 1a-d). The peaks maxima were at 30th-32nd minute, which corresponded to biomolecules of MM of 11-18 kDa (Table 3) and the elution time of two MT-standards (Table 2). This finding confirmed the well-known affinity of MTs for Cd binding, as one of the most important mechanisms for its detoxification (Roesijadi et al., 1992; McGeer et al., 2012). Obtained profiles, which differences referred only to increase of peak height following the increase of cytosolic Cd concentrations, without specific spatial and seasonal patterns, suggested high detoxification rate of Cd when present in low and moderate concentrations. However, slight association of Cd with HMM region was observed in samples with highest cytosolic concentrations (in Prussian carp from Trebež village), indicating possible higher susceptibility to Cd toxicity after its higher bioaccumulation. Generally, the presumable Cd-MT peaks were higher in the Prussian carp than brown trout, probably owing to considerably higher intestinal Cd cytosolic concentrations in that fish (Fig. 1a-d, Table 1). Dominant binding of Cd to MTs has already been described in the liver of brown trout (Dragun et al., 2018b) and liver and gills of Prussian carp (Dragun et al., 2020) from the same samplings. Comparison of intestinal Cd distribution with the distributions in the other two organs from these species revealed variability only in heights of the existing peaks, as a consequence of different cytosolic Cd concentrations (Dragun et al., 2018b, 2020). Probable binding of Cd to MTs was also observed in the liver of variety of fish species (Goenaga Infante et al., 2003; van Campenhout et al., 2010; Krasnići et al., 2013, 2018; Urien et al., 2018), indicating dominant Cd detoxification by MTs.

3.2.2. Copper

Copper is an essential element known to have an important role in fish metabolic processes involving its catalytic and structural function in various enzymes and metalloproteins (Festa and Thiele, 2011). Although the dominant LMM peak was almost the same as in the case of Cd (maxima at 11-18 kDa; Table 3) indicating predominant Cu binding to MTs, there was an additional peak in MMM biomolecules range of both fish species (Fig. 1e-h) at elution times from 24th to 30th minute (biomolecules MM range from 18 to 85 kDa). That peak has encompassed many other proteins known to contain Cu, such as albumin (66 kDa), superoxide dismutase (32 kDa) and carbonic anhydrase (29 kDa) (Szpunar and

Lobinski, 1999; Table 2), but it was much less pronounced in all cases than presumable Cu-MT-peak (Fig. 1e-h). Specific seasonal and spatial patterns were generally not observed, with the exception of peaks in brown trout from the Krka River source which, due to the lowest cytosolic Cu concentrations, were not so clear and defined as in the fish from the other sites, but still revealed the similar distribution patterns (Fig. 1e). Variability of Cu distribution mainly referred to increase of peak height and area of Cu LMM-peak following the increasing Cu cytosolic concentrations (Fig. 1e-h, Table 1), with peak widening which possibly indicated Cu association to the other cytosolic biomolecules when present in the cell in higher concentrations. In the liver of brown trout the predominant binding of Cu to LMM region was also previously confirmed, suggesting the crucial role of MTs, but additional peaks in HMM biomolecule range were also observed when Cu was present in higher cytosolic concentrations (Dragun et al., 2018b). An indication of such peaks was also visible in the brown trout intestine at elution time from 13th to 17th minute in the samples with higher Cu intestinal concentrations (Fig. 1e, f). Comparison of Cu distribution in the intestine of Prussian carp with liver and gills of the same species indicated that intestinal Cu distribution was more similar to liver than to gills, with majority of Cu eluted in LMM region (Dragun et al., 2020). Generally, many studies confirmed dominant Cu elution at the elution time of MTs in many other fish species and tissues (van Campenhout et al., 2008; Krasnići et al., 2013, 2014, 2018; Caron et al., 2018; Urien et al., 2018).



3.2.3. Cobalt

Cobalt distribution included four separate Co-containing peaks in both fish species (Fig. 2a-d, Table 3), but there were differences in predominant peaks between the species. In brown trout, predominant binding of Co to HMM molecules was observed (85-235 kDa). This finding was consistent with Co distribution in hepatic cytosol of brown trout from the Krka River where even higher increase of Co quantity was observed in the HMM peak (Dragun et al., 2018b), similar as in the liver and gills of European and Vardar chub (Krasnići et al., 2013, 2014, 2018), and liver of juvenile yellow perch (Caron et al., 2018). Only smaller part of Co in brown trout intestine binded to MMM (30-85 kDa) or VLMM (0.5-18 kDa) biomolecules (Fig. 2a,b). The MMM-peak included the elution time of the bovine albumin standard (Table 2), known to have a role in binding and transport of metals, including Co (Sadler et al., 1994). Two less clear peaks, especially in samples with the lowest Co cytosolic concentrations, corresponded to VLMM biomolecules region (elution times from 30th to 38th minute, and from 39th to 44th minute; Table 3). The opposite trend was revealed in Prussian carp from the Ilova River, where similar distribution occurred, but predominant binding to VLMM biomolecules was observed at both investigated sites (Fig. 2c, d). The first VLMM-peak was higher and corresponded to molecules of 2.4-18 kDa. The other smaller VLMM-peak corresponded to biomolecules of 0.7-1.8 kDa (Table 3), and included MM of cobalamine (vitamin B12, 1.3 kDa; Kirschbaum, 1981), confirming the role of essential Co in building cobalamine structure (Blust, 2012). Although this possible link with cobalamine was more pronounced in Prussian carp, it was also observed in the intestine of brown trout, contrary to the liver of brown trout where this peak was not clearly visible (Dragun et al., 2018b). In Prussian carp, almost negligible portion of Co was associated with HMM and MMM regions (Fig. 2c, d). The significant impact of season or site on the obtained profiles was not noticed, and only the increase of Co quantity in HMM region was observed due to the increasing cytosolic Co concentrations (Fig. 2a-d), with the highest cytosolic concentration and peak heights observed in brown trout from the Town of Knin (Fig. 2b). In Prussian carp, the differences between the peaks were not that pronounced, and only slightly increased Co elution in VLMM region was observed with higher Co bioaccumulation (Fig. 2c, d).

3.2.4. Iron

As essential element, Fe has important physiological roles in oxygen transport as integral part of hemoglobin or cytochrome c oxidase, which participates in mitochondrial

respiratory chain (Bury et al., 2012). Further, it is involved in DNA synthesis and metabolism of collagen, fatty acids or tyrosine (Kuhn et al., 2016), as well as in protection against bacterial infections. Iron distribution profiles indicated its presence in two or three areas of MM, depending on the fish species. The first peak in the HMM area (182-1088 kDa) and the second one in the MMM area (18-109 kDa) were common for both fish species. Maximum of the HMM peak was associated to biomolecules of 400-500 kDa and likely presented binding to ferritin (450 kDa; Szpunar and Lobinski, 1999), Fe storage protein, whereas MMM peak covered MM of several Fe-containing biomolecules, such as blood protein hemoglobin (65 kDa) or its subunits (Krasnići et al., 2019), transport proteins transferrin (80 kDa; Asmamaw, 2016) and ferroportin (63 kDa), or enzyme catalase subunits (each of 60 kDa) (Martin-Antonio et al., 2009). Dominant peak in brown trout was observed in MMM biomolecule range (Fig. 2e, f), whereas the first peak in HMM region was dominant in Prussian carp (Fig. 2g, h). The clear third peak was observed only in brown trout in the LMM/VLMM range, covering an area of 1.8–14 kDa (Fig. 2e, f), which suggested possible binding to nucleotides, amino acids, pyrophosphates, and Fe complexes (Beard et al., 1996). In Prussian carps, there was no peak in VLMM biomolecule range, but a slight indication of a peak in LMM biomolecule region (5.1-14 kDa) was visible in a few samples (Fig. 2g, h). Intestinal Fe distribution in brown trout was comparable with hepatic distribution in the same species, with three peaks observed (Dragun et al., 2018b). Only difference was that in hepatic samples HMM peak was dominant, probably due to higher Fe concentrations in liver than intestine, suggesting more important role of the liver in Fe storage (Walker and Fromm, 1976; Dragun et al., 2018b). In Prussian carp, two peaks observed in the intestine were also observed in the liver and gills, where MMM peak was shown to be predominant in the gills and HMM peak in liver, similar to the intestine (Dragun et al., 2020). We further observed similarity of intestinal Fe distributions of Prussian carp with hepatic and gill distributions in European and Vardar chub, where binding of Fe in VLMM region was also not observed (Krasnići et al., 2013, 2014, 2018) suggesting some similar mechanisms of Fe binding in species belonging to the same fish family (Cyprinidae). Specific seasonal and spatial trends were not observed, but connection with different levels of bioaccumulation. In both fish species, elevated Fe concentrations were accompanied by an increase in the HMM or MMM peak, depending on the specimen.

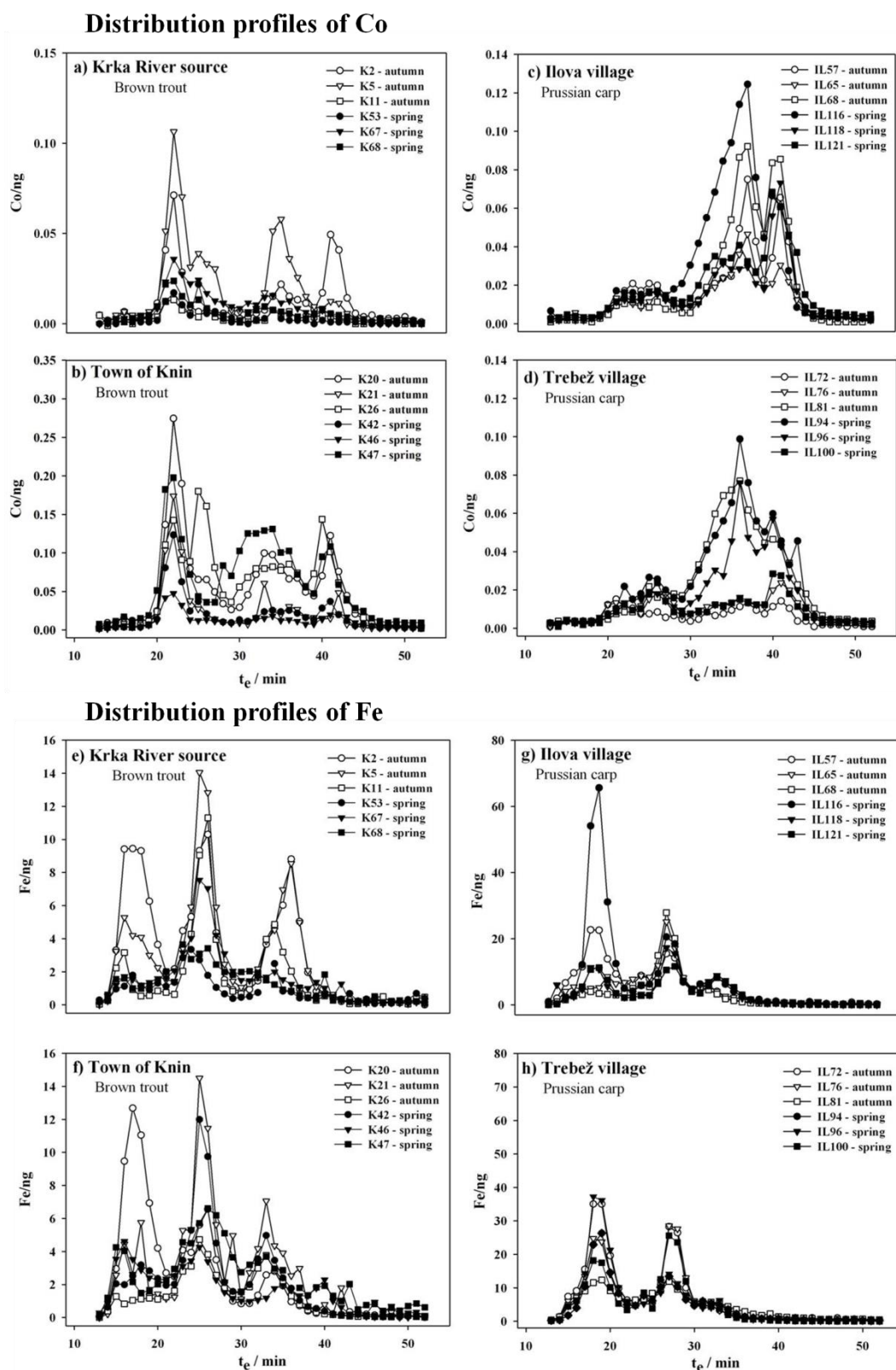


Figure 2. Distribution profiles of two selected elements (Co: a-d; Fe: e-h) among cytosolic biomolecules of different molecular masses in the intestine of brown trout from the two sites of the Krka River (Krka River source and Town of Knin) and Prussian carp from the two sites of the Ilova River (Ilova village and Trebež village) in two seasons (autumn and spring)

3.2.5. Molybdenum

Molybdenum serves as a cofactor in various enzymes including Fe-Mo flavoprotein xanthine oxidase (275 kDa, Truglio et al., 2002), aldehyde oxidase (130 kDa, Uchida et al., 2003) or sulfite oxidase (120 kDa; Johnson and Rajagopalan, 1976). Its distribution was similar in the intestine of both investigated fish species with two well shaped and clear peaks in HMM (~100-400 kDa, Table 3) and VLMM (~2-8 kDa, Table 3) biomolecule range, and the VLMM peak was visibly predominant in both species (Fig. 3a-d). Generally, Mo cytosolic concentrations were higher in Prussian carp than in brown trout which affected peak heights and quantity of eluted Mo (Fig. 3a-d, Table 1). Although MMs of all above mentioned enzymes were encompassed by the observed HMM peak, this peak was much smaller compared to VLMM peak, suggesting less significant Mo binding to enzymes in the intestine. For comparison, HMM peak was dominant and much more pronounced than VLMM peak in the liver of both brown trout and Prussian carp (Dragun et al., 2018b, 2020), as well as of European and Vardar chub (Krasnići et al., 2013, 2018), confirming the organ-specificity of these enzymes that have important roles in detoxification of xenobiotics, drugs and progesterone (Kisker et al., 1997), which mostly takes place in the liver as main detoxifying and metabolic organ (van Campenhout et al., 2008). The dominant peak in our research, in the intestine of both Prussian carp and brown trout, was located in VLMM region (maximum at 5.1 kDa; Fig. 3a-d, Table 3), same as observed in the gills of Prussian carp (Dragun et al., 2020), suggesting higher similarity in function of intestine with gills as uptake organs, than with the liver. Krasnići et al. (2019) have shown that VLMM Mo-binding biomolecules were heat-stable and determined their exact mass to be 3.3 kDa, which corresponded well to the estimated MM of predominant Mo-binding biomolecules in the intestine of brown trout and Prussian carp. The significant impact of season or site on the obtained profiles was not noticed and in both species, the increase of Mo elution in samples with higher cytosolic concentrations was seen in both peaks, but it was much more pronounced in the second, VLMM peak (Fig. 3a-d). Evidently, variability in different organs of the same species can be attributed to their different bioaccumulation capacities and different role of Mo in each organ. Presence in the intestine as a site of dietborne metal uptake, and gills as the site of waterborne metal uptake probably reflect recent Mo uptake and indicate binding to small metallochaperons or nonprotein compounds (Dragun et al., 2020).

3.2.6. Selenium

Selenium biological role is primarily related to incorporation into proteins in a form of selenocysteine, and is found, for example, in glutathione peroxidase, thioredoxin reductase and vitamin E (Watanabe et al., 1997). We noticed several species-specific differences in Se distribution in our research which included differences in number of peaks and peak predominance between the two species (Fig. 3e-h, Table 3). Distribution profiles of Se in the intestine of the brown trout showed its predominant presence within two peaks in VLMM range, one covering biomolecular region from 1.8-5.1 kDa (maximum at 3.9 kDa) and another one covering biomolecules of less than 1.5 kDa (Fig. 3e, f, Table 3). This second peak was predominant at both locations of the Krka River (Fig. 3e, f). In some brown trout specimens, two barely visible peaks were also observed in HMM region (500-100 kDa and 65-235 kDa; Fig. 3e, f). In the liver of brown trout, Se association with biomolecules below 1.5 kDa was also the most pronounced (Dragun et al., 2018b). Major binding of Se to biomolecules below 2 kDa was already reported in gills of European and Vardar chub (Krasnići et al., 2014, 2018), but not in the liver (Krasnići et al., 2013, 2018). Such Se elution in VLMM region could refer to forms of free selenocysteine (167 Da) or selenomethionine (196 Da) in the cytosols, as fish mostly accumulate Se in these forms and gastrointestinal uptake is central to both nutritional Se requirements and its toxicity (Janz et al., 2010). Further, it may indicate binding to compounds active in defense against oxidative stress, such as selenoneine (0.5 kDa, Yamashita and Yamashita, 2010). In addition to two VLMM peaks, which in Prussian carp encompassed biomolecules of 0.4–11 kDa, possibly including low MM selenoprotein SelW (~10 kDa), protein that possibly participates in antioxidant function (Lopez Heras et al., 2011), in this species Se was also comparably eluted in HMM region (30-303 kDa; maximum at 110 kDa; Fig. 3g, h). This HMM range includes known selenoproteins, such as glutathione peroxidase (85 kDa, Shulgin et al., 2008) or thioredoxin reductase (66 kDa), as well as selenoprotein SelP (50 kDa) identified in zebra fish (*Danio rerio*), primarily synthesized in liver and involved in the transport and delivery of Se to remote tissues (Kryukov and Gladyshev, 2000). As in brown trout, in some samples of Prussian carp additional small HMM peak was indicated at elution time from 14th to 18th minute (Fig. 3g, h). In Prussian carp, the increase in Se accumulation at the Ilova village was mainly reflected in its increased presence in the HMM biomolecule region, while at Trebež village higher amount of Se was bounded to VLMM biomolecules (Fig. 3g, h), regardless of the fact that Se in the intestine cytosol was present in a relatively narrow concentration range (Table 1). In brown trout, the

increase in intestinal cytosolic Se concentrations was not site-specific and resulted in a more pronounced presence in the VLMM biomolecule region, precisely in the second VLMM peak (<2 kDa) at both sites. Previous research on Prussian carp showed the existence of four peaks in gills with major part of Se being eluted in two VLMM peaks, whereas in the liver majority of Se was eluted within one HMM peak and only minor part was eluted within two VLMM peaks (Dragun et al., 2020). Therefore, the intestinal distribution profiles of Se in Prussian carp unite the characteristics of both gills and liver, with observed Se elution being almost comparable in HMM and VLMM regions (Fig. 3g, h). Recorded species-specific differences between brown trout and Prussian carp could be associated to the variability of their feeding behavior (Maher, 1987), and to differences in Se metabolism between the species.

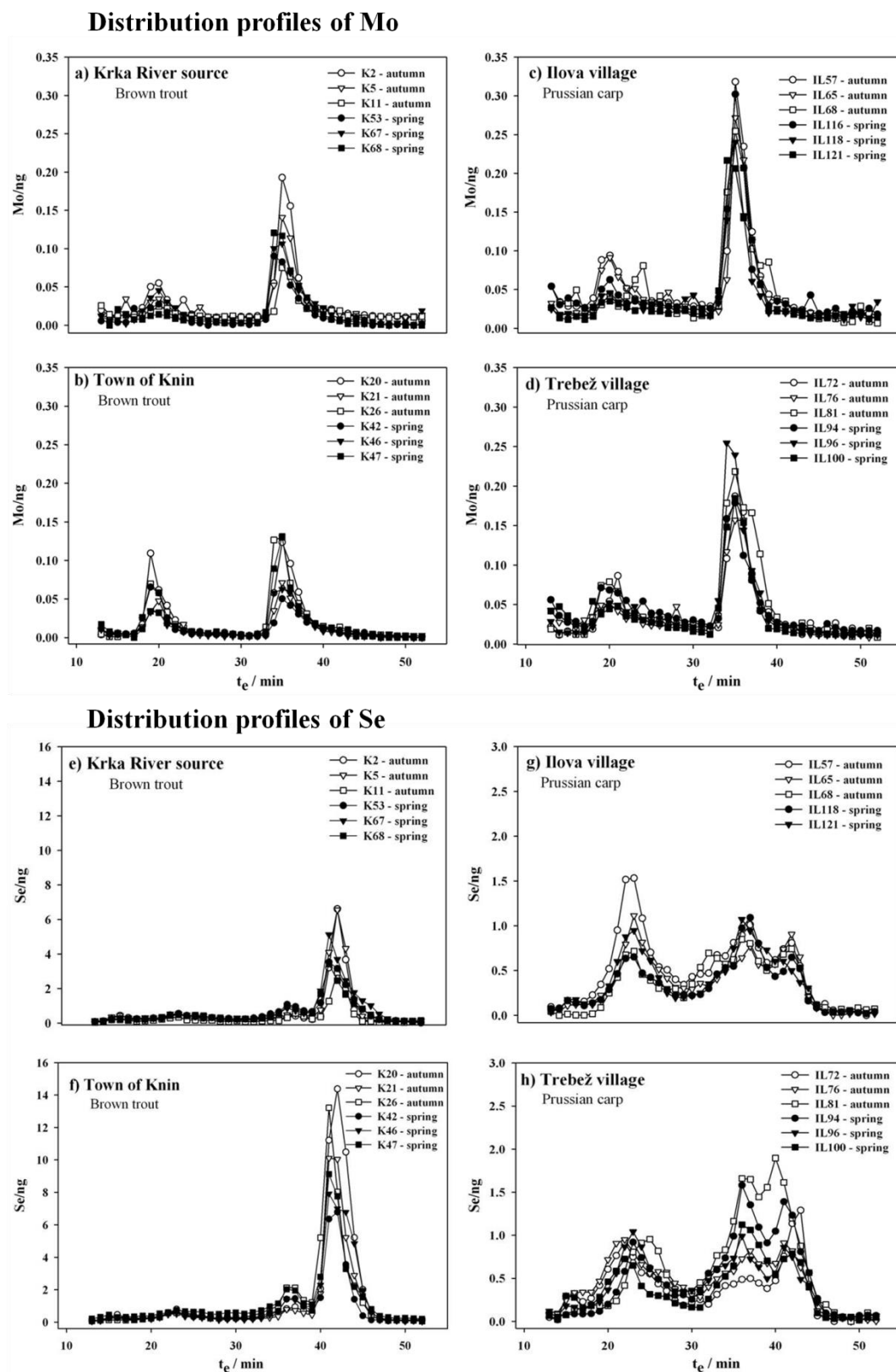


Figure 3. Distribution profiles of two selected elements (Mo: a-d; Se: e-h) among cytosolic biomolecules of different molecular masses in the intestine of brown trout from the two sites of the Krka River (Krka River source and Town of Knin) and Prussian carp from the two sites of the Ilova River (Ilova village and Trebež village) in two seasons (autumn and spring)

3.2.7. Zinc

Zinc essentiality is reflected by its role in metabolism of different biomolecules including proteins, carbohydrates and lipids, but also in cell signalization, protection of the immune system and neurotransmission, therefore encompassing catalytic, regulatory, and structural functions (Coelman, 1992). Therefore, its distribution in relatively wide range of MM was not unexpected. Moreover, species-specific differences between brown trout and Prussian carp were observed (Fig. 4). In brown trout, Zn was eluted in two clear peaks in HMM biomolecule region. First and dominant peak covered the biomolecules of 392-1088 kDa, and the second one of 30-235 kDa, with the maxima at ~600-800 kDa and 85-109 kDa, respectively (Table 3). This range could suggest binding to different proteins such as albumin (66 kDa, Table 2) or transferrin (80 kDa), carbonic anhydrase (29 kDa, Table 2), Zn superoxide dismutase (32 kDa, Table 2) and alcohol dehydrogenase (150 kDa, Table 2). Similar distribution, with the predominant peak at 20-400 kDa which corresponded well to our second HMM peak, but with the additional LMM peak (9-19 kDa), which coincided with the elution time of MT standard, was observed in the hepatic cytosols of brown trout (Dragun et al., 2018b). In Prussian carp, in addition to above mentioned two HMM peaks (maxima at 843 kDa and 109 kDa, respectively), elution in VLMM region was also observed (Fig. 4c, d, Table 3). Although Zn in VLMM region was eluted in broad range, by careful insight two separate peaks could be distinguished in most samples (Fig. 4c, d), the first at 1.8-14 kDa, and the second one at 0.7-1.8 kDa, and the predominant binding to VLMM was mostly observed for specimens with the higher Zn intestinal cytosolic concentrations (Fig. 4c, d). Therefore, in Prussian carp there was an indication of possible binding to MT (the tail of the first VLMM peak), but this was not clear and pronounced. More dominant Zn association with MT region was observed in liver of Prussian carp, while hepatic Zn HMM peaks appeared at MMs of 30–300 kDa and above 400 kDa (Dragun et al., 2020) comparable to intestine. The significant impact of season or site was not noticed. Intestine was, as in the case of some other elements, confirmed to be more similar to gills as the uptake tissue, in which clear binding to MTs was also not observed in Prussian carp, but neither in European nor Vardar chub (Krasnići et al., 2014, 2018; Dragun et al., 2020). In addition, two VLMM peaks were also observed in both the gills and the liver of Prussian carp (Dragun et al., 2020), as well as of European chub (<5 kDa), especially in the specimens with higher cytosolic Zn concentrations (Krasnići et al., 2013). Elution in VLMM biomolecule range could indicate role of Zn in antioxidative defense by binding to glutathione (GSH, 307 Da), intracellular thiol compound composed of cysteine, glutamic acid and glycine, which can be free in the cells or bound to proteins (Iwasaki et al.,

2009). This mechanism of detoxification by GSH might occur in the fish intestine, as GSH quantity was shown as quite high in the intestine of brown trout and Prussian carp (Mijošek et al., 2019a, 2021). Zinc distribution profiles slightly differed for different species or different tissues, due to differences in biology and ecology of the species, their different accumulation and detoxification mechanisms, as well as tissue composition or specific role.

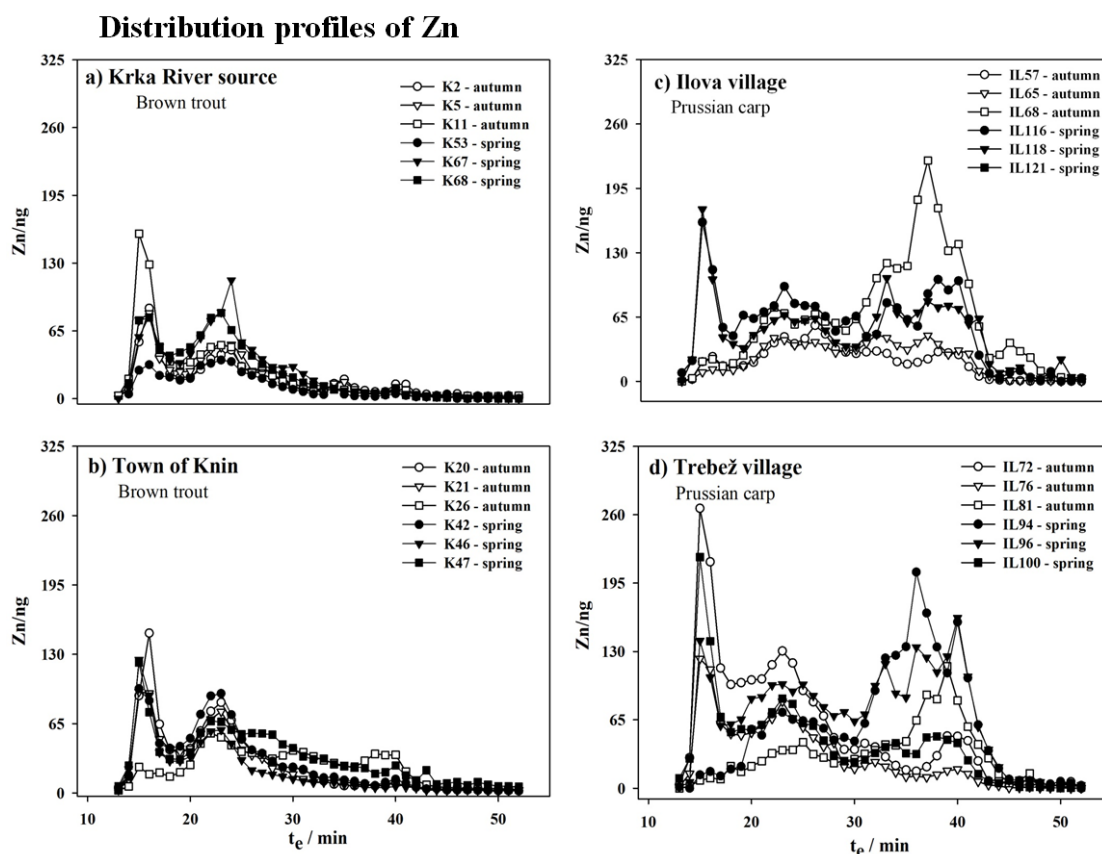


Figure 4. Distribution profiles of Zn among cytosolic biomolecules of different molecular masses in the intestine of brown trout from the two sites of the Krka River (Krka River source and Town of Knin) and Prussian carp from the two sites of the Ilova River (Ilova village and Trebež village) in two seasons (autumn and spring)

4. Conclusions

Applied methodology enabled us to define, for the first time, the molecular mass ranges of cytosolic molecules that bind Cd, Co, Cu, Fe, Mo, Se and Zn in the intestines of brown trout and Prussian carp from two moderately impacted rivers. Although we considered spatial and temporal variability at each river, comparison of the obtained profiles indicated that distribution of trace elements among different biomolecules was mostly dependent on level of exposure and consequent bioaccumulation. Significant differences associated to seasons were

not observed, and trace elements under all studied conditions were associated to the same biomolecules, and only the proportions associated to specific cytosolic compounds changed as a consequence of different concentrations of elements. Well-established link of Cd and Cu to MTs was confirmed for the intestine of both fish species, suggesting efficient detoxification of these elements, as well as functional association in case of Cu. However, association of Zn to MTs was not observed in the intestine, contrary to previously established presence of Zn-MT binding in the liver of brown trout and Prussian carp. Further, Fe, Se and Zn showed some considerable species-specific differences in our research. Specifically, Fe elution in VLMM biomolecule range was observed only in brown trout, while Se was eluted in HMM and Zn in VLMM biomolecule range only in Prussian carp. Additionally, Co was found to predominantly bind to biomolecules of MM of 85-235 kDa in brown trout, but to biomolecules of MM <18 kDa in Prussian carp, but the same peaks were observed in both species. Tissue-specific differences were additionally observed for Fe, Se and Zn distribution between intestinal, hepatic and gills cytosols. They reflected different functions of these organs, showing some similarities of the intestine with both gills and liver, but more clear with gills, as both being the uptake organs. Comparison with other fish species also indicated species-specific variability, due to the different ecology of the species, their accumulation capacities and specific metal handling strategies. As there is no other available data on cytosolic distribution of elements in the fish intestine, obtained results present a significant contribution to better understanding the fate of the elements, their detoxification mechanisms and behavior in general in fish intestinal tissue, specifically in brown trout and Prussian carp. Comparison with other tissues suggested the importance of considering few different tissues, as their primary role has significant impact on metal handling strategies. Future research should involve additional separation methods and mass spectrometry to accurately identify the exact metal binding molecules. This would enable the clear recognition of detoxification mechanisms, but also the estimation of metal/nonmetal toxicity. Such comprehensive methodology is the base of development of new biomarkers of metal exposure, similar to MTs, which are extremely important in the assessment of metal contamination.

5. Acknowledgements

The study was conducted within the project “Accumulation, subcellular mapping and effects of trace metals in aquatic organisms” (project no.: IP-2014-09-4255), financed by Croatian Science Foundation. Authors are thankful for assistance in fish sampling to colleagues from

Laboratory for Aquaculture and Pathology of Aquatic Organisms from the Ruđer Bošković Institute.

6. References

- Asmamaw, B., 2016. Transferrin in fishes: A review article. *J. Coast. Life Med.* 4, 176-180.
- Beard, J.L., Dawson, H., Pifero, D.J., 1996. Iron metabolism: a comprehensive review. *Nutr. Rev.* 54, 295–317.
- Blust, R., 2012. Cobalt. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), *Fish Physiology: homeostasis and toxicology of essential metals*. Vol. 31A. Academic, London, pp. 291–326.
- Bury, N.R., Boyle, D., Cooper, C.A., 2012. Iron. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), *Fish Physiology: homeostasis and toxicology of essential metals*. Vol. 31A. Academic, London, pp. 201–251.
- Caron, A., Rosabal, M., Drevet, O., Couture, P., Campbell, P.G.C., 2018. Binding of trace elements (Ag, Cd, Co, Cu, Ni, and Tl) to cytosolic biomolecules in livers of juvenile yellow perch (*Perca flavescens*) collected from lakes representing metal contamination gradients. *Environ. Toxicol. Chem.* 37, 576–586.
- Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. *J. Exp. Biol.* 203, 2455–2466.
- Coleman, J.E., 1992 Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. *Annu. Rev. Biochem.* 61, 897–946.
- Dragun, Z., Filipović Marijić, V., Krasnići, N., Ivanković, D., Valić, D., Žunić, J., Kapetanović, D., Vardić Smrzlić, I., Redžović, Z., Grgić, I., Erk, M., 2018a. Total and cytosolic concentrations of twenty metals/metalloids in the liver of brown trout *Salmo trutta* (Linnaeus, 1758) from the karstic Croatian river Krka. *Ecotox. Environ. Safe.* 147, 537–549.
- Dragun, Z., Krasnići, N., Kolar, N., Filipović Marijić, V., Ivanković, D., Erk, M., 2018b. Cytosolic distributions of highly toxic metals Cd and Tl and several essential elements in the liver of brown trout (*Salmo trutta* L.) analyzed by size exclusion chromatography and inductively coupled plasma mass spectrometry. *Chemosphere* 207, 162–173.

- Dragun, Z., Krasnići, N., Ivanković, D., Filipović Marijić, V., Mijošek, T., Redžović, Z., Erk, M., 2020. Comparison of intracellular trace element distributions in the liver and gills of the invasive freshwater fish species, Prussian carp (*Carassius gibelio* Bloch, 1782). *Sci. Total Environ.* 730, 138923.
- Erdogan, Z., Koc, H.T., Gungor, S., and Ulunehir, G. 2014. Age, growth and reproductive properties of an invasive species *Carassius gibelio* (Bloch, 1782) (Cyprinidae) in the Ikizcetepeler Dam Lake (Balikesir), Turkey. *Period. Biol.* 116(3), 285-291.
- Festa, R.A., Thiele, D.J., 2011. Copper: an Essential Metal in Biology. *Curr. Biol.* 21, 877-883.
- Goenaga Infante, H., Van Campenhout, K., Schaumlöffel, D., Blust, R., Adams, F.C., 2003. Multi-element speciation of metalloproteins in fish tissue using size-exclusion chromatography coupled “on-line” with ICP-isotope dilution-time-of-flight-mass spectrometry. *Analyst* 128, 651–657.
- Holm, R.H., Kennepohl, P., Solomon, E.I., 1996. Structural and functional aspects of metal sites in biology. *Chem. Rev.* 96, 2239-2314.
- HRN EN 14011, 2005. Fish Sampling by Electric Power [uzorkovanje riba električnom strujom].
- Iwasaki, Y., Saito, Y., Nakano, Y., Mochizuki, K., Sakata, O., Ito, R., Saito, K., Nakazawa, H., 2009. Chromatographic and mass spectrometric analysis of glutathione in biological samples. *J. Chromatogr. B* 877, 3309–3317.
- Janz, D.M., 2012. Selenium. Homeostasis and Toxicology of Essential Metals. *Fish Physiology* vol. 31A. Elsevier Inc, pp. 327–374
- Johnson, J.L., Rajagopalan, K.V., 1976. Purification and properties of sulphite oxidase from human liver. *J. Clin. Invest.* 58, 543-550.
- Kirschbaum, J., 1981. Cyanocobalamin. In: Florey, K. (Ed.), *Analytical profiles of drug substances*. Vol. 10. Academic, New York, pp. 183–288.
- Kisker, C., Schindelin, H., Rees, D.C., 1997. Molybdenum-cofactor-containing enzymes: structure and mechanism. *Annu. Rev. Biochem.* 66, 233-267.

Krasnići, N., Dragun, Z., Erk, M., Raspor, B., 2013. Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se and Zn) and nonessential (Cd, Pb) trace elements among protein fractions from hepatic cytosol of European chub (*Squalius cephalus* L.). Environ. Sci. Pollut. Res. 20, 2340–2351.

Krasnići, N., Dragun, Z., Erk, M., Raspor, B., 2014. Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (*Squalius cephalus* L.). Environ. Sci. Pollut. Res. 21, 13512–13521.

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 northeastern Macedonia. Sci. Total Environ. 613-614, 1055–1068.

Krasnići, N., Dragun, Z., Kazazić, S., Muharemović, H., Erk, M., Jordanova, M., Rebok, K., Kostov, V., 2019. Characterization and identification of selected metal-binding biomolecules from hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928) using various techniques of liquid chromatography and mass spectrometry. Metallomics 11, 1060–1078.

Kryukov, G.V., Gladyshev, V.N., 2000. Selenium metabolism in zebrafish: multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. Genes Cells 5, 1049–1060.

Kuhn, D.E., O'Brien, K.M., Crockett, E.L., 2016. Expansion of capacities for iron transport and sequestration reflects plasma volumes and heart mass among white-blooded notothenioid fishes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 311, 649–657.

Lavradas, R.T., Chávez Rocha, R.C., Dillenburg Saint' Pierre, T., Godoy, J.M., Hauser-Davis, R.A., 2016. Investigation of thermostable metalloproteins in *Perna perna* mussels from differentially contaminated areas in Southeastern Brazil by bioanalytical techniques. J. Trace Elem. Med. Biol. 34, 70–78.

Lopez Heras, I., Palomo, M., Madrid, Y., 2011. Selenoproteins: the key factor in selenium essentiality. State of the art analytical techniques for selenoprotein studies. Anal. Bioanal. Chem. 400, 1717–1727.

- Maher, W.A., 1987. Distribution of selenium in marine animals: relationship to diet. *Comp. Biochem. Physiol.* 86C, 131–133.
- Martin-Antonio, B., Jimenez-Cantizano, R.M., Salas-Leiton, E., Infante, C., Manchado, M., 2009. Genomic characterization and gene expression analysis of four hepcidin genes in the red banded seabream (*Pagrus auriga*). *Fish Shellfish Immunol.* 26, 483–491.
- Mason, A.Z., Jenkins, K.D., 1995. Metal detoxification in aquatic organisms. In: Tessier, A., Turner, D. (Eds.), *Metal speciation and bioavailability in aquatic systems*. IUPAC, Wiley, New York, pp 479–512.
- McGeer, J.C., Niyogi, S., Smith, D.S., 2012. Cadmium. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), *Fish Physiology: Homeostasis and toxicology of nonessential metals*, vol. 31B. Elsevier Academic, London, pp. 125-184.
- 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. *Ecol. Indic.* 105, 188–198.
- 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. *Sci. Total Environ.* 660, 1079–1090.
- Mijošek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Sertić Perić M., Vdović, N., Bačić, N., Dautović, J., Erk, M., 2020. The assessment of metal contamination in water and sediments of the lowland Ilova River (Croatia) impacted by anthropogenic activities. *Environ. Sci. Pollut. Res.* 27, 25374-25389.
- Mijošek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Erk, M., 2021. Intestine of invasive fish Prussian carp as a target organ in metal exposure assessment of the wastewater impacted freshwater ecosystem. *Ecol. Indic.* 122, 107247
- NN 55, 2013. Ordinance on the Protection of Animals Used for Scientific Purposes [Pravilnik o zaštiti životinja koje se koriste u znanstvene svrhe].

- Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat. Toxicol.* 22, 81–114.
- Sadler, P.J., Tucker, A., Viles, J.H., 1994. Involvement of a lysine residue in the N-terminal Ni²⁺ and Cu²⁺ binding site of serum albumins: comparison with Co²⁺, Cd²⁺, Al³⁺. *Eur. J. Biochem.* 220, 193-200.
- Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.M., Aït-Aïssa, S., 2005. Copper induced oxidative stress in the three-spined stickleback: relationship with hepatic metal levels. *Environ. Toxicol. Pharmacol.* 19, 177–183.
- 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. *Ecotoxicol. Environ. Saf.* 163, 125–135
- Shulgin, K.K., Popova, T.N., Rakhmanova, T.I., 2008. Isolation and purification of glutathione peroxidase. *Appl. Biochem. Microbiol.* 44, 247–250.
- Strižak, Ž., Ivanković, D., Pröfrock, D., Helmholz, H., Cindrić, A.-M., Erk, M., Prange, A., 2014. Characterization of the cytosolic distribution of priority pollutant metals and metalloids in the digestive gland cytosol of marine mussels: seasonal and spatial variability. *Sci. Total Environ.* 470(471), 159–170.
- Szpunar, J., Lobinski, R., 1999. Species-selective analysis for metal-biomacromolecular complexes using hyphenated techniques. *Pure Appl. Chem.* 71, 899-918.
- Szpunar, J., 2004. Metallomics: A new frontier in analytical chemistry. *Anal. Bioanal. Chem.* 378, 54-56.
- Truglio, J.J., Theis, K., Leimkühler, S., Rappa, R., Rajagopalan, K.V., Kisker, C., 2002. Crystal structures of the active and alloxanthine inhibited forms of xanthine dehydrogenase from *Rhodobacter capsulatus*. *Structure* 10, 115-125.
- Uchida, H., Kondo, D., Yamashita, A., Nagaosa, Y., Sakurai, T., Fujii, Y., Fujishiro, K., Aisaka, K., Uwajima, T., 2003. Purification and characterization of an aldehyde oxidase from *Pseudomonas* sp. KY 4690. *FEMS Microbiol. Lett.* 229, 31–36.

Urien, N., Jacob, S., Couture, P., Campbell, P.G.C., 2018. Cytosolic distribution of metals (Cd, Cu) and metalloids (As, Se) in livers and gonads of field-collected fish exposed to an environmental contamination gradient: an SEC-ICP-MS analysis. *Environments* 5 (102), 1–17.

van Campenhout, K., Goenaga Infante, H., Goemans, G., Belpaire, C., Adams, F., Blust, R., Bervoets, L., 2008. A field survey of metal binding to metallothionein and other cytosolic ligands in liver of eels using an on-line isotope dilution method in combination with size exclusion (SE) high pressure liquid chromatography (HPLC) coupled to inductively coupled plasma time-of-flight mass spectrometry (ICP-TOFMS). *Sci. Total Environ.* 394, 379–389.

van Campenhout, K., Goenaga Infante, H., Hoff, P.T., Moens, L., Goemans, G., Belpaire, C., Adams, F., Blust, R., Bervoets, L., 2010. Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (*Carassius auratus gibelio*) from metal-impacted habitats. *Ecotoxicol. Environ. Safe.* 73, 296–305.

Vijver, A.G., van Gestel, C.A.M., Lanno, R.P., van Straalen, N.M., Peijnenburg, W.J.G.M., 2014. Internal metal sequestration and its ecotoxicological relevance: a review. *Environ. Sci. Technol.* 38, 4705–4712.

Vutukuru, S.S., Chintada, S., Madhavi, K.R., Rao, J.V., Anjaneyulu, Y., 2006. Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. *Fish Physiol. Biochem.* 32, 221–229.

Walker, R.L., Fromm, P.O., 1976. Metabolism of iron by normal and iron deficient rainbow trout. *Comp. Biochem. Physiol.* 55A, 311–318.

Wallace, W.G., Lee, B.-G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol. Prog. Ser.* 249, 183–197.

Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. *Aquaculture* 151, 185–207.

Yamashita, Y., Yamashita, M., 2010. Identification of a novel selenium-containing compound, selenoneine, as the predominant chemical form of organic selenium in the blood of a bluefin tuna. *J. Biol. Chem.* 285, 18134–18138.

Prilog 2. Citosolske koncentracije elemenata u vrstama *G. fossarum* i *G. roeselii* iz rijeke Ilove iz dvaju uzorkovanja (jesen i proljeće). Rezultati su prikazani kao srednja vrijednost \pm standardna devijacija. Statistički značajne razlike (Mann-Whitney *U* test, $p < 0,05$) između sezona su označene zvjezdicom (*).

	<i>Gammarus fossarum</i>		<i>Gammarus roeselii</i>	
	Listopad 2017.	Svibanj 2018.	Listopad 2017.	Svibanj 2018.
As	86,95 \pm 16,90	73,47 \pm 13,40	115,12 \pm 23,47	115,00 \pm 21,69
Cd	23,71 \pm 4,19	23,43 \pm 3,02	23,23 \pm 1,99*	27,06 \pm 4,34*
Co	37,69 \pm 8,10	31,93 \pm 3,33	43,57 \pm 10,08	39,19 \pm 4,65
Cu	6787,72 \pm 701,32*	9649,24 \pm 2333,32*	8783,66 \pm 1226,53	9715,41 \pm 1902,60
Mn	4025,31 \pm 1337,37	4293,02 \pm 1490,53	6864,21 \pm 2365,98	5275,04 \pm 1165,57
Mo	54,32 \pm 10,54*	40,73 \pm 7,34*	48,79 \pm 3,61*	42,86 \pm 4,68*
Rb	572,96 \pm 49,58*	784,04 \pm 42,90*	612,03 \pm 49,54*	758,98 \pm 79,31*
Se	201,31 \pm 32,24*	263,58 \pm 49,48*	184,16 \pm 31,94	220,65 \pm 50,76
Sr	8151,43 \pm 679,90*	6743,05 \pm 691,32*	8917,27 \pm 1049,66*	6259,61 \pm 724,90*
Tl	0,42 \pm 0,09*	0,53 \pm 0,11*	0,68 \pm 0,15	0,65 \pm 0,23
V	9,44 \pm 3,46	11,29 \pm 2,29	11,47 \pm 2,58	10,55 \pm 1,50
Zn	6769,64 \pm 777,40*	7915,87 \pm 432,40*	7224,28 \pm 528,62*	8670,51 \pm 870,00*
Ca	3604,85 \pm 293,18*	2948,57 \pm 346,02*	3986,52 \pm 481,14*	3060,58 \pm 407,40*
K	2034,50 \pm 154,51	1812,47 \pm 140,18	2034,50 \pm 137,65	1901,62 \pm 150,47
Mg	287,86 \pm 19,51	264,21 \pm 27,83	355,83 \pm 28,03	359,96 \pm 45,70
Na	1261,87 \pm 39,69*	1192,70 \pm 82,00*	1278,21 \pm 92,27	1301,57 \pm 111,67

Prilog 3. Biometrijska obilježja (srednja vrijednost \pm standardna devijacija) potočnih pastrva (*S. trutta*) ulovljenih u rijeci Krki na referentnoj (izvor rijeke Krke) i onečišćenoj postaji (grad Knin) u dva uzorkovanja i osnovne epidemiološke značajke kukaša iz probavila potočnih pastrva. Statistički značajne razlike (Mann-Whitney *U* test, $p < 0,05$) između sezona na pojedinoj postaji su označene zvjezdicom (*), a između dviju postaja u jednoj sezoni s različitim velikim tiskanim slovima (A i B).

	Izvor rijeke Krke		Grad Knin	
	jesen 2015. n = 12	proljeće 2016. n = 13	jesen 2015. n = 13	proljeće 2016. n = 13
Ukupna dužina (cm)	24,45 \pm 4,46*	18,22 \pm 2,10*	24,27 \pm 3,14*	20,05 \pm 2,90*
Tjelesna masa (g)	161,2 \pm 85,1*	65,65 \pm 21,36* ^A	164,7 \pm 53,9*	102,1 \pm 44,7* ^B
FCI (g cm⁻³*100)	1,01 \pm 0,08 ^A	1,06 \pm 0,06 ^A	1,11 \pm 0,09* ^B	1,20 \pm 0,09* ^B
HSI (%)	1,01 \pm 0,22*	1,32 \pm 0,31*	0,94 \pm 0,26*	1,57 \pm 0,49*
GSI (%)	4,42 \pm 2,36*	0,40 \pm 0,34* ^A	3,56 \pm 2,68*	0,14 \pm 0,05* ^B
Spol (Ž/M/ND)	4/7/1	5/8/0	5/8/0	7/6/0
Epidemiološke značajke kukaša				
Zastupljenost (broj i % pastrva invadiranih nametnicima)	12, 100%	13, 100%	13, 100%	12, 92,3%
Prosječni intenzitet invazije	42,0 \pm 19,0	20,7 \pm 8,9	51,5 \pm 53,3	5,9 \pm 6,7
Ukupan broj nametnika u uzorkovanim ribama	504	269	669	76

Prilog 4. Koncentracije metala/metaloida/nemetala (srednja vrijednost \pm standardna devijacija) u bioindikatorskim organizima iz rijeke Krke s referentne (izvor rijeke Krke) i onečišćene postaje (grad Knin) u dvjema sezonama (jesen i proljeće). Za svaki element u prvom redu su prikazane koncentracije u kukašima, u drugom redu u rakušcima, a u trećem koncentracije metala u probavilu riba. Statistički značajne razlike (Mann-Whitney *U* test, $p < 0,05$) između sezona na pojedinoj postaji su označene zvjezdicom (*), a između dviju postaja u jednoj sezoni s različitim velikim tiskanim slovima (A i B).

Element/postaja ($\mu\text{g kg}^{-1}/\text{mg kg}^{-1}$)	Izvor rijeke Krke		Grad Knin	
	jesen 2015.	proljeće 2016.	jesen 2015.	proljeće 2016.
Cd ($\mu\text{g kg}^{-1}$)	458,0 \pm 366,5 ^{*A}	1047,0 \pm 533,6 ^{*A}	119,8 \pm 86,8 ^B	171,0 \pm 98,2 ^B
	140,1 \pm 51,6 ^A	139,3 \pm 36,4 ^A	28,1 \pm 12,9 ^B	19,6 \pm 7,5 ^B
	107,7 \pm 141,0	114,9 \pm 122,7 ^A	10,48 \pm 12,00	2,74 \pm 1,10 ^B
Co ($\mu\text{g kg}^{-1}$)	10,28 \pm 5,11 ^{*A}	23,23 \pm 11,07 ^{*A}	61,24 \pm 57,19 ^B	106,2 \pm 66,5 ^B
	34,02 \pm 12,30 ^A	39,30 \pm 10,71 ^A	85,74 \pm 23,58 ^B	116,9 \pm 50,8 ^B
	12,15 \pm 10,86	14,51 \pm 8,31 ^A	39,23 \pm 28,83	31,25 \pm 8,62 ^B
Cs ($\mu\text{g kg}^{-1}$)	4,97 \pm 1,59	4,11 \pm 1,37	3,88 \pm 2,10	5,04 \pm 1,96
	8,09 \pm 3,85 ^A	8,47 \pm 2,13	14,15 \pm 6,09 ^B	9,42 \pm 4,91
	10,59 \pm 1,16 ^{*A}	6,37 \pm 1,99 [*]	4,37 \pm 2,93 ^B	5,99 \pm 2,07
Pb ($\mu\text{g kg}^{-1}$)	224,7 \pm 154,1 ^A	404,5 \pm 271,6	659,7 \pm 464,9 ^B	346,3 \pm 298,7
	41,07 \pm 29,28 ^A	33,90 \pm 18,10 ^A	88,86 \pm 39,05 ^B	85,16 \pm 53,85 ^B
	41,21 \pm 20,96	49,11 \pm 11,97	30,91 \pm 13,10 [*]	59,25 \pm 24,00 [*]
Cu (mg kg^{-1})	6,50 \pm 4,62	12,06 \pm 10,08	7,15 \pm 6,53	18,35 \pm 16,64
	6,55 \pm 1,56 ^A	7,00 \pm 1,21	8,89 \pm 1,81 ^B	7,14 \pm 1,91
	0,844 \pm 0,429	0,693 \pm 0,206	1,07 \pm 0,42	0,725 \pm 0,132
Fe (mg kg^{-1})	10,26 \pm 4,78 ^A	8,57 \pm 2,75	28,61 \pm 8,70 ^B	15,93 \pm 12,94
	22,13 \pm 13,72 ^A	25,44 \pm 11,93 ^A	71,81 \pm 37,58 ^B	70,74 \pm 49,72 ^B
	14,52 \pm 5,19 [*]	7,54 \pm 2,94 [*]	14,80 \pm 4,39	12,27 \pm 5,37
Mn (mg kg^{-1})	3,07 \pm 0,53	3,36 \pm 0,40 ^A	3,65 \pm 0,98	4,26 \pm 1,10 ^B
	1,99 \pm 0,85 ^A	1,81 \pm 0,53 ^A	5,43 \pm 1,47 ^B	4,16 \pm 1,85 ^B
	0,47 \pm 0,12 ^A	0,57 \pm 0,07	0,71 \pm 0,15 ^B	0,72 \pm 0,30
Rb (mg kg^{-1})	2,08 \pm 0,45 ^A	1,70 \pm 0,57 ^A	1,35 \pm 0,52 ^{*B}	2,34 \pm 0,61 ^{*B}
	1,82 \pm 0,15 [*]	1,63 \pm 0,14 ^{*A}	1,80 \pm 0,11 [*]	1,42 \pm 0,19 ^{*B}
	4,20 \pm 1,33 ^{*A}	2,34 \pm 0,76 [*]	2,56 \pm 0,97 ^B	2,93 \pm 0,76
Se (mg kg^{-1})	0,590 \pm 0,140	0,698 \pm 0,245	0,610 \pm 0,110	0,726 \pm 0,225
	1,95 \pm 0,53	1,45 \pm 0,48	1,90 \pm 0,55	1,78 \pm 0,50
	0,857 \pm 0,217	0,668 \pm 0,107 ^A	0,927 \pm 0,204	0,895 \pm 0,216 ^B
Sr (mg kg^{-1})	0,706 \pm 0,724 ^A	0,842 \pm 0,482 ^A	3,63 \pm 1,05 ^B	2,41 \pm 0,89 ^B
	53,26 \pm 17,04 [*]	30,44 \pm 6,03 ^{*A}	50,49 \pm 16,42	51,12 \pm 17,95 ^B
	0,106 \pm 0,027 ^A	0,093 \pm 0,041 ^A	0,260 \pm 0,063 ^B	0,337 \pm 0,222 ^B
Zn (mg kg^{-1})	13,82 \pm 2,56	14,10 \pm 8,44 ^A	16,73 \pm 7,76	19,77 \pm 5,12 ^B
	8,73 \pm 0,93 ^A	8,44 \pm 2,15	9,93 \pm 1,06 ^B	9,63 \pm 2,23
	182,4 \pm 57,7	109,4 \pm 67,3	130,8 \pm 95,3	114,4 \pm 48,2
Ca (mg kg^{-1})	510,9 \pm 496,3 ^A	487,3 \pm 251,6 ^A	869,3 \pm 348,9 ^B	1045,4 \pm 434,8 ^B
	24370,9 \pm 5823,6	26371,2 \pm 3118,2	23561,5 \pm 4917,8	22125,8 \pm 7765,9
	119,1 \pm 35,2	130,2 \pm 60,3	124,8 \pm 30,2	193,8 \pm 164,7
K (mg kg^{-1})	1854,7 \pm 359,1	1954,2 \pm 245,3 ^A	1963,3 \pm 294,2 [*]	2441,8 \pm 289,9 ^{*B}
	1496,8 \pm 294,8 ^A	1435,6 \pm 132,0	2110,3 \pm 114,4 ^{*B}	1508,4 \pm 155,7 [*]
	2993,1 \pm 318,8	2924,2 \pm 219,5	2906,0 \pm 276,9	2742,9 \pm 333,3

Prilog 4 - nastavak,

Mg (mg kg⁻¹)	164,5±50,3 ^A	174,8±23,1 ^A	200,6±30,81 ^B	209,2±27,2 ^B
	443,1±125,0	421,3±42,2	429,1±76,7	430,1±125,8
	164,4±28,1	184,8±19,9	167,7±26,8	173,3±54,9
Na (mg kg⁻¹)	1822,2±357,3 [*]	1413,6±261,4 [*]	1523,5±216,5	1359,7±255,1
	1377,0±313,4	1329,6±223,3	1290,8±272,6	1209,2±215,9
	1192,1±137,1	1140,2±135,4 ^A	1041,6±145,4	962,2±137,5 ^B

Prilog 5. Biokoncentracijski faktori za kukaše (*D. truttae*) izračunati u odnosu na rakušce (*G. balcanicus*) kao međudomadare i probavno tkivo potočnih pastrva (*S. trutta*) kao domadara.

Element/ postaja	Kukaši/rakušci				Kukaši/probavilo riba			
	Izvor rijeke Krke		Grad Knin		Izvor rijeke Krke		Grad Knin	
	jesen	proljeće	jesen	proljeće	jesen	proljeće	jesen	proljeće
Cd	3,27	7,52	4,27	8,72	4,25	9,11	11,43	62,40
Co	0,30	0,59	0,71	0,91	0,85	1,60	1,56	3,40
Cs	0,61	0,49	0,27	0,54	0,47	0,65	0,89	0,84
Pb	5,47	11,93	7,42	4,07	5,45	8,24	21,34	5,84
Cu	0,99	1,72	0,80	2,57	7,70	17,40	6,68	25,31
Fe	0,46	0,34	0,40	0,23	0,71	1,14	1,93	1,30
Mn	1,54	1,86	0,67	1,03	6,53	5,89	5,14	5,92
Rb	1,14	1,04	0,75	1,65	0,50	0,73	0,53	0,80
Se	0,30	0,48	0,32	0,41	0,69	1,04	0,66	0,81
Sr	0,01	0,03	0,07	0,05	6,66	9,00	13,96	7,15
Zn	1,58	1,67	1,68	2,05	0,08	0,13	0,13	0,17
Ca	0,02	0,02	0,04	0,05	4,29	3,74	6,97	5,39
K	1,24	1,36	0,93	1,62	0,62	0,67	0,68	0,89
Mg	0,37	0,41	0,47	0,49	1,00	0,95	1,20	1,21
Na	1,32	1,06	1,18	1,12	1,53	1,24	1,46	1,41

Prilog 6. Fish soft tissues, calcified structures and intestinal parasites, acanthocephalans as indicators of short and long term metal exposure in the karst freshwater ecosystem

Vlatka Filipović Marijić^{1, §}, Tatjana Mijošek^{1,, §}, Zrinka Dragun¹, Anika Retzmann², Andreas Zitek³, Thomas Prohaska², Niko Bačić¹, Zuzana Redžović¹, Ivana Grgić⁴, Nesrete Krasnići¹, Damir Valić¹, Damir Kapetanović¹, Jakov Žunić⁵, Dušica Ivanković¹, Irena Vardić Smrzlić¹, Marijana Erk¹*

¹Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička cesta 54, 10000 Zagreb, Croatia

²Montanuniversität Leoben, Chair of General and Analytical Chemistry, Franz-Josef-Strasse 18, 8700 Leoben, Austria

³FFoQSI—Austrian Competence Centre for Feed and Food Quality, Safety & Innovation, FFoQSI GmbH, Technopark 1C, 3430 Tulln, Austria

⁴Ruđer Bošković Institute, Division of Physical Chemistry, Bijenička cesta 54, 10000 Zagreb, Croatia

⁵Vodovod d.o.o., Spire Brusine 17, 23000 Zadar, Croatia

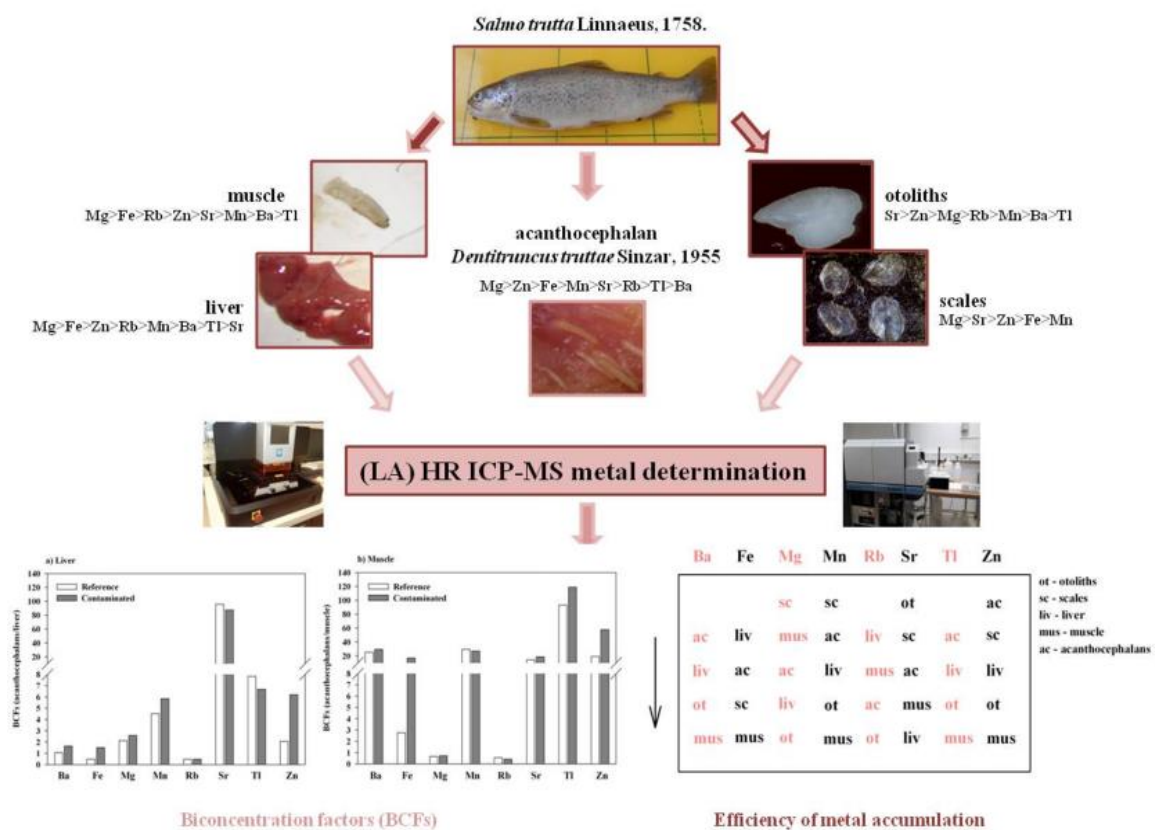
*Corresponding author:

Tatjana Mijošek, tmijosek@irb.hr

Laboratory for Biological Effects of Metals, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia.

[§] These authors contributed equally to this work

Graphical abstract



Abstract

Although there are common and well established bioindicator organisms and tissues, there is still a need of reliable and sensitive bioindicators, which will rapidly reflect short and long term metal changes in the aquatic environment. In addition to commonly used soft tissues, in the present pilot-study fish calcified structures and intestinal parasites were applied as combination of long term and rapid indicators of metal exposure, respectively. Patterns of metal accumulation and distribution in soft (muscle, liver) and hard (scales, otoliths) tissues of brown trout (*Salmo trutta* Linnaeus, 1758) and their intestinal parasites, acanthocephalans (*Dentitruncus truttae* Sinzar, 1955) from the Krka River influenced by industrial and municipal wastewaters were estimated and compared. The novel multi-indicator approach required the application of advanced analytical techniques, HR ICP-MS for metal measurements in soft tissues and laser ablation system to assess metal content in otoliths and scales. Levels of most elements were higher in acanthocephalans, scales and liver than muscle and otoliths, and therefore indicated differences in metal uptake routes, tissue function and metabolic activity. Despite recorded differences in metal contents, hard and soft fish tissues and acanthocephalans reflected environmental conditions in a similar way. Most elements were accumulated in all investigated tissues in higher levels at the contaminated than at the reference site. Acanthocephalans were confirmed as sensitive bioindicators in metal exposure assessment due to effective metal accumulation capacity, while combination of soft and hard tissues provided extended temporal information on recent to long-term metal exposure. Estimation of wastewater impact was evidenced as moderate metal pollution by all applied indicators and therefore pointed to present but also long term disturbances in the karst Krka River and importance of continuous monitoring and protective actions.

Keywords: otoliths, scales, muscle, liver, Krka River, metal contamination

1. INTRODUCTION

Metals are among the major chemical toxicants polluting the environment due to their prolonged persistence and complex interactions with organisms in aquatic ecosystems (Boyd et al., 2010; Authman et al., 2015; de Paiva Magalhães et al., 2015). Consequently, changes in metal levels can be reflected in aquatic organisms, which serve as biological indicators of metal exposure (Geffen et al., 2003). The advantage of applying indicator organisms and organs is that metals are retained in tissues for longer period than in the water, so biological responses indicate long-term metal variability. That is especially valid for hard tissues, like fish scales and otoliths, which offer a permanent record of metal exposure over fish life span. To date fish hard tissues were mostly applied for stock discrimination (Campana et al., 1994; Milton et al., 2008), movement studies (Tabouret et al., 2012; Prohaska et al., 2016) and only to a lesser extent as environmental indicators of pollution (Sawhney and Johal., 1999; Adami et al., 2001; Saquet et al., 2002; Darafsh et al., 2008; Ranaldi and Gagnon, 2008) which were mostly represented by fish soft tissues, such as liver, gills and muscle (Jarić et al., 2011; Krasnići et al., 2013; Dragun et al., 2018).

Otoliths are calcified structures in the inner ear of teleost fish (Ranaldi and Gagnon, 2008), while scales are composed of a thin, hard, external, well-mineralized layer in which annular structure incorporate metals over time. As covering the surface of the fish body, application of scales in biomonitoring studies represents a nonlethal alternative in metal exposure assessment (Muhlfeld and Marotz, 2005). Since fish calcified structures are acellular and metabolically inert, any elements that incorporate into their surface stay conserved permanently and reflect conditions in the surrounding habitat. In soft tissues, apart from environmental conditions, the influence of fish physiology, mechanisms of elimination of potentially toxic metals and tissue regeneration might have a significant impact on metal levels as well (Bath et al., 2000; Filipović Marijić et al., 2014). Therefore, application of soft tissues requires continuous monitoring and consideration of all these factors which might interfere with responses to metal exposure. In addition, powerful analytical technology, LA ICP-MS enables highly sensitive elemental analysis directly on solid samples, such as calcified structures. Application of acanthocephalans as bioindicators of metal exposure has gained increasing interest due to their effective metal accumulation, orders of magnitude higher than in other commonly used aquatic indicator organisms, such as fish, bivalves and crustaceans (Filipović Marijić et al., 2013, 2014; Sures et al., 2017). So far, few research groups dealing with parasites as indicators of environmental health has mainly been focused

on reporting and comparing metal levels in parasites and other bioindicator organisms (Sures, 2001, 2004; Nachev and Sures, 2016) but studies on their application as indicators in metal exposure assessment are rare (Thielen, 2004, Filipović Marijić et al., 2013, 2014). One of the reasons is high variability of metal levels among parasite individuals, which was explained by fish mobility and different age (Sures et al., 1999; Filipović Marijić et al., 2014).

The studies on application of scales and otoliths in metal exposure assessment of the freshwater ecosystem and their comparison to soft tissues as bioindicators are rare (Darafsh et al., 2008). Thus, the goal of the present study was to assess whether the metal accumulation in hard tissues (scales, otoliths) reflects metal exposure in correspondence to soft tissues (liver, muscle) of brown trout (*Salmo trutta* Linnaeus, 1758) and their intestinal parasites acanthocephalans (*Dentitruncus truttae* Sinzar, 1955). We have chosen 8 elements to measure; Fe, Mg, Mn and Zn as essential elements and Ba, Rb, Sr and Tl as nonessential elements for organisms which was based on results obtained for metal concentrations in water and the fact that one of our main goals was to compare response to metal exposure in soft and hard tissues. Therefore, it was important to use elements which could be measured in (almost) all of these tissues. Further, most of these elements (Ba, Fe, Mg, Mn and Sr) were already found to correlate significantly between the two fish otoliths (Campana et al., 1994; Campana, 2000; Rooker et al., 2001; Huxham et al., 2007). Some toxic elements like Cd or Hg could not be considered due to the lack of available reference data for fish hard tissues which would disable our goal to compare soft and hard tissues. Fish were collected at a reference (river source) and a contaminated site impacted by the wastewater outlets (Krka Knin) of the Krka River, a typical karst river in the Republic of Croatia. Lower part of the Krka River was proclaimed National Park in 1985, but only 2 km upstream of the park borderline technological and municipal wastewaters have a direct impact on the river water.

Since total metal content in tissues do not necessarily represent metabolically available metal fraction, known to be able to cause possible toxic effects, but comprise the complete amount of accumulated metal, our study included both fractions, total metal levels in muscle and metals bound to cytosolic biomolecules in liver, representing the metabolically available metal fraction (Wallace et al., 2003; Rainbow et al., 2011; Urien et al., 2018; Mijošek et al., 2019b). This way, we applied new approach against conventional biomonitoring studies and achieved the following specific objectives: a) evaluation of the fish calcified tissues as tracers of environmental metal exposure, especially scales as noninvasive alternative; b) comparison of metal levels and spatial differences in the river water, fish soft (liver, muscle) and hard tissues (scales, otoliths) and intestinal parasites (acanthocephalans); c) estimation of the

wastewaters impact regarding water, fish and acanthocephalans to get conclusion on the quality status of the karst freshwater ecosystem, Krka River.

2. MATERIALS AND METHODS

2.1. Study area and sampling procedure

The study was conducted in the Krka River watercourse, influenced by two contamination sources, technological wastewaters from the screw factory and municipal wastewaters from the Town of Knin (11,000 inhabitants), which are released without adequate treatment in the river water. Sampling of the river water ($n=3$ per site) and fish was conducted in April 2015 at two locations, reference (Krka River source; $n=18$) and wastewater impacted (Krka Knin, located downstream of the wastewater outlets near the Town of Knin; $n=17$) (Fig. 1). More detailed description of the sampling sites was given by Filipović Marijić et al. (2018) and Sertić Perić et al. (2018).

The river water was collected in triplicates in acid-cleaned polyethylene bottles and immediately filtered through 0.45 μm pore diameter cellulose acetate filter (Sartorius, Germany) mounted on syringes. Aliquots of filtered samples were transferred into acid pre-cleaned 20 mL polyethylene bottles and acidified with concentrated nitric acid (Rotipuran® Supra 69%, Carl Roth, Germany) and stored at +4 °C until metal measurements.

Individuals of brown trout were sampled in April in order to avoid physiology related metal variability during the spawning period, which occurs in the late autumn. Sampling was performed by electrofishing, according to the Croatian standard HRN EN 14011 (2005). The electric field does not kill fish but only temporarily stuns them so captured fish were kept alive in aerated water tank until further processing in the laboratory. This way all fish survived transport to the laboratory and physiological disturbances were minimized.



Figure 1. The map of the Krka River with indicated sampling locations (1 – Krka River source; 2 – Krka Knin) and its position in the Republic of Croatia.

2.2. Dissection of fish tissues

After specimens were anesthetized with freshly prepared anaesthetic tricaine methanesulphonate (MS 222, Sigma Aldrich) in accordance to the Ordinance on the protection of animals used for scientific purposes (NN 55/2013) and sacrificed, fish total length and body mass were recorded. Fish soft tissues (liver and muscle) were dissected and samples were individually stored at $-80\text{ }^{\circ}\text{C}$ for further analyses. Fish intestinal parasites, acanthocephalans, were manually isolated from the intestine using tweezers, counted in each specimen, and stored at $-80\text{ }^{\circ}\text{C}$. Gonads were used for sex determination and calculation of fish gonadosomatic index.

Hard tissues, scales and otoliths, were taken from each fish and stored in small paper bags. Around 15-20 scales were removed from the area closely above the lateral line and below the dorsal fin. The head of the fish was cut off directly behind the gills and the skullcap was opened to remove the saggital otoliths which were cleaned from adherent tissue. Elemental fingerprints are suggested to be consistent between right and left otoliths (Campana et al., 2000), therefore only one otolith per fish was analyzed. Few other studies already confirmed significant correlation between concentrations of elements like Al, Ba, Fe, Mg, Mn and Sr in left and right otoliths of the fish (Campana et al., 1994; Rooker et al., 2001; Huxham et al., 2007).

2.3. Preparation of hepatic cytosolic fraction

Hepatic samples ($n=18$ for the reference and $n=15$ for the contaminated site) were homogenized by addition (w/v 1:5) of cooled homogenization buffer (100 mM Tris-HCl/Base (Merck, Germany, pH 8.1 at 4 °C) supplemented with reducing agent (1 mM dithiotreitol; DTT, Sigma, USA)). Homogenization was performed in ice cooled tubes by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA). Resulting homogenates were centrifuged (Avanti J-E centrifuge, Beckman Coulter, USA) at $50,000\times g$ for 2 h at +4 °C to obtain hepatic soluble cytosolic fractions (Dragun et al., 2018), which were stored at -80°C until further metal analysis.

2.4. Acid digestion of muscle tissue and acanthocephalans

Digestion was performed in a dry oven at 85 °C for 3.5 h, using concentrated HNO₃ (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H₂O₂ (Suprapur®, Merck, Germany) in appropriate volumes for muscle tissues ($n=6$ per site) and acanthocephalans ($n=10$ per site). Considering the low mass of the acanthocephalan specimens, individuals from the same fish were pooled together in order to perform reliable measurements. After digestion, the clear, colourless solutions of muscle and acanthocephalans were left to cool and afterwards were stored at +4°C.

2.5. Hard tissues preparation

Fish otoliths (one sagittus from individual fish, $n=3$ per site) were rinsed and sonicated in Type I reagent-grade water (18 MΩ cm) (F+LGmbH, Vienna, Austria) for 5 minutes and let to dry completely for about an hour. Dried otoliths were placed on small glass slides using adhesive Krazy glue (InstantKrazy Glue Pen, Elmer's Products (Distributor), Westerville, Ohio, USA). After 24 h of hardening, otoliths were carefully ground and polished in small circular movements using lapping films of 30 μm and 3 μm for grinding and polishing (lapping film 266X, 3M™). Polishing remains were removed under the air flow. Scales of the same three fish per site were rinsed and sonicated in tubes in Type I reagent-grade water for 5 minutes and then completely cleaned in Type I reagent-grade water using brush under the light microscope and left to dry. Each sample contained 4-6 scales, which were mounted on small glass slides using two-side adhesive tape. They were afterwards observed under the microscope and one scale with the most visible growth zones was chosen per sample for the subsequent lasering procedure. Selected scales and otoliths were photographed, marked and

stored in small plastic bags until further analysis by laser ablation inductively coupled plasma mass spectrometer (LA ICP-MS).

2.6. Determination of metal content in water, fish soft tissues and acanthocephalans

High resolution ICP-MS (HR ICP-MS, Element 2; Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (ElementalScientific, USA) was used to analyze macro and trace elements in water, acanthocephalans and fish soft tissues. Prior to measurement, river water samples were 10 times diluted with Type I reagent-grade water for the determination of Mg due to its higher levels, whereas trace elements were measured directly in the prepared water samples. Hepatic cytosols were 100 times diluted for Mg and 10 times for trace element analyses, whereas digested muscle and acanthocephalans were 20 times diluted with Type I reagent-grade water for Mg analyses and 5 times for the measurements of trace elements.

Depending on the element, the measurement was operated in low (^{85}Rb and ^{205}Tl) or medium (^{24}Mg , ^{55}Mn , ^{56}Fe , ^{63}Cu , ^{66}Zn , ^{86}Sr , and ^{138}Ba) resolution mode. Multielement 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 as calibration standard for the measurement of macro element Mg. Multielement standard solution for trace elements (Analytika, Czech Republic) supplemented with Rb (Sigma-Aldrich, Germany) was used for the external calibration for the trace element analyses. Indium ($1\text{ }\mu\text{g L}^{-1}$, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to all solutions as an internal standard. The accuracy and the precision of HR ICP-MS measurements was tested using quality control sample for macro-elements (QC Minerals, Catalog number 8052, UNEP GEMS, Burlington, Canada) and for trace elements (QC trace metals, catalog number 8072, UNEP GEMS, Burlington, Canada). Good agreement was observed between certified values and our data, resulting in the following average recoveries obtained during measurements of water, liver, muscle and acanthocephalans samples: Ba: $100.3\pm 2.7\%$, Mg: $95.9\pm 4.7\%$, Mn: $93.9\pm 11.8\%$, Sr: $99.9\pm 3.0\%$, Tl: $102.6\pm 5.5\%$.

2.7. Determination of metal contents in hard tissues

Laser ablation measurements were conducted by connecting a laser ablation system (NWR193, Electro Scientific Industries, Portland, USA) to an HR ICP-MS (Element XR; Thermo Finnigan, Germany). Prior to LA-ICP-MS measurement, the instrument was optimized using the solution set-up in a daily routine for maximum intensity while maintaining low oxid and doubly-charged ion rates. Cross-sectional line scans were taken

through the whole length of the otoliths and scales and going through the core. The time-resolve information of these line scans had been object to a different study. The elements of interest showed minor variations as compared to the relative uncertainty of the LA-ICP-MS measurement (approx. 30%). Given limited habitate changes, average metal mass fractions have been considered to study their accumulation in these hard tissues. Measurement involved isotopes ^{24}Mg , ^{55}Mn , ^{57}Fe , ^{63}Cu , ^{64}Zn , ^{85}Rb , ^{88}Sr , ^{138}Ba , ^{205}Tl and ^{208}Pb according to the following laser instrumental parameters: spot size 100 μm , scan speed 5 $\mu\text{m s}^{-1}$, repetition rate 15 Hz and energy of 50 %. The otolith and scale samples were analyzed within one batch. At the beginning and the end of the batch, the following certified reference materials were ablated: (i) FEBS-1 (Otolith Certified Reference Material for Trace Metals, National Research Council Canada) and MACS-3 (Calcium carbonate standard, United States Geological Survey, 189 USA) were used as reference materials for the calculations of metal concentrations in otoliths. (ii) In addition, NIST SRM 1400 (Bone ash, National Institute of Standards and Technology, Gaithersburg, MD, USA) and NIST SRM 1486 (Bone meal, National Institute of Standards and Technology, Gaithersburg, MD, USA) were used in the case of the fish scales. Nano-pellets of FEBS-1 and MACS-3 were prepared by μ standards according to standard protocols (Garbe-Schönberg and Müller, 2014). In-house pressed reference pellets of NIST SRM 1400 and NIST SRM 1486 were prepared using hydraulic press (10 tons per cm^2). All reference pellets were prepared without addition of any binders. Calcium, as a main element in the aragonite matrix of otoliths (approx. 38 %, Sturgeon et al., 2005) and in the hydroxyapatite matrix in scales (approx. 25 %, Holá et al., 2010), was used as internal standard because it shows only small variations in mass fractions. Final mass fractions in the otolith samples were calculated via MACS-3. FEBS-1 was used as quality control for the quantification. For the final calculation of metal mass fractions in scales, NIST SRM 1400, chosen as most suitable material, was used as a reference material. NIST SRM 1486 was used as quality control for the quantification. Differences in Ca content between the reference material used for calibration and the sample were considered in the calibration strategy. Good agreement was observed between certified values and our data, resulting in the following average recoveries obtained during LA-ICP-MS measurements of otoliths and hydroxyapatites samples and were within measurement uncertainties: Ba: 97 \pm 15%, Mg: 108 \pm 29%, Mn:89 \pm 31%, Rb: 121 \pm 18%, Sr:126 \pm 37%, Zn: 123 \pm 33%.

2.8. Data processing and statistical analyses

Biological data

Fulton condition index was calculated as: $FCI = (W / L^3) \times 100$ (Ricker, 1975), hepatosomatic index as: $HSI = (LW / W) \times 100$ (Heidinger and Crawford, 1977), and gonadosomatic index as: $GSI = (GW / W) \times 100$ (Wootton, 1990), where W is the body mass (g), L is the total length (cm), LW is the liver mass (g) and GW is the gonad mass (g).

Level of parasite infection was quantified by calculating prevalence (the percentage of infected fish) and mean intensity (parasite number per host individual) according to Bush et al. (1997).

Chemical data

Metal levels were presented as mean values \pm standard deviations for all types of samples and expressed according to IUPAC terminology: mass fraction ω ($\mu\text{g g}^{-1}$) and mass concentration γ (g L^{-1}). Therefore, elemental contents in water samples were expressed as mass concentration ($\mu\text{g L}^{-1}$), and as mass fraction ($\mu\text{g g}^{-1}$) in fish hard structures (dry mass) and in soft parts (wet mass, w.m.). In muscle and acanthocephalans, total metal contents were measured, while the biologically available metal fraction was quantified in the cytosols of fish hepatic tissues.

For the purpose of comparison of soft and hard tissues, metal contents in soft tissues were expressed as $\mu\text{g g}^{-1}$ of dry mass (d.m.) using the respective ratios between the wet and dry tissue masses. For the muscle tissue we applied factor of 4.75 based on the mean value of wet:dry ratios in muscles of 8 freshwater fish species from more than 20 locations in Croatia (Mikac et al., 2018). Considering hepatic samples, the mean value of 5.4 was used as reported for the brown trouts from Asturian rivers in Spain (5.14-6.83) (Linde et al., 1998), while for acanthocephalans conversion factor of wet:dry mass of 4.3 was determined based on 4 independent measurements during this study.

Bioconcentration factors (BCFs) were determined according to Sures et al. (1999), as the ratio of the element content in the parasites and the host tissue.

For the calculation of mean metal content in otoliths and scales, data were accumulated through the whole lines of the structures. Starting and ending point of the lines were determined by the inspection of Ca and Sr intensities to prevent the “edge effect” and to assure that all analyzed points were within the scale/otolith area.

Statistics

SigmaPlot 11.0 (Systat Software, USA) for Windows was used for statistical analysis and creation of graphs. Nonparametric statistical tests were used, because assumptions of

normality and homogeneity of variance were not always met. Variability of metal levels between two data sets was tested by Mann-Whitney U test and statistically significant differences in metal concentrations between the two locations at $p < 0.05$, $p < 0.01$ or $p < 0.001$ level were indicated.

3. RESULTS AND DISCUSSION

3.1. Biometric characteristics of *S. trutta* sampled in the Krka River

There were no significant differences between biometric data of fish from the two sampling sites, although mean fish total length and body mass were higher at the contaminated (24.4 cm, 339.4 g) than those at the reference location (20.4 cm, 114.4 g) (Table 1). Such trend might be related to the presence of more organic matter and food sources downstream of the wastewater outlets (Couture and Rajotte, 2003). The opposite trend was observed for gonadosomatic and Fulton condition indices, which were slightly higher at the reference than contaminated site, but not significantly. Usually, decreased values of FCI can indicate the need for inducing additional defense mechanisms in fish, which requires a lot of energy and consequently lowers the fish condition. Trend of lower FCI values in fish from the metal polluted locations was already observed, for example in wild yellow perch *Perca flavescens* (Couture and Rajotte, 2003) and Prussian carp *Carassius gibelio* (Zhelev et al., 2016, 2018). However, in our study the impact of present contamination on fish condition was not significant.

Total number, prevalence and mean intensity of infection with acanthocephalans were higher in fish from the reference than those from the contaminated site (Table 1). Altogether, 685 acanthocephalans were isolated from the intestines of 17 infected fish individuals from the Krka River source, and 417 from 13 individuals from the Krka Knin. Accordingly, prevalence of 94% and 76% and mean intensity of infection of 40.3 and 32.1 were recorded at the Krka River source and at the Krka Knin, respectively (Table 1). Such trend was in accordance with the sampling campaigns conducted in autumn 2015 and spring 2016 at the same research sites of the Krka River, when prevalence of 83-100% was reported (Mijošek et al., 2020). Vardić Smrzlić et al. (2013) reported average prevalence of 73% for *D. truttae* in brown trouts from the few sites along the Krka River during 11 sampling campaigns from 2005 to 2008. In Italy, prevalence of *D. truttae* in *S. trutta* from the Tirino River was comparable to our results (90.9–100%) (Paggi et al., 1978), as well as the prevalence (81.2%) and the mean intensity of infection (46.2) in brown trouts from the Lake Piediluco (Dezfuli et

al., 2008). Generally, it was reported that decrease in species richness, as well as the abundance of endoparasites with indirect life cycles, including acanthocephalans, might indicate pollution impact and stressful environmental conditions (Marcogliese, 2004). In our research, the decrease of around 20% at the wastewater impacted site indicated moderate pollution, as already reported regarding the physico-chemical water parameters and dissolved metal levels (Filipović Marijić et al., 2018; Sertić Perić et al., 2018).

Table 1. Biometric characteristics (mean± S.D.) of *S. trutta* caught in the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka Knin) and epidemiological characteristics of acanthocephalans *D. truttae* hosted in *S. trutta*: prevalence (number and percentage of infected fish), mean intensity of infection (average ±S.E.) and total number of parasite individuals.

	Krka River source <i>n</i> =18	Krka Knin <i>n</i> =17
Point sources of pollution	Reference site- unknown pollution sources	Contaminated site- screw factory, industrial and municipal wastewaters, agricultural runoff
Total length (cm)	20.4±4.2	24.4±13.5
Body mass (g)	114.4±81.2	339.4±601.4
HSI (%)	1.3±0.5	1.3±0.6
GSI (%)	0.37±0.24	0.25±0.16
FCI (g cm ⁻³ *100)	1.2±0.4	1.1±0.3
Sex (M/F/ND*)	9/9/0	5/10/2
Prevalence (number and % of trouts infected with parasites)	17; 94%	13; 76%
Mean intensity of infection (mean ± S.E.)	40.3±8.9	32.1±11.3
Total number of parasite individuals in sampled fish	685	417

* ND - gender not determined

3.2. Metal content in the river water

Spatial variability of dissolved metal levels in the river water indicated higher metal levels at the Krka Knin than at the river source for the most of the measured metals, being significant for Ba, Fe, Mn, Rb, Sr and Zn (Table 2). Exceptions were Mg and Tl, which levels were higher at the reference location. Dissolved metal levels in the Krka River water followed the order at the reference site: Sr>Mg>Ba>Zn>Fe>Rb>Mn>Tl and at the anthropogenically

impacted location Krka Knin: Sr>Zn>Fe>Mg>Mn>Ba>Rb>Tl, indicating higher presence of metals often used in industrial manufacturing (Zn, Fe, Mn) downstream of the screw factory. Iron, Mn and Zn were among elements with the most pronounced difference between the two investigated sites, with Mn being 673, Fe 34 and Zn 9 times higher at the contaminated than the reference site (Table 2). Such high metal increase could be related to metal production facility, as Fe and Mn are often used in the manufacture of iron and steel alloys, and manganese compounds, respectively (WHO, 2011; Sertić Perić et al., 2018). Zinc is one of the most commonly used metals in the world due to its reducing and anti-corrosive properties, so it has a high importance in the industrial production. Therefore, we have included the same elements in the analysis of biological samples, either due to their toxicity, essentiality or interesting patterns in water samples. Despite recorded spatial differences, metal contents of the Krka River water were still comparable with the other karst ecosystems (Dossi et al., 2007; Cukrov et al., 2008) and, according to Filipović Marijić et al. (2018) and Sertić Perić et al. (2018), mostly below environmental quality standards and metal concentrations in the other rivers of technological or rural catchments. However, disturbances of environmental conditions were obvious at the contaminated site Krka Knin regarding conductivity, chemical oxygen demand, levels of ammonium, total nitrogen, total phosphorus, nitrate and bacteria counts, which have not satisfied the requirements for the good water quality status (Filipović Marijić et al., 2018). As already described, such karst ecosystems are characterized by the effective self-purification process, which reduce the effect of pollution impact in the Krka River (Cukrov et al., 2008). Therefore, metal levels at the location impacted by the wastewater outlets indicated obvious metal input but could be considered as indication of moderate pollution impact.

Table 2. Elemental content (mean \pm S.D.) in the water ($\mu\text{g L}^{-1}$) of the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka Knin).

	Krka River source	Krka Knin
Ba ($\mu\text{g L}^{-1}$)	4.38 \pm 0.11*	5.69 \pm 0.10*
Fe ($\mu\text{g L}^{-1}$)	0.340 \pm 0.060*	11.62 \pm 1.89*
Mg ($\mu\text{g L}^{-1}$)	11630 \pm 130*	11100 \pm 180*
Mn ($\mu\text{g L}^{-1}$)	0.010 \pm 0.004*	6.73 \pm 0.10*
Rb ($\mu\text{g L}^{-1}$)	0.280 \pm 0.005*	0.460 \pm 0.001*
Sr ($\mu\text{g L}^{-1}$)	88.42 \pm 1.72*	186.2 \pm 1.0*
Tl ($\mu\text{g L}^{-1}$)	0.006 \pm 0.000	0.005 \pm 0.000
Zn ($\mu\text{g L}^{-1}$)	3.57 \pm 0.62*	30.03 \pm 4.53*

Statistically significant differences (Mann-Whitney U test, $p < 0.05$) between the two sites are assigned with asterisk (*).

3.3. Metal content in fish soft tissues and acanthocephalans

Due to the dynamic nature of aquatic environments, metal concentrations in water may significantly vary over time, so extent of metal exposure was further evaluated on aquatic organisms, applying commonly used fish liver and muscles and rarely used intestinal parasites, acanthocephalans as bioindicators. Liver was selected as the main metabolic and detoxification site in the organism, which might reflect chronic exposure to metals (Linde et al., 1998; Krasnići et al., 2013), whereas muscle, due to its importance in human consumption, might represent a health risk for humans in case of the elevated metal content (Carvalho et al., 2005; Jarić et al., 2011). Spiny-headed worms, acanthocephalans, are already described as organisms of rapid and high metal accumulation capacity, which is an order of magnitude higher than in the other aquatic organisms (Sures et al., 1999; Nachev and Sures, 2016; Sures et al., 2017). Due to high variability of metal levels among parasite individuals, their application as bioindicators in metal exposure assessment is still under question (Sures, 2004; Sures et al., 2017).

In our study, the pattern of higher metal levels at the contaminated than the reference site was observed for Sr, Fe, Zn, Mn and Ba in liver, Mg, Mn, Ba, Rb, Sr and Zn in muscle and Zn, Fe, Sr, Mg, Mn and Ba in acanthocephalans (Table 3). The exceptions were Fe, Rb and Tl, showing around 1.6 higher Fe values in muscle of fish from the reference than contaminated site, 1.2-1.8 times higher Tl in soft tissues and acanthocephalans and 1.3 times higher Rb in liver and acanthocephalans (Table 3). Other studies also conducted in the Krka River confirmed elevated Rb and Tl contents, as well as Cs and Cd, in liver (Dragun et al., 2018) and the intestine of brown trouts and gammarids from the river source than downstream locations (Mijošek et al., 2019a, 2019b). Although higher metal accumulation in organisms could be an indication of higher exposure level in the water, in our research, levels of Tl were comparable, and of Rb and Fe even significantly higher in the water at the contaminated than the reference site (Table 2). Therefore, the cause of the significantly higher content of these few elements in fish from the reference site could not simply be explained by waterborne uptake and requires further research considering river sediment and food as possible metal sources, i.e. dietborne metal uptake, which can even be a major route of exposure to some metals, including Tl (Clearwater, 2000; Lapointe and Couture, 2009).

In general, contents of analyzed metals mostly followed the comparable order in both soft tissues and parasites, with the highest levels of Mg, Fe, Zn and Rb and the lowest of Ba and Tl. The exceptions were the lowest Sr contents in hepatic samples and lower Rb accumulation in acanthocephalans (Table 3). Regarding metal levels in fish parasites, their

values were much more variable compared to fish soft tissues (Table 3), confirming previous findings that metal contents among acanthocephalan individuals show high variability, possibly as a result of host mobility, different age and consequently different exposure times (Sures et al., 1999; Filipović Marijić et al., 2014). In addition, our results confirmed that metal levels in acanthocephalans were mostly higher than those in the fish soft tissues, as already reported in many studies involving parasites and fish liver, intestine, kidney or muscle (Sures et al., 1999; Filipović Marijić et al., 2013; Nachev and Sures, 2016; Sures et al., 2017). That was confirmed by bioconcentration factors (BCFs), which were calculated to express the relation of metal concentrations in acanthocephalans and two host tissues (Fig. 1). High ratio between metal levels in acanthocephalans and fish tissues indicates more recent increase in metal exposure, whereas low ratio (comparable metal levels in both organisms) points to longer and continuous exposure (Sures et al., 1999; Filipović Marijić et al., 2013). That is based on the fact that metal accumulation is faster in parasites due to their shorter life span of 50–140 days (Kennedy, 1985), compared to the average life span of fish which usually ranges from 10-15 years (Kottelat and Freyhof, 2007). Following lower contents of most elements in muscle than liver of brown trout, BCFs were higher for all metals with respect to muscle, except for Mg and Sr (Fig. 2). Overall, the highest values of BCFs were observed for Sr (88-96) and Tl (7-8) regarding liver (Fig. 2a) and for Tl (93-119) and Zn (19-58) regarding muscle (Fig. 2b). It means that in our research BCFs pointed to possible recent increase in Mn, Sr, Tl and Zn exposure, opposite to more continuous exposure of Rb, Ba, Fe and Mg (Fig. 2). Further, BCF values were higher at the contaminated than reference site for most elements in both liver and muscle (Fig. 2), suggesting even faster and recent accumulation in acanthocephalans exposed to anthropogenic impact which confirmed their potential as sensitive bioindicators of bioavailable metal levels and rapid response to metal changes in the environment. Sampling campaigns during autumn 2015 and spring 2016 also indicated high BCFs of Tl with respect to the muscle in fish from the same area as in our study, ranging 49-112 depending on the site and season (Mijošek et al., 2020). To our knowledge, there is no other literature data available regarding BCFs on this acanthocephalan (*D. truttae*) and fish (*S. trutta*) species, but studies using other freshwater species reported comparable BCFs ranges for many elements (reviewed in Sures, 2004 and Sures et al., 2017).

Estimation of spatial variability of metals in fish soft tissues and in intestinal parasites revealed similar trends and indicated that not only total metal burden but also cytosolic metal levels might be useful indicators of metal exposure in the aquatic environment, while

acanthocephalans were shown as effective metal accumulators which reflect biologically available metal fraction absorbed from fish intestine over their whole lifespan.

Table 3. Elemental content (mean \pm S.D., $\mu\text{g g}^{-1}$ w.m.) in soft tissues (liver, muscle) and intestinal parasites (acanthocephalans) of brown trout from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka Knin).

	Liver		Muscle		Acanthocephalans	
	Krka River source	Krka Knin	Krka River source	Krka Knin	Krka River source	Krka Knin
Ba	0.311 \pm 0.088	0.365 \pm 0.109	0.013 \pm 0.001*	0.021 \pm 0.08*	0.325 \pm 0.160*	0.592 \pm 0.255*
Fe	27.79 \pm 9.25	33.60 \pm 13.71	4.74 \pm 1.42*	2.98 \pm 0.62*	13.04 \pm 3.75***	50.70 \pm 26.05***
Mg	93.02 \pm 5.36	91.56 \pm 8.15	300.3 \pm 14.2*	328.8 \pm 17.8*	197.9 \pm 21.9	235.2 \pm 61.4
Mn	0.716 \pm 0.115	0.785 \pm 0.124	0.111 \pm 0.024	0.170 \pm 0.090	3.25 \pm 0.30	4.56 \pm 2.02
Rb	4.93 \pm 2.02	3.73 \pm 1.79	4.12 \pm 0.40	4.13 \pm 1.32	2.27 \pm 0.37*	1.74 \pm 0.59*
Sr	0.013 \pm 0.010*	0.037 \pm 0.048*	0.079 \pm 0.089	0.189 \pm 0.204	1.15 \pm 0.92**	3.50 \pm 2.07**
Tl	0.192 \pm 0.099	0.164 \pm 0.096	0.016 \pm 0.005*	0.009 \pm 0.005*	1.49 \pm 1.22	1.07 \pm 0.83
Zn	19.44 \pm 9.2	20.72 \pm 5.18	2.11 \pm 0.60	2.23 \pm 0.84	39.95 \pm 14.38*	128.6 \pm 151.9*

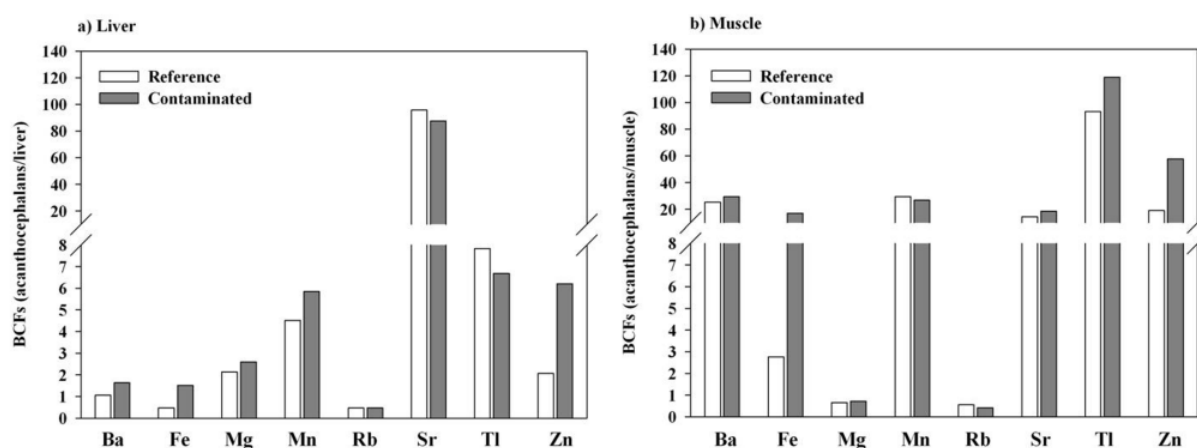


Figure 2. Bioconcentration factors for *Dentitruncus truttae* calculated with respect to the soft tissues of the brown trout from the Krka River: a) liver and b) muscle.

3.4. Metal content in fish scales and otoliths

To get an insight into the long-term metal exposure, metal levels were measured in fish scales and otoliths using LA-ICP-MS, recognized as the most representative technique for precise analysis of several trace elements in solid samples at the same time, while measurements across laser lines give information on pollution exposure over the whole life history of a fish (Zitek et al., 2010; Prohaska et al., 2016). In the present pilot-study, for the first time conducted in freshwater fish from the Croatian rivers, the focus is put on the

estimation of soft and hard tissue responses to the actual industrial and municipal contamination in the Krka River.

Fewer elements were reported for fish scales (Mg, Sr, Zn, Fe, Mn) than otoliths (Sr, Zn, Mg, Mn, Rb, Ba, Tl) due to the lack of suitable reference material. Those reported in both scales and otoliths had mostly higher levels in fish scales (Table 4), in accordance to few other studies (Wells et al., 2000; Ramsay et al., 2011; Kalantzi et al., 2019). The largest difference between the two hard tissues was observed for Mg with around 53 times higher values in scales than in otoliths at both locations, followed by 10-13 times higher Mn and around 4 times higher Zn levels in scales (Table 4). Similar or even higher differences in Mg and Mn levels between scales and otoliths were reported by Wells et al. (2003) for westslope cutthroat trout from the Coeur d'Alene River. Given the integral role of Mg in apatite formation and its significant amount in biologic hydroxyapatite, high values are expected to appear in scales (Bigi et al., 1992; Kalvoda et al., 2009). Mean Mg values of around 4000 $\mu\text{g g}^{-1}$ found in the scales of the brown trout in our research are comparable with the values reported for the scales of grass carp, common carp, and tench from the Czech Republic, and about two times higher than those reported in European perch (Holá et al., 2009, 2011; Kalvoda et al., 2009). Higher contents of Mn in fish scales than in otoliths were also consistent with the high affinity of both synthetic and biogenic apatites for Mn (Bosticket al., 2003; Wells et al., 2000). Higher Mn values than in brown trout from our research (9.26-20.81 $\mu\text{g g}^{-1}$) were reported in the scales of other freshwater fish species (80-450 $\mu\text{g g}^{-1}$) (Holá et al., 2009, 2011). Further, higher metal burden in the scales in general could be due to their direct contact with water, possibly increasing the direct metal uptake, as well as due to known high ion-exchange properties of hydroxyapatite structures for metals and radionuclides and different metal uptake (Goto and Sasaki, 2014). Opposite trend between the calcified structures of brown trout from the Krka River was visible for Sr content with 4 times higher values obtained in otoliths than scales (Table 4), as already observed in different studies (Ramsay et al., 2011; Kalantzi et al., 2019), probably due to the significant chemical association of Sr with Ca, which is the main component of otoliths (Campana, 1999).

In both scales and otoliths, mean levels of Sr, Mn and Zn tended to be higher at the contaminated than the reference site, Sr and Mn around 2-2.5 and Zn around 1.5 times, whereas in otoliths Rb and Tl were around 1.5 times higher at the reference site (Table 4), but lack of significant differences is probably a consequence of only 3 samples per site. Hard tissues mostly confirmed patterns recorded in fish soft tissues and acanthocephalans (Table 3). As in the case of soft tissues, resulting trends might be connected with significantly elevated

Fe, Mn, Zn and Sr contents in the water samples from the contaminated site, whereas some other metal sources, and not the water, should be considered at the river source as a cause of the increased Tl and Rb levels in the otoliths. However, due to the limited number of samples in our study, significant spatial or tissues-specific differences were hard to detect, especially due to the variability of metal levels observed in both hard tissues. Differences in metal accumulation in fish calcified structures was explained by the differences in ion precipitation, which are in scales incorporated into the crystal lattice from the blood (Veinott et al., 1999), while ions precipitate in otoliths directly from the endolymph fluid (Campana, 1999), which shows less variations in ion levels than the blood (Campana et al., 2000). Considering metal variability in our study, both calcified structures showed relatively high variability between fish individuals, as already stated in some other studies (Wells et al., 2003; Ramsay et al., 2011). Variability was especially emphasized for Mn and Sr contents (Table 4), although in our research such finding should be considered with caution due to the small number of samples and further investigation is required.

Table 4. Elemental content (mean \pm S.D.) in hard tissues (scales, otoliths; $\mu\text{g g}^{-1}$) of brown trout from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: Krka Knin).

	Scales		Otoliths	
	Krka River source	Krka Knin	Krka River source	Krka Knin
Ba	n.d.	n.d.	0.910 \pm 0.220	0.980 \pm 0.100
Fe	69.14 \pm 27.94	76.60 \pm 38.12	n.d.	n.d.
Mg	4300 \pm 469	4094 \pm 317	80.75 \pm 2.29	77.51 \pm 6.17
Mn	9.26 \pm 3.14	20.81 \pm 10.16	0.860 \pm 0.210	1.57 \pm 0.81
Rb	n.d.	n.d.	1.64 \pm 0.68	1.23 \pm 0.46
Sr	87.84 \pm 22.76	205.7 \pm 74.4	335.0 \pm 56.6	789.2 \pm 563.3
Tl	n.d.	n.d.	0.400 \pm 0.060	0.230 \pm 0.020
Zn	72.15 \pm 25.36	113.8 \pm 29.8	20.97 \pm 7.83	26.43 \pm 6.85

n.d. – not determined

In connection to our specific goals on the hard tissues application, we confirmed mostly higher metal accumulation capacity of scales compared to otoliths as in the few other studies (Wells et al., 2000; Ramsay et al., 2011; Kalantzi et al., 2019). Using scales rather than otoliths for the analysis could be advantageous for several reasons: elemental levels are generally higher which reduces errors and increases the precision of measurements, especially for the elements present in very low contents; they are easy to collect and present a nonlethal

alternative which is especially beneficial if investigating some rare and endangered species; their preparation for the measurement on LA ICP-MS is easier and quicker and due to the scale growth, does not require grinding procedure, which reduces the possibility of contamination and false element presence from the grinding material. However, there is one analytical and one biological disadvantage that need to be overcome to completely and successfully apply scales in metal exposure assessments. The analytical problem refers to the lack of completely suitable reference material which would enable precise calculations of contents of more metals in scales, although in the literature different approaches with NIST 1400, NIST 1486, NIST 610, NIST 612 or NIST 613 reference materials were used (Clarke et al., 2007; Holá et al., 2008; Ramsay et al., 2011). From the biological point of view, regeneration and resorption of scales in case of injuries or scale removal, represent the possible problem as such scales are distinguishable only under the microscope which is not always available and practical at the field sampling. These new, regenerated scales then do not show concentric pattern in the middle, and consequently cannot be used in time resolved manner because they do not represent the information on the whole life history. Nevertheless, even regenerated scales may be useful in interpreting environmental changes over a recent, short time span if the age of regenerated scale is known (Hammond and Savage, 2009).

3.5. Comparison of metal accumulation in soft and hard fish tissues and acanthocephalans

Our final approach was to compare metal accumulation capacities among all used tissues of brown trout, liver, muscle, otoliths and scales, and their intestinal parasites, acanthocephalans. For that purposes, the results of metal contents in soft tissues and acanthocephalans were expressed as $\mu\text{g g}^{-1}$ d.m. to be comparable with hard tissue metal levels, using the conversion factors on wet:dry ratios from our previous research and the literature data as already explained in Materials and Methods section. Based on this approach, it was obvious that metals showed the most efficient accumulation in acanthocephalans and liver (Fe, Zn, Rb, Tl, Ba), followed by fish scales (Zn, Mg, Mn), whereas in muscle and otoliths the accumulation was lower, except of Sr in hard tissues and Mg and Rb in muscle.

As already stated, acanthocephalans are known as organisms of high accumulation capacity, while liver plays a main role in metal metabolism, uptake, storage and detoxification/elimination processes (Linde et al., 1998; Kalantzi et al., 2019). In contrast, fish muscle represents tissue with the low metabolic rate (Jarić et al., 2011; Nachev and Sures, 2016), resulting in the lowest metal levels. Further, large mass of muscle in comparison with the total fish mass contributes to the dilution of metals when compared to the other organs

such as liver (Kalantzi et al., 2019). Scales showed the highest accumulation of Mg and Mn, in accordance to the study of Kalantzi et al. (2019) who investigated elemental distribution in the different tissues of brood stock from Greek hatcheries. Magnesium is a major constituent of hard structures, whereas Mn is known to be mainly accumulated in bone tissues (Wells et al., 2000). The research of Kalantzi et al. (2019) is, to our knowledge, the only similar research on the distribution of metals and elements in the different soft and hard tissues of freshwater fish species and comparable to our research, results indicated higher metal contents in the liver, kidney, bone and scales compared to the other body tissues and organs (gills, gonads, otoliths, stomach and muscle) of brood stocks. Rubidium, which was relatively high in muscle in our research, was also elevated in the muscles of brood stocks compared to other tissues (Kalantzi et al., 2019). Otoliths were also found to contain the lowest levels of most elements, except highly dominant Sr. Although otoliths are calcified tissues as well, they are mostly composed of aragonite which has a lower metal affinity than apatite structures (Adey et al., 2009; Goto and Sasaki, 2014; Kalantzi et al., 2019).

4. CONCLUSIONS

Despite observed variability in metal levels and their accumulation patterns, which is dependent on tissue function, the uptake route and metabolism, ecological needs and physiology of fish, as well as chemistry of each element comparison of spatial metal differences in all tissues revealed similar trends. Mostly higher Mn, Fe and Zn contents confirmed the impact of the screw factory near the contaminated site, since these elements are often used in that type of industry, while Mg, Rb and Tl were mostly higher at the reference site, pointing to the importance of considering not only the waterborne but also dietborne uptake routes. Therefore, hard tissues reflected the influence of metal contamination from different environmental sources such as water, food or sediments in correspondence to the fish soft tissues and fish intestinal parasites, and might be considered as valuable traces of environmental pollution exposure.

However, there were differences in accumulation efficiency, acanthocephalans accumulated Ba, Tl and Zn most effectively compared to the fish tissues representing the combination of short- and long- term metal exposure, which confirmed their great potential and value as bioindicators in metal exposure assessment. Liver as the main metabolically active and detoxifying fish organ, showed the highest accumulation of Fe and Rb, whereas Mg and Mn accumulated mostly in scales. On the other hand, metal accumulation, except for

Sr, was the lowest in muscle or otoliths, both considered as more metabolically inert tissues. Hence, acanthocephalans accumulated most metals more effectively than the fish soft tissues and therefore were shown as rapid and sensitive indicators of bioavailable metal levels and their changes in the environment. Possible application of scales as nonlethal tool in monitoring programmes showed strengths, as high accumulation rate and easy handling, but also a high variability in metal levels between the scales and the lack of reliable reference materials to calibrate concentrations of more elements.

Altogether, moderately elevated metal accumulation was obvious in hard (scales, otoliths), but also soft tissues (liver, muscle) of brown trouts, especially from the contaminated site Krka Knin. It indicated recent and still present, but also longer and continuous metal contamination of the Krka River, which was also supported by BCFs values of Rb, Ba, Fe and Mg. Altogether, metal contents in biota and water of the Krka River still showed moderate contamination, but differences between the sites pointed to existing disturbances at location near the Town of Knin and showed the need of strict monitoring of this sensitive area.

5. ACKNOWLEDGMENTS

This work was supported by the Adris Foundation for the project “Evaluation of the Krka River water quality and potential risk to the Krka National Park by application of new bioindicators and biomarkers“ and Croatian Science Foundation (project no. IP-2014-09-4255 “AQUAMAPMET”). Croatian-Austrian Bilateral Project “Investigation of novel bioindicators of metal exposure in the aquatic ecosystems: fish intestinal parasites, cell cytosol and calcified structures” and CEEPUS scholarship programme are acknowledged for the scientific collaboration and scholarship stay at the BOKU University. We acknowledge the support by the COMET-K1 competence centre FFoQSI funded by the Austrian ministries BMVIT, BMDW and the Austrian provinces Niederoesterreich, Upper Austria and Vienna within the scope of COMET—Competence Centers for Excellent Technologies. The programme COMET is handled by the Austrian Research Promotion Agency FFG. Special thanks are due to Christine Opper, Melanie Diesner and Anastassiya Tchaikovsky for the technical support and checking the data and calculations on the hard tissues.

6. REFERENCES

- Adami, G., Miletić, M., Siviero, P., Barbieri, P., Reisenhofer, E., 2001. Metal contents in tench otoliths: relationships to the aquatic environment. *Ann Chim* 91, 401-408.
- Adey, E.A., Black, K.D., Sawyer, T., Shimmield, T.M., Trueman, C.N., 2009. Scale microchemistry as a tool to investigate the origin of wild and farmed *Salmo salar*. *Mar Ecol Prog Ser* 390, 225–235.
- Authman, M.M.N., Zaki, M.S., Khallaf, E.A., Abbas, H.H., 2015. Use of fish as bioindicator of the effects of heavy metals pollution. *J Aquac Res Development* 6, 328-340.
- Bath, G. E., Thorrold, S.R, Jones, C.M, Campana, S.E., McLaren, J.W., Lam, J.W.H., 2000. Strontium and barium uptake in aragonitic otoliths of marine fish. *Geochim Cosmochim Acta* 64, 1705–1714.
- Bigi, A., Foresti, E., Gregorini, R., Ripamonti, A., Roveri, N., Shah, J.S., 1992. The role of magnesium on the structure of biological apatites. *Calcif Tissue Int* 50, 439–444.
- Bostick, W.D., Stevenson, R.J., Harris, L.A., Peery, D., Hall, J.R., Shoemaker, J.L., Jarabek, R.J., Munday, E.B., 2003. Use of apatite for chemical stabilization of subsurface contaminants. Tech Rep submitted to U.S. Department of Energy, National Energy Technology Laboratory.
- Boyd, R.S., 2010. Heavy Metal Pollutants and Chemical Ecology: Exploring New Frontiers. *J Chem Ecol* 36, 46-58.
- Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets ecology on its own terms: Margolis *et al.* revisited. *J Parasitol* 83, 575–583.
- Campana, S.E., Fowler, A.J., Jones, C.M., 1994. Otolith elemental fingerprinting for stock identification of Atlantic cod (*Gadus morhua*) using laser ablation ICP MS. *Can J Fish Aquat Sci* 51, 1942-1950.
- Campana, S.E., 1999. Chemistry and composition of fish otoliths: pathways, mechanisms and applications. *Mar Ecol-Prog Ser* 188, 263–297.
- Campana, S.E., Chouinard, G.A., Hanson, J.M., Frechet, A., Bratney, J. 2000. Otolith elemental fingerprints as biological tracers of fish stocks. *Fish Res* 46, 343–357.

- Carvalho, M.L., Santiago, S., Nunes, M.L., 2005. Assessment of the essential element and heavy metal content of edible fish muscle. *Anal Bioanal Chem* 382, 426-432.
- Clarke, A.D., Telmer, K.H., Shrimpton, J.M., 2007. Elemental analysis of otoliths, fin rays and scales: a comparison of bony structures to provide population and life-history information for the Arctic grayling (*Thymallus arcticus*). *Ecol Freshw Fish* 16, 354-361.
- Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. *J Exp Biol* 203, 2455-2466.
- Couture, P., Rajotte, J.W., 2003. Morphometric and metabolic indicators of metal stress in wild yellow perch (*Perca flavescens*) from Sudbury, Ontario: a review. *J Environ Monit* 5, 216-221.
- Cukrov, N., Cmuk, P., Mlakar, M., Omanović, D., 2008. Spatial distribution of trace metals in the Krka River, Croatia. An example of the self-purification. *Chemosphere* 72, 1559-1566.
- Darafsh, F., Mashinchian, A., Fatemi, M., Jamili, S. 2008. Study of the application of fish scale as bioindicator of heavy metal pollution (Pb, Zn) in the *Cyprinus carpio* of the Caspian Sea. *Res J Environ Sci* 2 (6), 438- 444.
- de Paiva Magalhães D., da Costa Marques M.R., Baptista D.F., Buss, D.F., 2015. Metal bioavailability and toxicity in freshwaters. *Environ Chem Lett* 2015, 69-87.
- Dezfuli, B.S., Giovinazzo, G., Lui, A., Giari, L., 2008. Inflammatory response to *Dentitruncus truttae* (Acanthocephala) in the intestine of brown trout. *Fish Shell fish Immunology* 24, 726-733.
- Dossi, C., Ciceri, E., Giussani, B., Pozzi, A., Galgaro, A., Viero, A., Vigano, A., 2007. Water and snow chemistry of main ions and trace elements in the karst system of Monte Pelmo massif (Dolomites, Eastern Alps, Italy). *Mar Freshw Res* 58, 649-656.
- 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. *Ecotoxicol Environ Saf* 147, 537-549.

Filipović Marijić, V., Vardić Smrzlić, I., Raspor, B., 2013. Effect of acanthocephalan infection on metal, total protein and metallothionein concentrations in European chub from a Sava River section with low metal contamination. *Sci Total Environ* 463–464, 772–780.

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.

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. *Environ. Sci Pollut Res* 25, 4715–4727.

Garbe-Schönerg, D., Müller, S., 2014. Nano-particulate pressed powder tablets for LA-ICP-MS. *J Anal At Spectrom* 29, 990-1000.

Geffen, A.J., Jarvis, K., Thorpe, J.P., Leah, R.T., Nash, R.D.M., 2003. Spatial differences in the trace element concentrations of Irish Sea plaice *Pleuronectes platessa* and whiting *Merlangius merlangus* otoliths. *J Sea Res* 50, 247–254.

Goto, T., Sasaki, K., 2014. Effects of trace elements in fish bones on crystal characteristics of hydroxyapatite obtained by calcination. *Ceram Int* 40(7), 10777-10785.

Hammond, M.P., Savage, C., 2009. Use of regenerated scales and scale marginal increments as indicators of recent dietary history in fish. *Estuar Coast* 32(2), 340-349.

Heidinger, R.C., Crawford, S.D., 1977. Effect of temperature and feeding rate on the liver-somatic index of largemouth bass, *Micropterus salmoides*. *J Fish Res Board Can* 34, 633–638.

Holá, M., Kalvoda, J., Bábek, O., Brzobohatý, R., Holoubek, I., Kanický, V., Škoda, R., 2009. LA-ICP-MS heavy metal analyses of fish scales from sediments of the Oxbow Lake Certak of the Morava River (Czech Republic). *Environ Geol* 58, 141–151.

Holá, M., Kalvoda, J., Nováková, J.H., Škoda, R., Kanický, V., 2011. Possibilities of LA-ICP-MS technique for the spatial elemental analysis of the recent fish scales: Line scan vs. depth profiling. *Appl Surf Sci* 257, 1932–1940.

HRN EN 14011, 2005. Fish Sampling by Electric Power [Uzorkovanje riba električnom strujom].

<https://iupac.org/wp-content/uploads/2019/05/IUPAC-GB3-2012-2ndPrinting-PDFsearchable.pdf>

Huxham, M., Kimani, E., Newton, J., Augley, J., 2007. Stable isotope records from otoliths as tracers of fish migration in a mangrove system. *J Fish Biol* 70, 1554-1567.

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. *Microchem J* 98, 77-81.

Kalantzi, I., Mylona, K., Pergantis, S.A, Coli, A., Panopoulos, S., Tsapakis, M., 2019. Elemental distribution in the different tissues of brood stock from Greek hatcheries. *Aquaculture* 503, 175-185.

Kalvoda, J., Novak, M., Bábek, O., Brzobohatý, R., Holá, M., Holoubek, I., Kanický, V., Škoda, R., 2009. Compositional changes in fish scale hydroxylapatite during early diagenesis; an example from an abandoned meander. *Biogeochemistry* 94, 197-215.

Kennedy, C.R., 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.' (Publications Kottelat: Cornol, Switzerland)

Krasnići, N., Dragun, Z., Erk, M., Raspor, B., 2013. Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se, and Zn) and nonessential (Cd, Pb) trace elements among protein fractions from hepatic cytosol of European chub (*Squalius cephalus* L.). *Environ Sci Pollut Res* 20, 2340-2351.

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*). *Arch Environ Contam Toxicol* 57, 571-580.

Linde, A.R., Sánchez-Galán, S., Izquierdo, J.I., Arribas, P., Marañón, E., García-Vázquez, E., 1998. Brown trout as biomonitor of heavy metal pollution: effect of age on the reliability of the assessment. *Ecotoxicol Environ Saf* 40, 120–125.

Marcogliese, D.J., 2004. Parasites: Small players with crucial roles in the ecological theater. *EcoHealth* 1, 151–164.

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. *Ecol Indic* 105, 188–198.

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. *Sci Total Environ* 660, 1079–1090.

Mijošek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Veseli, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matoničkin Kepčija, R., Erk, M., 2020. Thallium accumulation in different organisms from karst and lowland rivers of Croatia under wastewater impact. *Environ Chem* 17(2), 201-212.

Mikac, N., Filipović Marijić, V., Mustafić, P., Lajtner, J., 2018. Development of methodology for monitoring the elements of chemical status in biota of freshwater ecosystem [report in Croatian]

Milton, D., Halliday, I., Sellin, M., Marsh, R., Staunton-Smith, J., Woodhead, J. 2008. The effect of habitat and environmental history on otolith chemistry of barramundi *Lates calcarifer* in estuarine populations of a regulated tropical river. *Estuar Coast Shelf Sci* 78, 301–315.

Muhlfeld, C.C., Marotz, B., 2005. Seasonal movement and habitat use by subadult bull trout in the upper Flathead River system, Montana. *N Am J Fish Manag* 25, 797-810.

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. *Parasite Vector* 9, 300-308.

NN 55, 2013. Ordinance on the Protection of Animals Used for Scientific Purposes [Pravilnik o zaštiti životinja koje se koriste u znanstvene svrhe].

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.

Prohaska, T., Irrgeher, J., Zitek, A., 2016. Simultaneous multi-element and isotope ratio imaging of fish otoliths by laser ablation split stream ICP-MS/MC ICP-MS. *J Anal At Spectrom* 31, 1612-1621.

Rainbow, P.S., Luoma, S.N., Wang, W.X., 2011. Trophically available metal – a variable feast. *Environ Pollut* 159, 2347–2349.

Ramsay, A.L., Milner, N.J., Hughes, R.N., McCarthy, I.D., 2011. Comparison of the performance of scale and otolith microchemistry as fisheries research tools in a small upland catchment. *Can J Fish Aquat Sci* 68, 823–833.

Ranaldi, M.M., Gagnon, M.M., 2008. Trace metal incorporation in otoliths of black bream (*Acanthopagrus butcheri* Munro), an indicator of exposure to metal contamination. *Water Air Soil Pollut* 194, 31-43.

Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. *B Fish Res Board Can* 191, 1–382.

Rooker, J.R., Zdanowicz, V.S., Secor, D.H., 2001. Chemistry of tuna otoliths: assessment of base composition and postmortem handling effects. *Mar Biol* 139, 35-43.

Saquet, M., Halden, N.M., Babaluk, J., Campbell, J.L., Nejedly, Z., 2002. Micro- PIXE analysis of trace element variation in otoliths from fish collected near acid mine tailings: Potential for monitoring contaminant dispersal. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms* 189, 196–201.

Sawhney, A.K., Johal, M.S., 1999. Potential application of elemental analysis of fish otoliths as pollution indicator. *Bull Environ Contam Toxicol* 63, 698-702.

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. *Ecotoxicol Environ Saf* 163, 125–135.

Sturgeon, R.E., Willie, S.N., Yang, L., Greenberg, R., Spatz, R.O., Chen, Z., Scriver, C., Clancy, V., Lam, J.W., Thorrold, S., 2005. Certification of a fish otolith reference material in support of quality assurance for trace element analysis. *J Anal At Spectrom* 20, 1067-1071.

Sures, B., Siddall, R., Taraschewski, H., 1999. Parasites as accumulation indicators of heavy metal pollution. *Parasitol Today* 15, 16–21.

Sures, B., 2004. Environmental parasitology: relevancy of parasites in monitoring environmental pollution. *Trends Parasitol* 20, 170-177.

Sures, B., Nachev, M., Selbach, C., Marcogliese, D.J., 2017. Parasite responses to pollution: what we know and where we go in ‘Environmental Parasitology’. *Parasit Vectors* 10, 65-83.

Tabouret, H., Bareille, G., Claverie, F., Pécheyran, C., Prouzet, P., Donard, O.F.X., 2010. Simultaneous use of strontium:calcium and barium:calcium ratios in otoliths as markers of habitat: application to the European eel (*Anguilla anguilla*) in the Adour basin, South West France. *Mar Environ Res* 70, 35–45.

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. *Environ Pollut* 129, 421–429.

Urien, N., Cooper, S., Caron, A., Sonnenberg, H., Rozon-Ramilo, L., Campbell, P.C.G., 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. *Aquat Toxicol* 202, 105–116.

Vardić Smrzlić, I., Valić, D., Kapetanović, D., Dragun, Z., Gjurčević, E., Četković, H., Teskeredžić, E., 2013. Molecular characterisation and infection dynamics of *Dentitruncus truttae* from trout (*Salmo trutta* and *Oncorhynchus mykiss*) in Krka River, Croatia. *Vet Parasitol* 197, 604–613.

Veinott, G., Northcote, T., Rosenau, M., Evans, R.D., 1999. Concentrations of strontium in the pectoral fin rays of the white sturgeon (*Acipenser transmontanus*) by laser ablation sampling –

inductively coupled plasma- mass spectrometry as an indicator of marine migrations. *Can J Fish Aquat Sci* 56, 1981–1990.

Wallace, W.G., Lee, B.-G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar Ecol Prog Ser* 249, 183–197.

Wells, B.K., Thorrold, S.R., Jones, C.M., 2000. Geographic Variation in Trace Element Composition of Juvenile Weakfish Scales. *T Am Fish Soc* 129(4), 889-900.

Wells, B.K., Rieman, B.E., Clayton, J.L., Horan, D.L., Jones, C. M., 2003. Relationships between water, otolith, and scale chemistries of westslope cutthroat trout from the Coeur d'Alene River, Idaho: the potential application of hard-part chemistry to describe movements in freshwater. *T Am Fish Soc* 132, 409-424.

WHO, 2011. Manganese in Drinking-water. Background Document for Development of WHO Guidelines for Drinking-water Quality. World Health Organization, Geneva.

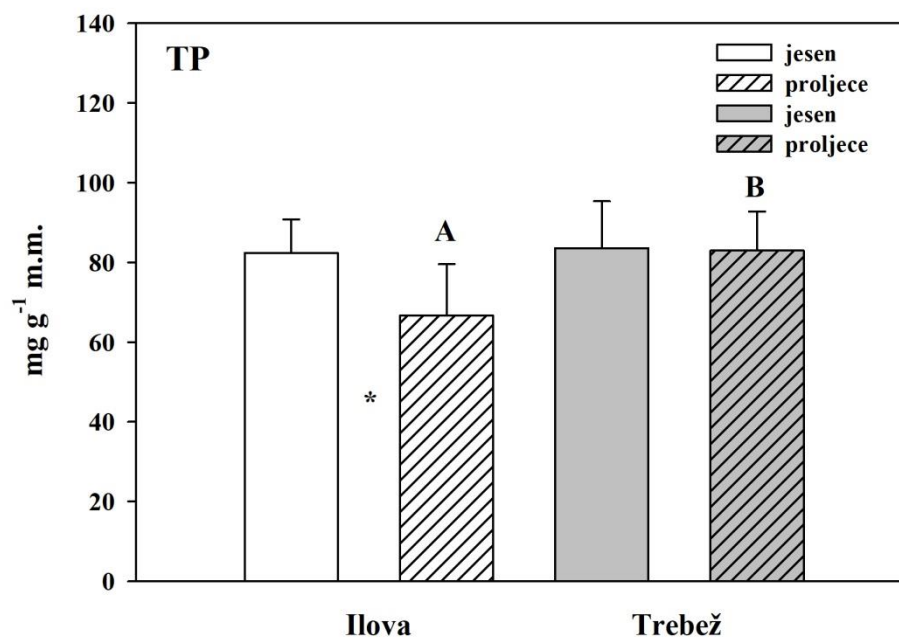
Wootton, R.J., 1990. Ecology of teleost fishes. Chapman and Hall, Fish and Fisheries Series 1. London, New York https://doi.org/10.1007/978-94-009-0829-1_9 (404 pp).

Zhelev, Zh., Mollova, D., Boyadziev, P., 2016. Morphological and hematological parameters of *Carassius gibelio* (Pisces: Cyprinidae) in conditions of anthropogenic pollution in Southern Bulgaria. Use hematological parameters as biomarkers. *Trakia J Sci* 14 (1), 1–15.

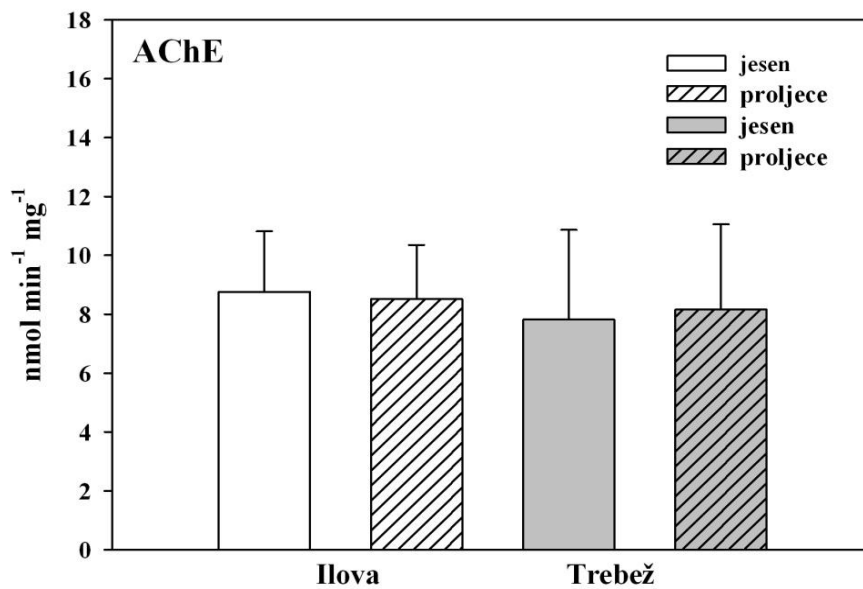
Zhelev, Zh.M., Tsonev, S.V., Boyadziev, P.S., 2018. Significant changes in morpho physiological and haematological parameters of *Carassius gibelio* (Bloch, 1782) (Actinopterygii: Cyprinidae) as response to sporadic effusions of industrial wastewater into the Sazliyka River, Southern Bulgaria. *Acta Zool Bulg* 70(4), 547–556.

Zitek, A., Sturm, M., Waidbacher, H., Prohaska, T., 2010. Discrimination of wild and hatchery trout by natural chronological patterns of elements and isotopes in otoliths using LA-ICP-MS. *Fish Manag Ecol* 17, 435–445.

Prilog 7. Koncentracije ukupnih proteina (TP) u probavnom tkivu babuški iz rijeke Ilove s dviju lokacija (referentna: selo Ilova, onečišćena: selo Trebež) i iz dviju sezona (jesen i proljeće). Statistički značajne razlike (Mann-Whitney U test, $p < 0,05$) između dviju sezona su označene zvijezdicom (*), a razlike između dviju lokacija u istoj sezoni velikim tiskanim slovima (A i B).



Prilog 8. Aktivnost acetilkolinesteraze (AChE) u probavnom tkivu babuški iz rijeke Ilove s dviju lokacija (referentna: selo Ilova, onečišćena: selo Trebež) i iz dviju sezona (jesen i proljeće).



8. ŽIVOTOPIS

Tatjana Mijošek je rođena 22.1.1992. u Zagrebu, a osnovnu i srednju školu je završila u Vrbovcu. Preddiplomski i diplomski studij biologije je završila na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu 2013., odnosno 2015. godine čime je stekla akademski naziv magistre eksperimentalne biologije (*mag. biol. exp.*). Na mjestu doktoranda u Laboratoriju za biološke učinke metala na Institutu Ruđer Bošković (IRB) zapošljava se u prosincu 2016. godine te u akademskoj godini 2016./2017. upisuje Poslijediplomski sveučilišni doktorski studij biologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu.

Njezin znanstveni interes se temelji na proučavanju bioloških učinaka metala na vodene organizme, okolišnom monitoringu i procjeni ekološkog rizika te ekotoksikologiji. Dobitnica je CEEPUS stipendija za dva jednomjesečna usavršavanja na BOKU Sveučilištu u Austriji u području laserske ablacije i mjerenja koncentracija metala u tvrdim strukturama riba, stipendije Francuske ambasade i Instituta Ruđer Bošković te EMBO stipendije za kratkoročne boravke na IPREM Institutu u Francuskoj s ciljem učenja metode NanoSIMS te obrade uzoraka i rezultata, kao i stipendije COST akcije TD1407 za sudjelovanje na treningu i radionici u Estoniji. Za znanstvene aktivnosti i dostignuća dodijeljene su joj godišnja nagrada IRB-a i Zavoda za istraživanje mora i okoliša IRB-a za dva znanstvena rada objavljena u časopisima visokog čimbenika odjeka za 2019. godinu. Predstavnik je znanstvenih novaka svojeg zavoda u Vijeću asistenata IRB-a te u Vijeću Zavoda za istraživanje mora i okoliša te je uključena u organizacijski odbor Otvorenog dana IRB-a od 2017. godine.

Do sada je autorica/suautorica na 11 znanstvenih radova u časopisima citiranim u Web of Science (WoS) bazi podataka, od kojih je 8 prvoautorskih. Suradnica je na više znanstvenih i gospodarskih projekata svojeg laboratorija. Aktivno je sudjelovala na nizu domaćih i međunarodnih kongresa, kao i radionica te je suautorica 14 sažetaka u zbornicima znanstvenih skupova.

Popis objavljenih znanstvenih radova

1. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Krasnići, N., Ivanković, D., Redžović, Z., Erk, M. (2021) First insight in trace element distribution in the intestinal cytosol of two freshwater fish species challenged with moderate environmental contamination. *Science of the Total Environment* 798, 149274.
2. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Erk, M. (2021) Intestine of invasive fish Prussian carp as a target organ in metal exposure assessment of the wastewater impacted freshwater ecosystem. *Ecological Indicators* 122, 107247.
3. Dragun, Z., Krasnići, N., Ivanković, D., Filipović Marijić, V., **Mijošek, T.**, Redžović, Z., Erk, M. (2020) Comparison of intracellular trace element distributions in the liver and gills of the invasive freshwater fish species, Prussian carp (*Carassius gibelio* Bloch, 1782). *Science of the Total Environment* 730, 138923.
4. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Sertić Perić, M., Vdović, N., Bačić, N., Dautović, J., Erk, M. (2020) The assessment of metal contamination in water and sediments of the lowland Ilova River (Croatia) impacted by anthropogenic activities. *Environmental Science and Pollution Research* 27, 25374-25389.
5. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Veseli, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matoničkin Kepčija, R., Erk, M. (2020) Thallium accumulation in different organisms from karst and lowland rivers of Croatia under wastewater impact. *Environmental Chemistry* 17(2), 201–212.
6. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Erk, M., Gottstein S., Lajtner, J., Sertić Perić, M., Matoničkin Kepčija, R. (2019) 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.
7. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Krasnići, N., Ivanković, D., Erk, M. (2019) 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.

8. Barišić, J., Filipović Marijić, V., **Mijošek, T.**, Čož-Rakovac, R., Dragun, Z., Krasnići, N., Ivanković, D., Kružlicová, D., Erk, M. (2018) Evaluation of architectural and histopathological biomarkers in the intestine of brown trout (*Salmo trutta* Linnaeus, 1758) challenged with environmental pollution. *Science of the Total Environment* 642, 656-664.
9. Ečimović, S., Velki, M., Vuković, R., Štolfa Čamagajevac, I., Petek, A., Bošnjaković, R., Grgić, M., Engelmann, P., Bodó, K., Filipović Marijić, V., Ivanković, D., Erk, M., **Mijošek, T.**, Lončarić, Z. (2018) Acute toxicity of selenate and selenite and their impacts on oxidative status, efflux pump activity, cellular and genetic parameters in earthworm *Eisenia andrei*. *Chemosphere* 212, 307-318.
10. **Mijošek, T.**, Erk, M., Filipović Marijić, V., Krasnići, N., Dragun, Z., Ivanković, D. (2018) Electrochemical determination of metallothioneins by the modified Brdička procedure as an analytical tool in biomonitoring studies. *Croatica chemica acta* 91, 475-480.
11. **Mijošek, T.**, Jelić, M., Mijošek, V., Maguire, I. (2017) Molecular and morphometric characterisation of the invasive signal crayfish populations in Croatia. *Limnologica* 63, 107-118.

Kongresna priopćenja

1. **Mijošek, T.**, Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Valić, D., Sertić Perić, M., Erk, M., Kljaković-Gašpić, Z., Filipović Marijić, V. Dugoročni trendovi koncentracija metala i kakvoće vode rijeke Krke u dijelu toka pod utjecajem otpadnih voda. *Simpozij studenata doktorskih studija PMF-a - Knjiga sažetaka*, Barišić, Dajana (ur.). Zagreb, Hrvatska: Prirodoslovno-matematički fakultet Sveučilišta u Zagrebu, 2021., str. 103-103 (predavanje).
2. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Krasnići, N., Ivanković, D., Redžović, Z., Erk, M. Thallium accumulation in the intestinal tissue, homogenate and cytosol of brown trout and Prussian carp from two Croatian rivers. *COST ACTION TD 1407 Final Meeting: Book of Abstracts*, Filella, Montserrat; Omanović, Dario; Dror, Ishai (ur.), Zagreb, Hrvatska, 2019., str. 58-58 (poster).
3. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Retzmann, A., Zitek, A., Prohaska, T., Bačić, N., Redžović, Z., Grgić, I., Krasnići, N., Ivanković, D., Erk, M., Valić, D., Žunić, J.,

Vardić Smrzlić, I., Kapetanović, D. Evaluation of metal levels in soft and hard tissues of brown trout and fish intestinal parasites as indicators of wastewater impact in the karst Krka River. *3rd Symposium of Freshwater Biology: Book of abstracts*, Ivković, Marija; Stanković, Igor; Matoničkin Kepčija, Renata; Gračan, Romana (ur.) Zagreb, Hrvatska, 2019., str. 23-23 (predavanje).

4. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Erk, M. Biological responses of Prussian carp (*Carassius gibelio*, Bloch 1782) to the impact of industrial wastewaters in the Ilova River. *11th Symposium for European Freshwater Sciences - Abstract Book*, Sertić Perić, Mirela; Miliša, Marko; Gračan, Romana; Ivković, Marija; Buj, Ivana; Mičetić Stanković, Vlatka (ur.) Zagreb, Hrvatska, 2019., str. 395-395 (predavanje).

5. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Veseli, M., Erk, M. Thallium bioaccumulation in different bioindicator organisms from the karst Krka River in Croatia. Book of Abstracts of the *Workshop on Technology Critical Elements in Ecosystem and Human Health*, Ospina-Alvarez, Natalia; Zimmermann, Sonja; Aruoja, Villem (ur.). Tallinn: NOTICE-COST action TD1407, 2018., str. 45-45 (poster).

6. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Erk, M. Elektrokemijsko određivanje razine metalotioneina metodom po Brdički u bioti iz rijeke Krke. *5. DAN ELEKTROKEMIJE & 8TH ISE SATELLITE STUDENT REGIONAL SYMPOSIUM ON ELECTROCHEMISTRY - Knjiga sažetaka*, Kraljić Roković, Marijana; Strmečki Kos, Slađana; Cvitešić Kušan, Ana; Ljubek, Gabrijela (ur.). Zagreb, Hrvatska, 2018., str. 18-18 (predavanje).

7. **Mijošek, T.**, Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Erk, M. Application of invasive Prussian carp in metal exposure assessment of the Ilova River. *Hrvatski simpozij o invazivnim vrstama s međunarodnim sudjelovanjem - Zbornik sažetaka*, Jelaska, Sven (ur.) Zagreb, Hrvatska, 2018., str. 29-29 (predavanje).

8. **Mijošek, T.**, Jelić, M., Mijošek, V., Maguire I. Genetička raznolikost invazivne strane vrste *Pacifastacus leniusculus* u Hrvatskoj. *Simpozij o biologiji slatkih voda - Knjiga sažetaka*, Gračan, Romana; Matoničkin Kepčija, Renata; Miliša, Marko; Ostojić, Ana (ur.) Zagreb, Hrvatska, 2017., str. 49-49 (poster).