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PRIRODOSLOVNO-MATEMATIČKI FAKULTET
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Nesrete Krasnići

RASPODJELA ODABRANIH METALA MEĐU CITOSOLSKIM BIOMOLEKULAMA ŠKRGA I JETRE KLENOVA (*Squalius cephalus* (Linnaeus) i *Squalius vardarensis* Karaman; Actinopterygii, Cyprinidae) IZ RIJEKA ONEČIŠĆENIH METALIMA

DOKTORSKI RAD

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University of Zagreb

FACULTY OF SCIENCE
DIVISION OF BIOLOGY

Nesrete Krasnići

DISTRIBUTION OF SELECTED METALS AMONG CYTOSOLIC BIOMOLECULES IN GILLS AND LIVER OF CHUB (*Squalius cephalus* (Linnaeus) and *Squalius vardarensis* Karaman; Actinopterygii, Cyprinidae) FROM METAL CONTAMINATED NATURAL WATERS

DOCTORAL THESIS

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Ovaj je doktorski rad izrađen u Laboratoriju za biološke učinke metala Instituta Ruđer Bošković u Zagrebu pod vodstvom dr. sc. Zrinke Dragun, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu.

INFORMACIJE O MENTORU

Dr. sc. Zrinka Dragun rođena je 6. lipnja 1971. godine u Vinkovcima, gdje je završila osnovnoškolsko te srednjoškolsko obrazovanje, matematičko-informatičkog smjera. Na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu diplomirala je 1996. godine te stekla zvanje dipl. inž. medicinske biokemije. Magistrirala je 2001. godine na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu (grana toksikologija) te doktorirala 2006. godine na istome fakultetu. Zaposlena je na mjestu više znanstvene suradnice na Institutu „Ruđer Bošković“ u Zagrebu, gdje radi od 2002. godine. Godine 2018. stekla je zvanje znanstvene savjetnice u znanstvenom području prirodnih znanosti (polje interdisciplinarne prirodne znanosti). Autor je i suautor brojnih znanstvenih i stručnih radova te priopćenja na znanstvenim skupovima, kao i voditelj nekoliko znanstvenih projekata te studentskih radova.

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Sveučilište u Zagrebu
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Biološki odsjek

Doktorski rad

RASPODJELA ODABRANIH METALA MEĐU CITOSOLSKIM BIOMOLEKULAMA ŠKRGA I JETRE KLENOVA (*Squalius cephalus* (Linnaeus) i *Squalius vardarensis* Karaman; Actinopterygii, Cyprinidae) IZ RIJEKA ONEČIŠĆENIH METALIMA

NESRETE KRASNIĆI

Institut Ruđer Bošković, Zagreb

Citosolske raspodjele nekoliko odabranih elemenata (Cd, Co, Cu, Fe, Mn, Mo, Pb, Se i Zn) u jetrima i škrgama dvaju slatkovodnih bioindikatorskih organizama, klena (*Squalius cephalus*) iz rijeke Sutle u Hrvatskoj i vardarskog klena (*Squalius vardarensis*) iz triju rijeka sjeveroistočne Sjeverne Makedonije, izučavane su primjenom tekućinske kromatografije visoke djelotvornosti s isključenjem po veličini (SEC-HPLC) i spektrometrije masa visoke rezolucije s induktivno spregnutom plazmom (HR ICP-MS). Određene su raspodjele metala/nemetala među citosolskim biomolekulama različitih molekulskih masa svojstvene uvjetima niske izloženosti metalima u riječnoj vodi, kao i promjene u raspodjelama koje su javljaju kao posljedica povišenih razina izloženosti. Primjena anionsko izmjenjivačke kromatografije (AEC-HPLC) kao drugog stupnja razdvajanja i pročišćavanja citosolskih biomolekula te dviju tehnika spektrometrije masa (MALDI-TOF-MS i LC-MS/MS) omogućila je identifikaciju ili precizno određivanje molekulskih masa nekoliko citosolskih biomolekula koje vežu metale u jetrima i škrgama vardarskog klena, poput izoformi metalotioneina koje vežu Cd, Cu i Zn, hemoglobinskih podjedinica koje vežu Fe te toplinski stabilnih biomolekula koje vežu Mo. Primijenjeni analitički pristup pokazao se korisnim u istraživanju unutarstanične sudbine i ponašanja metala u organima riba te otkrivanju potencijalnih novih biomarkera izloženosti i učinaka metala.

(190 stranica, 34 slike, 17 tablica, 340 literaturna navoda, jezik izvornika hrvatski)

Ključne riječi: klen, vardarski klen, metali, citosolske biomolekule, kromatografija, spektrometrija masa

Mentor: dr. sc. Zrinka Dragun, viša znanstvena suradnica

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izv. prof. dr. sc. Renata Matoničkin Kepčija

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Doctoral thesis

DISTRIBUTION OF SELECTED METALS AMONG CYTOSOLIC BIOMOLECULES IN GILLS AND LIVER OF CHUB (*Squalius cephalus* (Linnaeus) and *Squalius vardarensis* Karaman; Actinopterygii, Cyprinidae) FROM METAL CONTAMINATED NATURAL WATERS

NESRETE KRASNIĆI

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The cytosolic distributions of several selected elements (Cd, Co, Cu, Fe, Mn, Mo, Pb, Se, and Zn) in the liver and gills of two freshwater bioindicators, European chub (*Squalius cephalus*) from the Sutla River in Croatia and Vardar chub (*Squalius vardarensis*) from three rivers of north-eastern Macedonia, were studied using size-exclusion high performance liquid chromatography (SEC-HPLC) and high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). Distributions of metals/non-metal among the biomolecules of different molecular masses which are characteristic for low metal exposure in the river water were determined, as well as the changes in distribution which occur as a consequence of increased exposure to metals. The use of anion-exchange high performance liquid chromatography (AEC-HPLC) as the second step of separation and purification of cytosolic biomolecules, as well as the use of two mass spectrometry techniques (MALDI-TOF-MS and LC-MS/MS) have enabled the identification or exact determination of molecular masses of several cytosolic biomolecules that bind metals in the liver and gills of Vardar chub, such as the metallothionein isoforms that bind Cd, Cu and Zn, the hemoglobin subunits that bind Fe and heat stable biomolecules that bind Mo. Applied analytical approach has proven to be useful in the study of intracellular fate and behavior of metals in organs of fish, as well as in the process of discovery of potential new biomarkers of metal exposure and effects.

(190 pages, 34 figures, 17 tables, 340 references, original in Croatian)

Keywords: European chub, Vardar chub, metals, cytosolic biomolecules, chromatography, mass spectrometry

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SADRŽAJ

U OVOJ DOKTORSKOJ DISERTACIJI OBJEDINJENI SU SLJEDEĆI ZNANSTVENI RADOVI:

1. **Krasnići N**, Dragun Z, Erk M, Raspor B: Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se, 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 (2013), 4: 2340-2351. doi:10.1007/s11356-012-1105-8

2. **Krasnići N**, Dragun Z, Erk M, Raspor B: 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 (2014), 23: 13512-13521. doi:10.1007/s11356-014-3274-0

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

4. **Krasnići N**, Dragun Z, Kazazić S, Muharemović H, Erk M, Jordanova M, Rebok K, Kostov V: Characterization and identification of selected metal-binding biomolecules from hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928) by various techniques of liquid chromatography and mass spectrometry. Metallomics 11 (2019), 1060-1078. doi:10.1039/C9MT00036D

UVOD

1. UVOD

Onečišćenje prirodnih voda metalima u porastu je zbog tehnološkog napretka ljudske zajednice, pri čemu industrija, rudarstvo, poljoprivreda, otpad iz domaćinstava i promet predstavljaju njegove najznačajnije izvore. Za razliku od organskih zagađivala, metali se ne mogu kemijski niti biološki razgraditi pa tako jednom uneseni u vodotoke mogu promijeniti kemijski oblik te postati manje ili više toksični, ali se iz biogeokemijskog kruženja ne mogu ukloniti (Sadiq, 1992.). Neki metali u vodenim organizmima imaju važnu biološku ulogu, sastavni su dio enzima i drugih složenih biomolekula, tzv. metaloproteina (Smith i sur., 1997.), te su prozvani esencijalnim metalima (npr., Cu, Fe i Zn). Metaloproteini su uključeni u niz procesa, poput transporta elektrona i metala, skladištenja kisika, hidrolize kemijskih veza, redoks procesa i sinteza bioloških spojeva (Gellein i sur., 2007.). Cijeli niz drugih metala nema poznatih uloga u živim organizmima te su stoga prozvani neesencijalnim metalima (npr., Cd i Pb). Uslijed unosa neesencijalnih metala, koji su toksični već u vrlo niskim koncentracijama, ili prekomjernog unosa esencijalnih metala, koji također mogu biti toksični kada su u organizmu prisutni u povišenim koncentracijama, u vodenim organizmima može doći do poremećaja homeostaze i razvoja toksičnih učinaka (Livingstone, 1993.). Nakon unosa, metali se raspodjeljuju po organima i stanicama vodenih organizama te naposljetku i po različitim unutarstaničnim odjeljcima, kao što su citosol, granule, organele i stanične membrane, a njihova toksičnost većinom nastaje uslijed reakcija u citosolu, odnosno nespecifičnog vezanja metala na fiziološki važne molekule i njihove posljedične inaktivacije (Mason i Jenkins, 1995.). Stoga se u istraživanjima izloženosti metalima i njihovih toksičnih učinaka u vodenim ekosustavima često koriste bioindikatorski organizmi. Među najčešće korištene indikatorske organizme za praćenje posljedica onečišćenja slatkovodnih ekosustava metalima ubrajaju se ribe jer se nalaze na vrhu hranidbenog lanca, imaju potencijal za akumulaciju metala, prikladnu veličinu te dugi životni vijek (Dragun i sur., 2015.). U brojnim europskim vodotocima često se kao bioindikatorski organizam koristi klen (*Squalius cephalus* L., 1758), dok se, na primjer, u makedonskim rijekama može naći srodna vrsta, vardarski klen (*Squalius vardarensis* Karaman, 1928). Obje vrste kao svejedi odražavaju izloženost metalima iz raznih izvora, kako iz vode tako i iz hrane životinjskog i biljnog podrijetla. Najčešće korišteni organi za analize metala su jetra, kao najvažnije mjesto metabolizma, pohranjivanja i detoksikacije metala, te škrge, kao mjesto unosa metala putem vode (Giguère i sur., 2006.; Kraemer i sur., 2005.). Dosadašnja istraživanja izloženosti metalima i njihovih učinaka kod klena i vardarskog klena uključuju uglavnom određivanje ukupnih koncentracija

akumuliranih metala u organima klena (Dragun i sur., 2007; 2012; 2016; 2019) te ne daju informaciju o mogućem toksičnom učinku metala ili njihovoj detoksikaciji (de la Calle Guntińas i sur., 2002.). Stoga je potrebno podrobnije istražiti sudbinu metala nakon njihovog unosa u organizam, odnosno njihovu unutarstaničnu raspodjelu. Biološka funkcija, kao i mehanizmi toksičnosti i detoksikacije metala u mnogim organizmima još uvijek nisu dovoljno istraženi, dok su biomolekule koje vežu pojedine metale u ribama tek djelomično prepoznate i opisane (Janz, 2012.; McGeer i sur., 2012.). Prepoznavanje staničnih komponenata s kojima metali stupaju u interakcije pri različitim razinama izloženosti značajan je korak u predviđanju njihovih potencijalnih toksičnih učinaka. U skladu s tim, razvijeno je novo područje u istraživanju metala, nazvano metalomika, koje je Szpunar (2005.) definirala kao "sveobuhvatnu analizu svih oblika metala i metaloida u stanicama ili tkivima", a koje obuhvaća specijaciju metala u najširem smislu, uključujući izučavanje kompleksiranja elemenata, kao i utjecaja tog kompleksiranja na okoliš te posljedično i na ljudsko zdravlje. U području metalomike danas se koriste različite instrumentalne tehnike. Jedan je od najprihvaćenijih pristupa primjena kombinacije tekućinske kromatografije visoke djelotvornosti (HPLC; za razdvajanje citosolskih biomolekula) i spektrometrije masa s induktivno spregnutom plazmom (ICP-MS; za određivanje koncentracija metala) (Montes-Bayón i sur., 2003.). Brojne kromatografske tehnike razdvajanja mogu se koristiti za analizu raspodjele metala među citosolskim biomolekulama, poput kromatografije s isključenjem po veličini (SEC-HPLC) i anionsko-izmjenjivačke kromatografije (AEC-HPLC), koje se odlikuju visokom osjetljivošću i selektivnošću (Montes-Bayón i sur., 2003.; Szpunar, 2005.). Kombinirana primjena različitih kromatografskih tehnika i ICP-MS-a predstavlja vrijedan alat za razlučivanje citosolske raspodjele metala među proteinima i drugim molekulama različitih molekulskih veličina i naboja. Konačno prepoznavanje i karakterizacija biomolekula koje vežu metale, zahtjevan je korak u kojemu se primjenjuju razne tehnike spektrometrije masa (MS) za identifikaciju struktura molekula na koje su vezani metali, poput tekućinske kromatografije - tandemne spektrometrije mase (LC-MS/MS) te spektrometrije masa s analizatorom masa s vremenom leta (TOF) uz matricom potpomognutu desorpciju i ionizaciju laserskim zračenjem (MALDI) (Salekdeh i sur., 2002.). Ovakav višestruki analitički pristup povezuje detektore za elemente i molekularne detektore, sa svrhom olakšane identifikacije metaloproteina čak i u vrlo niskim koncentracijama (Gómez-Ariza i sur., 2004.). Promjene u raspodjeli metala među citosolskim biomolekulama, kao i prepoznavanje i karakterizacija biomolekula koje vežu metale nakon izloženosti organizama povišenim koncentracijama metala u vodi, predstavljaju osnovu za razvoj novih biomarkera izloženosti i toksičnih

učinaka metala, koji će se s vremenom moći ugraditi u sustave praćenja stanja prirodnih voda.

1.1. Ciljevi i svrha rada

Biološka funkcija i mehanizmi toksičnosti u različitim organizmima, a posebice u ribama, za mnoge metale još nisu dovoljno istraženi te je važno odrediti točan identitet citosolskih biomolekula na koje se ti metali vežu. U skladu s time, osnovni cilj ovoga istraživanja bio je produbljivanje razumijevanja unutarstanične sudbine metala nakon njihovog unosa u jetra i škrge dvaju odabranih bioindikatorskih organizama, dviju srodnih ribljih vrsta, klena (*S. cephalus*) i vardarskog klena (*S. vardarensis*), pri različitim razinama izloženosti tih organizama metalima u umjereno i izrazito metalima onečišćenim rijekama u Hrvatskoj i Sjevernoj Makedoniji. Istraživanje je bilo usmjereno na devet elemenata, sedam esencijalnih (Co, Cu, Fe, Mn, Mo, Se i Zn) i dva neesencijalna (Cd i Pb).

Cilj istraživanja ostvaren je kroz sljedeće aktivnosti:

- određivanje citosolskih koncentracija odabranih elemenata akumuliranih u jetrima i škrgama klena (*S. cephalus*) iz rijeke Sutle te vardarskog klena (*S. vardarensis*) iz triju rijeka sjeveroistočne Sjeverne Makedonije sa svrhom prepoznavanja jedinki s povećanom bioakumulacijom uslijed povećane izloženosti metalima u vodi;
- definiranje raspodjela odabranih elemenata među citosolskim biomolekulama različitih molekulskih masa u jetrima i škrgama klena (*S. cephalus*) i vardarskog klena (*S. vardarensis*) pri niskim izloženostima te definiranje promjena u raspodjelama koje nastaju uslijed povišene izloženosti metalima u riječnoj vodi primjenom SEC-HPLC-a i ICP-MS visoke rezolucije (HR);
- definiranje raspodjela odabranih elemenata među toplinski stabilnim biomolekulama u jetrima i u škrgama vardarskog klena (*S. vardarensis*) primjenom SEC-HPLC-a i HR ICP-MS;
- primjenu AEC-HPLC-a kao drugog stupnja razdvajanja citosolskih biomolekula na koje se vežu metali na temelju razlika u naboju, sa svrhom dodatnog pročišćavanja metaloproteina i razdvajanja njihovih izoformi iz jetara i škrga vardarskog klena (*S. vardarensis*);
- karakterizaciju i identifikaciju nekoliko odabranih citosolskih biomolekula koje vežu Fe, Cd, Cu, Zn i Mo u jetrima i škrgama vardarskog klena (*S. vardarensis*) primjenom dviju tehnika spektrometrije masa (MALDI-TOF-MS i LC-MS/MS).

1.2. Hipoteze istraživanja

Provođenjem istraživanja testirali smo sljedeće hipoteze:

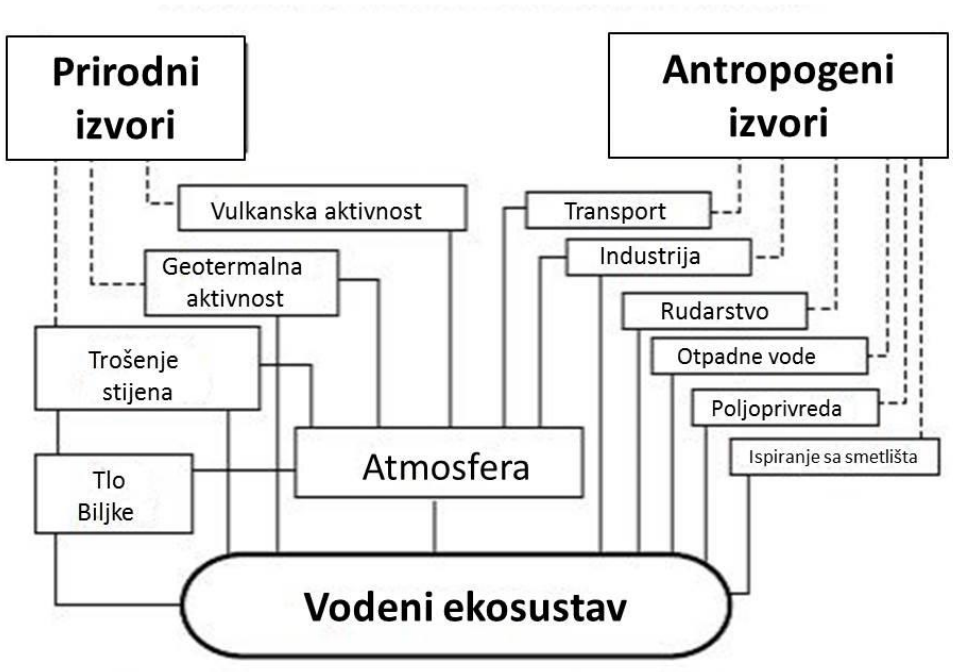
- raspodjela esencijalnih (Co, Cu, Fe, Mn, Mo, Se i Zn) i neesencijalnih elemenata (Cd i Pb) među citosolskim biomolekulama u jetrima i škrgama klena (*S. cephalus*) i vardarskog klena (*S. vardarensis*) mijenja se uslijed promjene razine izloženosti metalima/nemetalu u vodi;
- raspodjela esencijalnih elemenata (Co, Cu, Fe, Mn, Mo, Se i Zn) i neesencijalnog elementa Cd među citosolskim biomolekulama različita je u različitim organima (jetrima i škrgama) obiju istraživanih vrsta, klena i vardarskog klena;
- raspodjela istraživanih elemenata među citosolskim biomolekulama usporediva je u dvjema srodnim vrstama riba, klenu (*S. cephalus*) i vardarskom klenu (*S. vardarensis*), koje se stoga mogu usporedno koristiti u monitoringu.

LITERATURNI PREGLED

2. LITERATURNI PREGLED

2.1. Metali u slatkovodnim sustavima

U šarolikom rasponu onečišćivača metali imaju važnu ulogu jer mogu uzrokovati toksične učinke u vodenim organizmima (Maritim i sur., 2016.; Tessier i Turner, 1995.), iako brojni metali imaju i bitnu ulogu u funkcioniranju svih živih organizama te se prirodno nalaze u svim sastavnicama okoliša (Cukrov i sur., 2006.). Koncentracija metala u vodenom okolišu regulirana je procesima poput kemijskog i mehaničkog trošenja stijena i tla pod utjecajem vanjskih sila te njihovog ispiranja oborinskim vodama ili raznošenja vjetrom. Nadalje, prirodne koncentracije metala u velikoj mjeri ovise i o području u kojem se nalaze, odnosno o sastavu stijena i tla (Cukrov i sur., 2006.). Osim prirodnim putem, metali u vodu dospijevaju i iz brojnih antropogenih izvora, koji su u porastu zbog tehnološkog napretka ljudske zajednice, pri čemu razvoj industrije (proizvodnja plastičnih i električnih elemenata), rudarstvo, promet, napredna poljoprivreda (putem gnojiva) i gradske otpadne vode imaju najznačajniju ulogu u onečišćenju vodenih ekosustava metalima (Slika 1.) (Gaillardet i sur., 2004.).



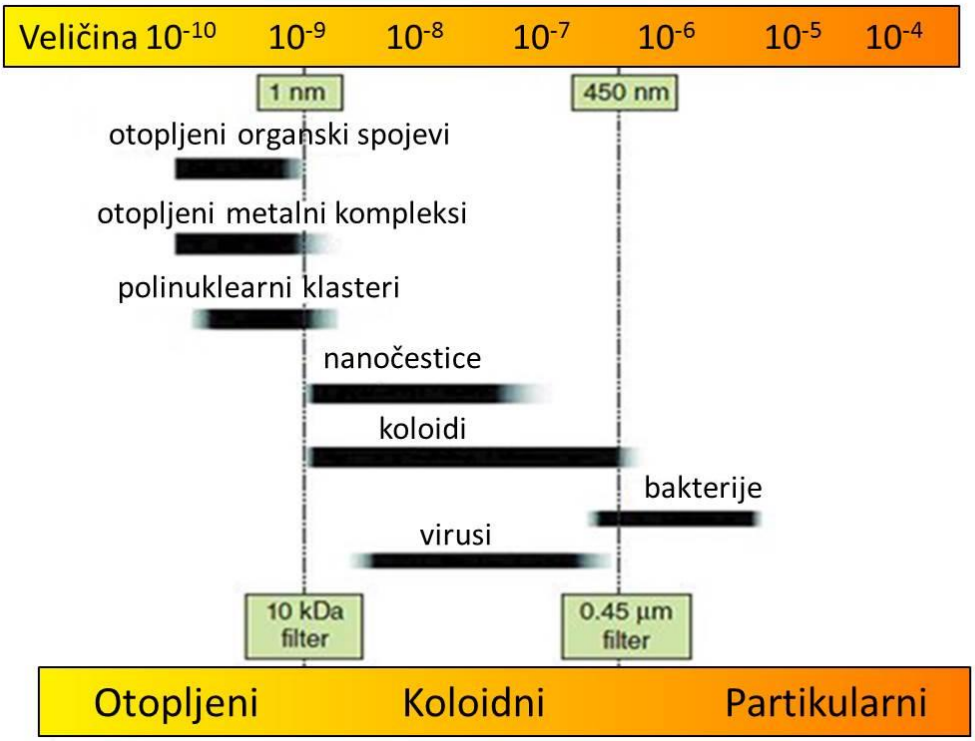
Slika 1. Izvori onečišćenja vodenog ekosustava metalima (preuzeto i prilagođeno prema Gaillardet i sur., 2004.).

Budući da su prirodne koncentracije većine metala u rijekama niske, od nekoliko nanograma do nekoliko mikrograma po litri, zbog čega su nazvani metalima u tragovima,

svaki dodatni unos utječe na njihove koncentracije i time mijenja prirodnu ravnotežu u vodenim ekosustavima (Gaillardet i sur., 2004.). Kada antropogeni unos nadmaši prirodni, dolazi do poremećaja prirodnog biogeokemijskog ciklusa metala, što za posljedicu može imati povećanu akumulaciju metala u vodenim organizmima te eventualno i prekomjerni unos u ljudski organizam (Flora i sur., 2008.; Mikac i sur., 2007.). U vodenim ekosustavima, metali se javljaju u raznovrsnim organskim i anorganskim oblicima, od hidratiziranih iona do velikih organskih kompleksa (Slika 2.), a njihova specijacija u vodi određuje njihovu bioraspoloživost, što predstavlja glavni čimbenik o kojemu ovisi utjecaj toksičnih metala na organizme. Naime, vrste metala u tragovima u vodi su raspodijeljene između različitih fizičkih i kemijskih oblika, npr. jednostavnih anorganskih i organskih spojeva, labilnih i inertnih kompleksa, kao i onih adsorbiranih na čvrstim i koloidnim česticama (Branica, 1999.).

Najjednostavnija kategorizacija metala u vodi zasniva se na podjeli na otopljenu i partikularnu fazu nakon filtriranja uzoraka vode kroz filter promjera pora 0,45 μm. Frakcija koja prođe kroz filter definirana je kao otopljena, dok je frakcija prikupljena na filteru definirana kao partikularna. Unatoč tome, kroz filter promjera pora 0,45 μm mogu proći i koloidni oblici metala koji obuhvaćaju čestice čija se veličina nalazi u rasponu 1 nm do 450 nm te mogu biti organski i anorganski (Gaillardet i sur., 2004.).

Nakon što metali dospiju u okoliš, mogu mijenjati kemijski oblik te tako postati više ili manje bioraspoloživi, a time i toksičniji za živi svijet u vodi. Na kemijski oblik u kojem se nalazi pojedini metal, odnosno na specijaciju metala u prirodnom okolišu utječe niz čimbenika, poput saliniteta, pH, temperature, prisustva organske tvari i otopljenog kisika (Mason, 2013.). Primjerice, pri nižim pH vrijednostima (kiseliji okoliš), više metala je prisutno u obliku hidratiziranog metalnog iona koji je pristupačniji i toksičniji za biotu (Campbell i Stokes, 1985.). Također, na primjeru kalifornijske pastrve (*Oncorhynchus mykiss*) dokazano je kako je toksičnost pojedinih metala (Cu, Zn, Pb i Hg) slabija u tvrdoj vodi, jer Ca ima veći afinitet za vezanje na membranu škrga riba, u odnosu na ostale katione u vodi, što smanjuje njihovu apsorpciju kroz škrge i posljedično akumulaciju u tkivima riba (Mason, 2002.). Utjecaj okolišnih čimbenika na metale u vodi, kao i činjenica da je voda dinamični medij (posebno u rijekama), ukazuje kako za procjenu utjecaja metala na biotu nije dovoljno odrediti samo ukupnu koncentraciju nekog metala u vodi. Ta koncentracija zapravo odražava trenutnu vrijednost u trenutku uzorkovanja i ne daje informaciju o biološki raspoloživoj koncentraciji toga metala, kao i o njegovim potencijalnim toksičnim učincima (Florence i Batley, 1977.).



Slika 2. Shematski prikaz sastavnica vode, prema njihovoj veličini, na temelju korištenja filtera promjera pora 0,45 µm (preuzeto i prilagođeno iz Aiken i sur., 2011.).

Radi pouzdane procjene onečišćenja vodenih sustava metalima i njegovog utjecaja na žive organizme u vodi te posljedično i na ljude putem prehrane, potrebno je istražiti posljedice dugoročne izloženosti biote metalima, izučavanjem bioakumulacije i toksičnih učinaka metala na vodene organizme. Kao primjer slatkovodnih sustava umjereno i izrazito onečišćenih metalima bit će prikazane rijeka Sutla u Hrvatskoj te tri rijeke sjeveroistočne Sjeverne Makedonije, Bregalnica, Kriva Reka i Zletovska Reka.

2.1.1. Rijeka Sutla u Hrvatskoj

Rijeka Sutla izvire u Sloveniji na južnim obroncima Maceljske gore te je granična rijeka između Hrvatske i Slovenije (Slika 3.), ukupne dužine oko 91 km, od toga u Hrvatskoj 89 km. Prema Okvirnoj direktivi o vodama Europske unije, rijeka Sutla svojom površinom porječja od 581 km^2 pripada kategoriji srednje velikih rijeka (EPCEU, 2000.; Dragun i sur., 2011.). U rijeku Savu, jednu od triju najdužih rijeka na teritoriju Republike Hrvatske, utječe kao lijeva pritoka kod Savskog Marofa. Nadalje, rijeka Sutla nalazi se u području Hrvatskog zagorja, koje je bogato termalnim izvorima i kupeljima (Teskeredžić i sur., 2009.).

Slika 3. Karta rijeke Sutle s označenim mjestima uzorkovanja (Hum na Sutli, Donje Brezno, Kumrovec, Klanjec te Drenje Brdovečko) (preuzeto iz Dragun i sur., 2011.).

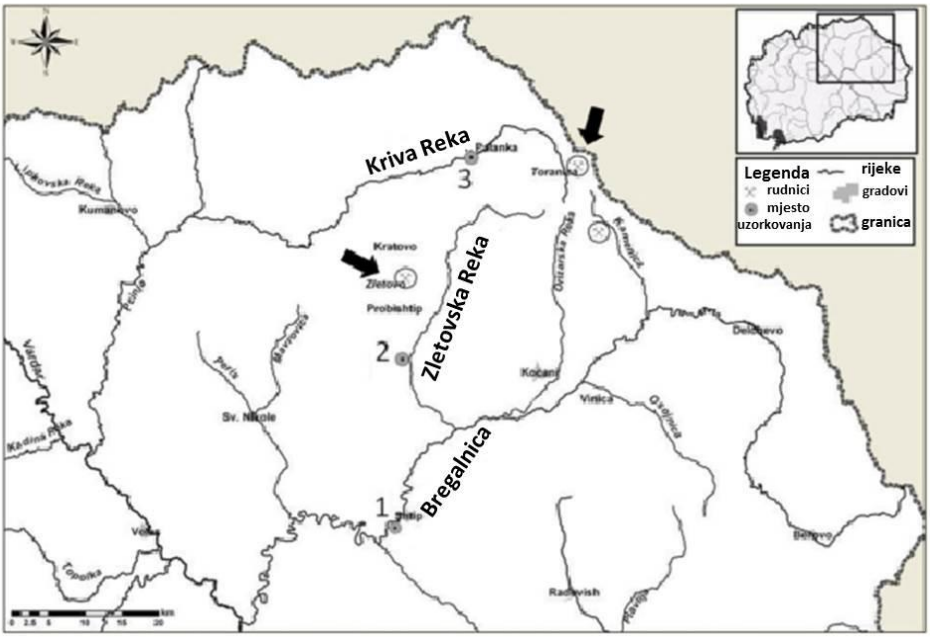
Rijeka Sutla smatra se umjereno onečišćenom metalima, a poznati izvori onečišćenja odnose se na male industrijske objekte, poput tvornice stakla, te na komunalne otpadne vode, poljoprivredne aktivnosti i ispuštanje otpadnih voda iz termalnih kupališta (Dragun i sur., 2011.). Zbog smanjene mogućnosti razrjeđenja unesenih toksičnih tvari uslijed niskog protoka vode (tijekom 2009. godine: 0,73-68,8 m^3s^{-1}; Dragun i sur., 2011.), mali vodotoci poput rijeke Sutle posebno su osjetljivi na onečišćenje. Istraživanje provedeno na rijeci Sutli u jesen 2009. godine (Dragun i sur., 2011.) pokazalo je kako su unatoč umjerenom antropogenom utjecaju koncentracije nekoliko otopljenih metala (Fe, Mn i Cd) na pojedinim postajama u ovoj rijeci bile uočljivo povišene, što je vidljivo iz širokog raspona njihovih koncentracija (Fe: 3,1-80,5 μg L^{-1}; Mn: 0,44-261,1 μg L^{-1}; Cd: 6-308 ng L^{-1}). Nasuprot tome, koncentracije otopljenog Zn i Cu bile su niske duž cijelog riječnog toka (Zn: <5 μg L^{-1}; Cu: 0,17-3,74 μg L^{-1}). Najviše su koncentracije metala zabilježene u riječnoj vodi gornjeg toka rijeke Sutle, uglavnom na postajama Hum na Sutli (utjecaj industrije stakla) i Donje Brezno

(mogući utjecaj otpadnih voda termalnih kupališta) (Dragun i sur., 2011.). Iako koncentracije metala izmjerene u rijeci Sutli još uvijek nisu premašile ograničenja koja se smatraju opasnima za život u vodi ili u konačnici za ljudsko zdravlje, uočena su značajna povećanja te se zabilježene koncentracije metala u vodi ove rijeke treba smatrati upozorenjem i poticajem za zaštitu malih i srednjih rijeka s ciljem sprječavanja budućeg pogoršanja, prema preporukama Okvirne direktive o vodama (ODV; EPCEU, 2008.)

2.1.2. Rijeke sjeveroistočnog dijela Sjeverne Makedonije

Otpadne vode rudnika ubrajaju se, uz difuzno onečišćenje izazvano poljoprivrednim aktivnostima, u najopasnije izvore onečišćenja koji mogu dovesti do degradacije slatkovodnih ekosustava širom svijeta (Byrne i sur., 2012.; Cerqueira i sur., 2011.; Environmental Agency, 2006.; Silva i sur. 2011. a, b, c). Uzrok toksičnosti otpadnih voda rudnika nalazi se u njihovoj izrazitoj kiselosti i visokim koncentracijama metala. U kiselim vodama metali su većinom prisutni u ionskom obliku, koji je biološki raspoloživiji te se lako akumulira u ribama i drugim vodenim organizmima, što značajno ugrožava njihovo zdravlje i vodi ka smanjenju bioraznolikosti (Jordanova i sur., 2016.; Stuhlberger, 2010.; Wojtkowska, 2013.).

Geološka i tektonska obilježja makedonskog teritorija bila su preduvjet za stvaranje raznovrsnih, brojnih i često veoma velikih ležišta metala, nemetala i energetskih mineralnih resursa (Spasovski i sur., 2011.). Posljednjih godina Sjeverna Makedonija je zauzela značajno mjesto u regiji u iskorištavanju ruda olova i cinka. Od brojnih nalazišta olovnih i cinkovih ruda posebno treba istaknuti najvažnija područja bogata tim mineralnim sirovinama, a to su područje Sasa - Toranica, te Kratovo i Zletovo. Ondje se nalaze tri velika rudnika olova i cinka: Zletovo, Sasa i Toranica. Ti rudnici predstavljaju sirovinsku bazu olova i cinka i pratećih nusproizvoda (srebra, bizmuta, kadmija, sumporne kiseline, superfosfata, itd.) (Spasovski i sur., 2011.). Zbog brojnih aktivnih rudnika, otpadne vode rudnika u Makedoniji još uvijek predstavljaju vrlo ozbiljan ekološki problem, uzimajući u obzir da su koncentracije Pb i Zn u rudama i ležištima i do tisuću puta više u odnosu na ostala područja (Alderton i sur., 2005.; Midžić i Silajdžić, 2005.). Ramani i sur. (2014.) procijenili su utjecaj dvaju trenutno aktivnih rudnika Pb i Zn (Zletovo i Toranica) na kakvoću vode dviju rijeka u sjeveroistočnom dijelu Sjeverne Makedonije (Slika 4.), vezano uz onečišćenje metalima i promjene fizikalno-kemijskih karakteristika riječne vode.



Slika 4. Karta područja istraživanja s označenim mjestima uzorkovanja u sjeveroistočnoj Sjeverne Makedoniji (Bregalnica, Zletovska Reka i Kriva Reka) (Ramani i sur., 2014.).

2.1.2.1. Rijeka Bregalnica

Rijeka Bregalnica druga je po veličini makedonska rijeka te je najduža lijeva pritoka rijeke Vardar, najveće rijeke u Republici Sjevernoj Makedoniji. Ukupna dužina rijeke je 225 km, a površina njenog sliva iznosi 4307 km². Rijeka Bregalnica je manje onečišćena metalima od ostalih rijeka sjeveroistočne Sjeverne Makedonije jer nije pod izravnim utjecajem rudnika. Onečišćenje ove rijeke karakteristično je za područja intenzivne poljoprivredne aktivnosti, a javlja se zbog ispiranja tla rižinih polja (Andreevska i sur., 2013.) te se odnosi na pojavu povišenih koncentracija nekoliko metala/metaloida (na primjer, As, Ba, Fe, Mo, U i V), nitrata i fosfata, kao i pojavu izrazitog fekalnog onečišćenja vode uslijed utjecaja poljoprivrednih farmi grada Štipa (Ramani i sur., 2014.). U ovoj rijeci nađene su i povišene koncentracije herbicida koji se koriste u uzgoju riže (Stipaničev i sur., 2017.).

2.1.2.2. Zletovska Reka

Ukupna dužina Zletovske Reke je 56 km, a površina njenog sliva iznosi 460 km². U gornjem dijelu toka ova rijeka protječe kroz strmu klisuru koju je sama izdubila u stijenama te zbog toga na pojedinim mjestima ima pad od 42%, puno brzaca i dva vodopada. Jedna je od najzagađenijih pritoka rijeke Bregalnice (Dolenec i sur., 2005.), a zagađenje pretežno potječe od rudnika Pb i Zn, Zletovo. Rudnik je aktivno radio od 1940.-ih, a njegova proizvodnja i

dalje traje, iako je bilo nekoliko kratkotrajnih prekida. Rude iz rudnika Zletovo, koje sadržavaju više od 9% Pb i 2% Zn te značajnu količinu Ag, Bi, Cd i Cu (Alderton i sur., 2005.), obrađuju se u malom gradiću Probištipu dok se otpad iz rudnika odlaže u susjednim dolinama. Pritoke Zletovske Reke, Kiselica i Koritnica, ispiru glavno područje iskopa ruda kod Probištipa, (Alderton i sur., 2005.) te tako metali dospijevaju i u druge rijeke. Osim toga, otpadne vode iz rudnika Zletovo, onečišćene metalima, ispuštaju se iz koncentracijskog postrojenja u rijeku Kiselicu, pritoku Zletovske Reke, bez prethodne obrade ili neutralizacije. Zbog toga u Kiselici nema mnogo života, odnosno prisutne su samo one vrste vodenih organizama koje su otpornije na onečišćenje te su u njima nađene visoke razine metala (Midžičić i Silajdžić, 2005.). U Zletovskoj Reci, nizvodno od rudnika Zletovo, nađene su iznimno visoke koncetracije Cd, Co, Cs, Cu, Li, Mn, Ni, Rb, Sn, Sr, Tl i Zn te sulfata i klorida. Osobito visoke koncentracije pojedinih metala bile su izmjerene u jesen 2012. godine, u vrijeme izrazito niskog vodostaja (npr., Cd 2,0 $\mu g\ L^{-1}$; Mn 2,5 $mg\ L^{-1}$; Zn 1,5 $mg\ L^{-1}$), što je jasan pokazatelj utjecaja rudnika na kakvoću riječne vode (Ramani i sur., 2014.). Pored onečišćenja rudarskim otpadom (Dragun i sur., 2019.; Ramani i sur., 2014.), Zletovska Reka je i pod utjecajem otpadnih voda grada Probištipa, uključujući i otpadne vode tvornice akumulatora (Spasovski i Dambov, 2009.).

2.1.2.3. Kriva Reka

Kriva Reka je najveća pritoka rijeke Pčinje, koja je lijevi pritok rijeke Vardar i najznačajnija rijeka u sjeveroistočnom dijelu Sjeverne Makedonije. Dužina ove rijeke je 78,7 km, dok površina sliva iznosi 968 km^2 (Ramani i sur., 2014.). Onečišćenje Krive Reke metalima potječe pretežno od otpada iz aktivnog rudnika Pb i Zn, Toranica. Proizvodnja Pb i Zn iz rudnika Toranica traje od 1987. godine, s nekoliko jednogodišnjih prekida nakon 2000. godine (Alderton i sur., 2005.). Rude iz rudnika Toranica sadržavaju oko 6,5% Pb+Zn s dodatno povišenim koncentracijama Cd, Cu, Mn, Ag i Bi (Serafimovski i sur., 2007.). Prema Ramani i sur. (2014.) u Krivoj Reci su zabilježene povišene koncentracije samo za Cd (0,270 $\mu g\ L^{-1}$) i Pb (1,85 μgL^{-1}) u proljeće 2012. godine te je, slično kao u Bregalnici, zamijećeno i izrazito fekalno onečišćenje, kao i povišene koncentracije amonijevih i fosfatnih iona, vjerojatno uslijed utjecaja otpada okolnih vrtova i obradivih površina.

2.2. Metali u vodenim organizmima

Unos metala u vodene organizme iz okolišne vode može se odvijati putem cijele vanjske površine organizma, putem respiratornih organa (škrge), putem probavnog epitela iz vode i hrane ili kombinacijom tih puteva (Brown i Depledge, 1998.). Nakon ulaska u izvanstanične tjelesne tekućine, unos metala u stanicu se vrši preko staničnih membrana procesima jednostavne difuzije, olakšane difuzije, aktivnog transporta i pinocitoze (Simkiss, 1998.), pri čemu je za unos u stanice najraspoloživiji hidratizirani ionski oblik metala. Ribe izlučuju štetne tvari iz organizma putem žuči, bubrega, škrga i kože (Heath, 1995). Akumulirana količina metala u stanicama odražava razliku između unosa i izlučivanja metala (Rainbow, 2018.).

S obzirom na svoju funkciju u živim organizmima metali se mogu podijeliti na esencijalne i neesencijalne. Metali koji predstavljaju važnu komponentu u metaboličkim procesima živih organizama, a dio su enzima ili vitamina, ubrajaju se u esencijalne ili biološki neophodne metale. To su, na primjer, makroelementi poput Ca, K, Mg i Na te mikroelemeti poput Zn, Cu, Co, Se i Fe, koji su ljudima i životinjama potrebni u malim količinama, ali prekomjernim unosom i oni postaju toksični (Verma i Dwiwedi, 2013.). One metale i metaloide za koje do sada nije otkrivena uloga u metaboličkim procesima živih organizama nazivamo neesencijalnima, a to su, na primjer, Pb, As, Hg, Ni i Cd koji su toksični već u vrlo malim količinama (Verma i Dwiwedi, 2013.). Međutim, za veći broj mikroelemenata ustanovljeno je da na organizam mogu djelovati kako u toksičnom tako i u stimulativnom smislu, ovisno o unesenoj koncentracijskoj razini (Luckey i sur., 1975.). Stoga oštro razgraničavanje metala/metaloida na apsolutno toksične ili netoksične nije u potpunosti prihvatljivo s obzirom na raznolikost fiziološko-biokemijskih mehanizama svojstvenih organizmima različitih dobi i vrsta. Reakcija organizma na metale koja ovisi o promjeni njihovih koncentracija može se opisati krivuljom koja prikazuje međuovisnost koncentracije i učinka, gdje se za svaki metal/metaloid može definirati područje tolerancije, s graničnim donjim i gornjim koncentracijama, koje čini prijelaz između stanja deficijencije i intoksikacije. To područje tolerancije za svaki pojedini metal ovisi o djelotvornosti regulacijskih mehanizama u organizmu, koji se znatno razlikuju među organizmima na različitom stupnju razvoja (Engel i Fowler, 1979.)

U vodenim organizmima metali mogu izazvati široki spektar učinaka koji se mogu pojaviti na raznim biološkim razinama: na razini stanice, organa, organizma, populacije, zajednice ili kombinacijama tih kategorija (Phillips i Rainbow, 1993.). Učinci na razini

organizma obično se dijele na morfološke (deformacije ljuštura, skeleta, škrga, itd.), bihevioralne (motoričke promjene, promjene hranjenja) i fiziološke. Fiziološki učinci obuhvaćaju promjene na razini cijelog organizma, na razini tkiva i organa, ali i stanične promjene (biokemijske i citokemijske). Svaki toksični učinak započinje biokemijskim procesima na razini stanice koji potom mogu dovesti do promjena na cijelom organizmu. Učinci metala na staničnoj razini su mnogobrojni, a neki od njih su: inhibiranje ili promjene funkcija proteina, oksidativni stres, inhibicija mitohondrijske aktivnosti, poremećaj propusnosti membrana (Phillips i Rainbow, 1993.; Dragun i sur., 2017.). Iako brojni metali u organizmima imaju važne biološke uloge, kao sastavni dio enzima ili drugih složenih molekula, takozvanih metaloproteina (Smith i sur., 1997.), oni svojim vezanjem na različite proteine mogu uzrokovati njihovu inhibiciju ili im promijeniti funkciju (Viarengo, 1985.). To se može dogoditi zamjenom esencijalnog metala vezanog na protein ili vezanjem na deaktivacijsko mjesto enzima. Štetni učinak metala može se očitovati i kroz oksidativni stres, odnosno stvaranjem reaktivnih oblika kisika. Reaktivnih oblici kisika obuhvaćaju radikale kisika (superoksidni, hidroksilni, peroksidni) i reaktivne neradikalne derivate kisika (vodikov peroksid, hipokloritna kiselina) te mogu izazvati inaktivaciju enzima, lipidnu peroksidaciju, oštećenja DNA, kao i povećati rizik od razvoja raka (Viarengo, 1985.). Nadalje, mitohondriji, organele odgovorne za aerobnu sintezu ATP-a, akumuliraju velike količine metala, između ostalog Hg, Cd, Zn i Fe. Inhibicija mitohondrijske aktivnosti i oksidativne fosforilacije ubrajaju se u moguće štetne učinke metala (Viarengo, 1985.). Poremećaj propusnosti membrana može se očitovati kroz poremećaj aktivnog i pasivnog transporta. Živa i Cd se primjerice mogu vezati na fosfatnu skupinu lipidnog sloja membrane i tako poremetiti pasivni transport iona. Aktivni transport se može narušiti izravno inhibicijom enzima Na/K ovisne ATP-aze, ili posredno, smanjenjem raspoloživosti ATP-a (Bouquegnau i Gilles; 1979; Viarengo, 1985.).

U organizmu, međutim, postoje različiti homeostatski mehanizmi za održavanje koncentracija metala u uskim rasponima, kao i detoksikacijski mehanizmi, koji sprječavaju, umanjuju ili poništavaju štetne interakcije metala s esencijalnim makromolekulama (Mason i Jenkins, 1995.), poput stvaranja netopljivih granula metala, odjeljivanja metala unutar lizosoma te vezanja metala za specifične biomolekule, pa do toksičnog učinka dolazi samo ukoliko je narušena ravnoteža (Viarengo i Nott, 1993.).

Uz sve navedeno, toksični utjecaj metala na organizam ovisi i o stanju organizma, odnosno o stupnju razvoja, starosti, veličini, spolu, prebivalištu i prehrani (Livingstone, 1993.), kao i o nizu vanjskih čimbenika, odnosno o uvjetima okoliša poput temperature,

saliniteta, pH vrijednosti, intenziteta svjetlosti, otopljenog kisika, itd. (Hamelink i sur., 1994.). Prisustvo drugih toksičnih tvari također može utjecati na djelovanje metala na organizme, izazivajući sinergistički ili antagonistički učinak.

2.3. Bioindikatori

U procjeni onečišćenja pojedinih ekosustava metalima koriste se prikladni bioindikatorski organizmi. Bioindikatori su organizmi koji svojom prisutnošću ili specifičnom reakcijom na određene čimbenike u okolišu mogu odražavati stanje okoliša. Bioindikatorske vrste, osim ekološkog stanja nekog vodenog sustava, mogu biti i dobar pokazatelj antropogenog utjecaja na taj ekosustav. Osnovne karakteristike indikatorskih organizama su široka rasprostranjenost i zastupljenost vrste, ograničeno područje kretanja, dovoljno dug životni vijek, dovoljna veličina za provođenje analiza te relativno jednostavna determinacija i dostupnost mjesta uzorkovanja (de Andrade i sur., 2004.). Kao bioindikatorski organizmi za praćenje izloženosti slatkovodnih ekosustava metalima najčešće se koriste ribe, školjkaši i raci (Dragun i sur., 2015). Prednost riba kao bioindikatora u tome je što se nalaze na vrhu prehrambenog lanca u slatkovodnim ekosustavima, imaju potencijal za akumuliranje metala, dug životni vijek i optimalnu veličinu za provođenje analiza, lako se uzorkuju te imaju veliku ekonomsku važnost (Dragun i sur., 2013.a i b). Budući da pripadaju kralježnjacima, pogodne su za izučavanje i zbog fiziološke sličnosti sa sisavcima, a k tome su i važan dio ljudske prehrane pa razina bioakumulacije metala iznad pravilnikom definiranih vrijednosti može predstavljati opasnost za ljudsko zdravlje (Baldwin i Kramer, 1994.). Zbog svih navedenih karakteristika ribe se smatraju jednim od najznačajnijih bioindikatora u slatkim vodama za procjenu onečišćenja metalima u tragovima (Evans i sur., 1993.; Barak i Mason, 1990.; Rashed, 2001.). Međutim, značajan problem kod upotrebe riba kao indikatorskih organizama ponekad može predstavljati njihova mobilnost, uslijed čega je teško precizno definirati mjesto i izvor onečišćenja, kao i vrijeme i trajanje izloženosti (Chovanec i sur., 2003.).

Riblja vrsta koja se često primjenjuje kao bioindikatorski organizam u europskim vodotocima je klen (*Squalius cephalus* L., 1758). Klen ima izvrsnu sposobnost prilagodbe tako da nastanjuje i tekućice i jezera te se ubraja u skupinu predatorskih riba (Habeković i sur., 1993.). Dok se u rijekama u Hrvatskoj može naći vrsta *S. cephalus*, u makedonskim rijekama obitava srodna vrsta istoga roda, odnosno vardarski klen (*Squalius vardarensis* Karaman, 1928).

2.3.1. Klen (*Squalius cephalus* L., 1758)

Klen (*Squalius cephalus* L., 1758) (Slika 5.), pripadnik porodice šaranki (Cyprinidae), slatkovodna je omnivorna riblja vrsta široko rasprostranjena u Europi s izrazitom otpornošću na kemijsko i fizikalno zagađenje (Gandolfi i sur., 1991.; Maitland i Campbell, 1992.; Vostradovsky, 1973.).

Znanstvena klasifikacija:

Carstvo:	Animalia
Koljeno:	Chordata
Razred:	Actinopterygii
Red:	Cypriniformes
Porodica:	Cyprinidae
Rod:	*Squalius*
Vrsta:	*Squalius cephalus* Linnaeus, 1758

Slika 5. Prikaz klena *Squalius cephalus* Linnaeus, 1758.
Autor fotografije: dr. sc. Damir Valić.

Naseljava mnoge stajaćice i tekućice nizinskog, prijelaznog i djelomično visinskog karaktera koje pripadaju slivu Sjevernog, Baltičkog, Crnog i Azovskog mora u Europi i dijelu Azije (Habeković, 1982.). Ima vretenasto snažno tijelo, prilagođeno plivanju i u pojačanim vodenim strujama (Habeković i sur., 1993.). Može narasti i do 80 cm dužine i 4 kg mase. Spolno sazrijeva u dobi od 4 do 5 godina pri dužini od 20 cm, a mrijesti se višekratno od travnja do lipnja kada temperatura vode dostigne 15°C, u plitkim zonama bogatim kisikom i čistog dna (Habeković, 1982.; Habeković i sur., 1993.). Unatoč velikoj sposobnosti

prilagođavanja, najčešće se zadržava ispod brana i drugih zapreka, gdje se hrani. S obzirom na sve navedeno, kao i na široku rasprostranjenost i relativno dug životni vijek, klen predstavlja pogodan bioindikatorski organizam za praćenje kakvoće slatkih voda (Kurtović i sur., 2007.; 2008.).

2.3.2. Vardarski klen (*Squalius vardarensis* Karaman, 1928)

U makedonskim rijekama može se naći druga vrsta iz roda *Squalius,* odnosno vardarski klen (*Squalius vardarensis* Karaman, 1928) (Slika 6.). Vardarski klen nastanjuje rijeke i potoke umjerenih tokova na području Sjeverne Makedonije i Grčke, a može narasti i do 60 cm ukupne dužine te se mrijesti od svibnja do lipnja u rijekama i potocima s čistom vodom (Kottelat i Freyhof, 2007.). Budući da je vrhunski grabežljivac, vardarski klen predstavlja izvrstan izbor za praćenje utjecaja kemijskih agenasa i metala na slatkovodnu biotu (Jordanova i sur., 2016.).

Znanstvena klasifikacija:

Carstvo:	Animalia
Koljeno:	Chordata
Razred:	Actinopterygii
Red:	Cypriniformes
Porodica:	Cyprinidae
Rod:	*Squalius*
Vrsta:	*Squalius vardarensis* Karaman, 1928

Slika 6. Prikaz vardarskog klena (*Squalius vardarensis* Karaman, 1928).
Autorica fotografije: dr. sc. Zrinka Dragun.

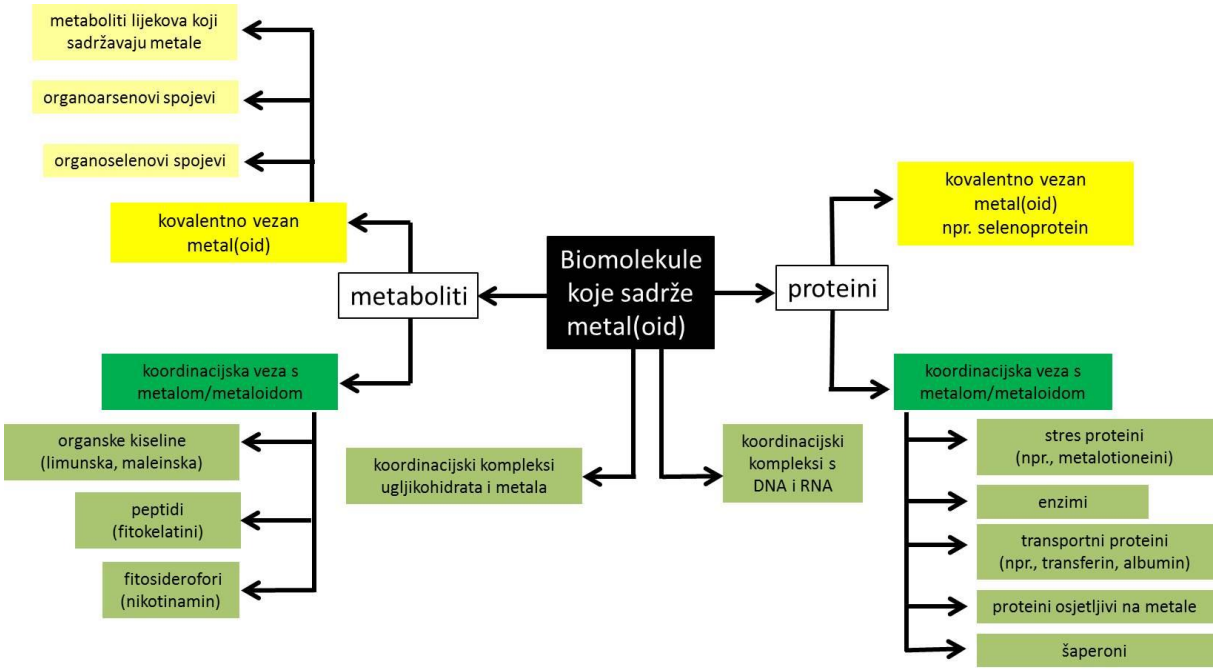
2.3.3. Ciljni organi za analizu metala

Metali se mogu akumulirati u različitim dijelovima organizma riba, ovisno o kojem se metalu radi. Primjerice, Pb ima veliki afinitet za kalcificiranim tkivima dok se u mekim tkivima najviše taloži u jetrima i bubrezima, nakon čega slijedi koža, mozak i mišići (Roberts i sur., 1976.; Sedak i sur., 2015.), Cd ima najveći afinitet za taloženje u jetrima (oko 50 % Cd) i bubrezima (Sedak i sur., 2015.; Siddiqui, 2010.), Zn u jetrima (Bawuro i sur., 2018.; Dragun i sur., 2019.), dok se Hg akumulira u mišićima, a k tome je jedan od rijetkih metala koji se i biomagnificira u hranidbenom lancu te može biti izrazito toksična za ljude putem prehrane (Frodello i sur., 2000.; Mason i sur., 2006.; Sedak i sur., 2015.). Za procjenu dugotrajne, kronične izloženosti riba metalima najbolje je analize provoditi u jetrima (Miller i sur., 1992.), budući da se radi o glavnom detoksikacijskom organu i metaboličkom centru organizma (Giguere i sur., 2004.) te organu koji raspolaže najučinkovitijom sposobnošću akumulacije raznih zagađivala (Papagiannis i sur., 2004.; Vukosav i sur., 2014.). Nadalje, hepatociti, dominantna vrsta stanica u jetrima, sadrže, kao obrambeni mehanizam, visoke razine unutarstaničnih proteina i peptida koji vežu metale i pomažu u njihovom uklanjanju, tako sprječavajući interakcije metala s potencijalno osjetljivim biomolekulama; to obilježje jetara omogućava proučavanje metala i detoksikacijskih mehanizama (Di Giulio i Hinton, 2008.; Heath, 1995.; Sigel i sur., 2009.). S druge strane, škrge su, kao važno mjesto izravnog unosa toksičnih tvari iz vode, prikladne za praćenje kratkotrajne izloženosti metalima te naglih promjena u razini izloženosti. Zbog relativno niske topljivosti kisika u vodi, ribe imaju vrlo veliku respiratornu površinu, propuštaju veliki volumen vode kroz škrge u jedinici vremena te imaju tanke epitelne membrane, što sve zajedno olakšava unos tvari iz vode, kao i njihov prijenos u krv (Chovanec i sur., 2003.; Heath, 1995.; Reid i McDonald, 1991.).

2.4. Metalomika

Metali se u živim organizmima javljaju vezani na cijeli niz različitih biomolekula (Slika 7.). Veliki broj proteina zahtijeva metal kao kofaktor, a obično se radi o prijelaznim metalima, poput Cu, Fe, Zn ili Mo (Mounicou i sur., 2009.; Tainer i sur., 1991.). Metalni ioni, nadalje, mogu kontrolirati regulaciju ekspresije proteina u stanicama, npr. metalotioneina, proteina bitnog u homeostazi i procesima detoksikacije (Maret, 2004.). Šaperoni metala štite i usmjeravaju metalne ione kroz citoplazmu (Rosen, 2005.; Rosenzweig, 2002.), dok su izvanstanični proteini poput albumina i transferina bitni kao transporteri metala u krvi (Mounicou i sur., 2009.). Stoga je unutarstanična koncentracija nekoliko metala, njihova

raspodjela među različitim staničnim odjeljcima te njihova ugradnja u metaloproteine strogo kontrolirana (Outten i O'Halloran, 2001.).

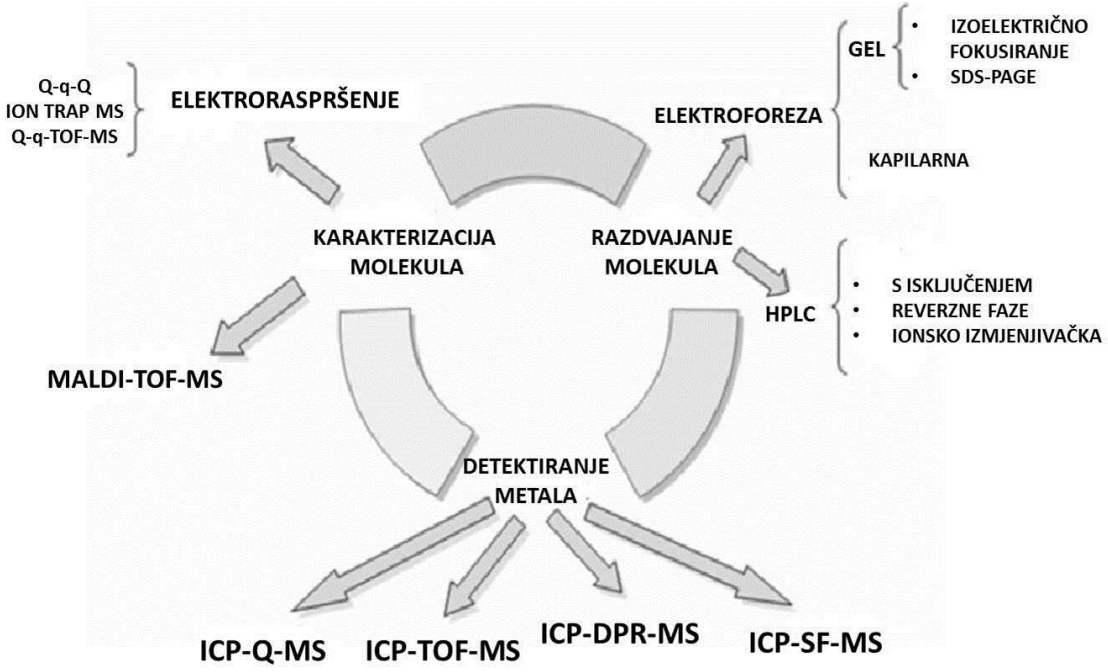


Slika 7. Različiti oblici metala u biološkom okruženju (Mounicou i sur., 2009.).

Zbog svega navedenog, sustavni pristup proučavanju sadržaja metala, njihove specijacije, lokalizacije i upotrebe unutar organizama i ekosustava postaje sve važniji (Thiele i Gitlin, 2008.). U prošlosti su istraživanja metala u okolišu bila pretežno usmjerena na fiziološke mehanizme unosa, toksičnosti i izlučivanja te se većina razvijenih metoda odnosila na mjerenje ukupnih koncentracija pojedinačnih metala u vodi i u organizmima, koje ne daju informaciju o metabolizmu i mehanizmima toksičnosti metala, kao ni o mehanizmima njihove detoksikacije (de la Calle Guntińas i sur., 2002.). Posljednjih godina razvijene su „omičke" tehnike, koje uključuju strukturnu genomiku, transkriptomiku, proteomiku, toksikoproteomiku i metalomiku te koje su usmjerene na sveobuhvatna izučavanja na molekularnoj i staničnoj razini, a koriste se i u okolišnim istraživanjima za prepoznavanje novih biomarkera učinaka raznih zagađivala na organizme (Shi i Chance, 2008.). U skladu s tim, metalomika 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 izučavanje kompleksiranja elemenata, kao i utjecaja tog kompleksiranja na okoliš i na ljudsko zdravlje. Metalomika se, nadalje, bavi proučavanjem i razjašnjavanjem fizioloških uloga i funkcija biomolekula koje vežu metalne ione u biološkim sustavima

(Monicou i sur., 2009.). Predstavlja transdisciplinarno istraživačko područje, koje povezuje razna druga znanstvena područja, poput geokemije, kliničke biologije, farmakologije, biljne i životinjske fiziologije (Monicou i sur., 2009.). Krajnji je cilj metalomike pružiti sveopće i sustavno razumijevanje unosa, prijenosa, uloge i izlučivanja metala u biološkim sustavima (Monicou i sur., 2009.). Istraživanja u području metalomike provode se pomoću različitih tehnoloških i metodoloških pristupa, no ne postoji nijedna tehnologija koja bi bila prikladna za samostalnu primjenu. Analize se sastoje od niza koraka koji zahtijevaju različite metodološke pristupe, a organizacija i integracija analitičkih koraka predstavljaju ključ uspjeha (Gómez-Ariza i sur., 2005.)

Metalomičke analize, dakle, zahtijevaju složene multidimenzionalne analitičke pristupe, uključujući (1) tehnike razdvajanja, poput različitih tehnika tekućinske kromatografije i elektroforeze, (2) detektore visoke osjetljivosti specifične za elemente te (3) molekularne detektore utemeljene na spektrometriji masa za karakterizaciju razdvojenih biomolekula koje vežu metale (Slika 8.) (Gómez-Ariza i sur., 2004.; Mounicou i sur., 2009.; Szpunar i Łobiński, 2002.).



Slika 8. Spregnute tehnike u analizama metala i biomolekula koje vežu metale (prilagođeno prema radu Mounicou i sur., 2009.).

2.4.1. Analitičke metode za razdvajanje i pročišćavanje biomolekula na koje se vežu metali

Za analizu metaloproteina i drugih biomolekula koje vežu metale razvijene su brojne metode razdvajanja, poput kromatografije i elektroforeze (kapilarna ili gel elektroforeze) (Gómez-Ariza i sur., 2005.; Monicou i sur., 2009.; Szpunar i Łobiński, 1999.).

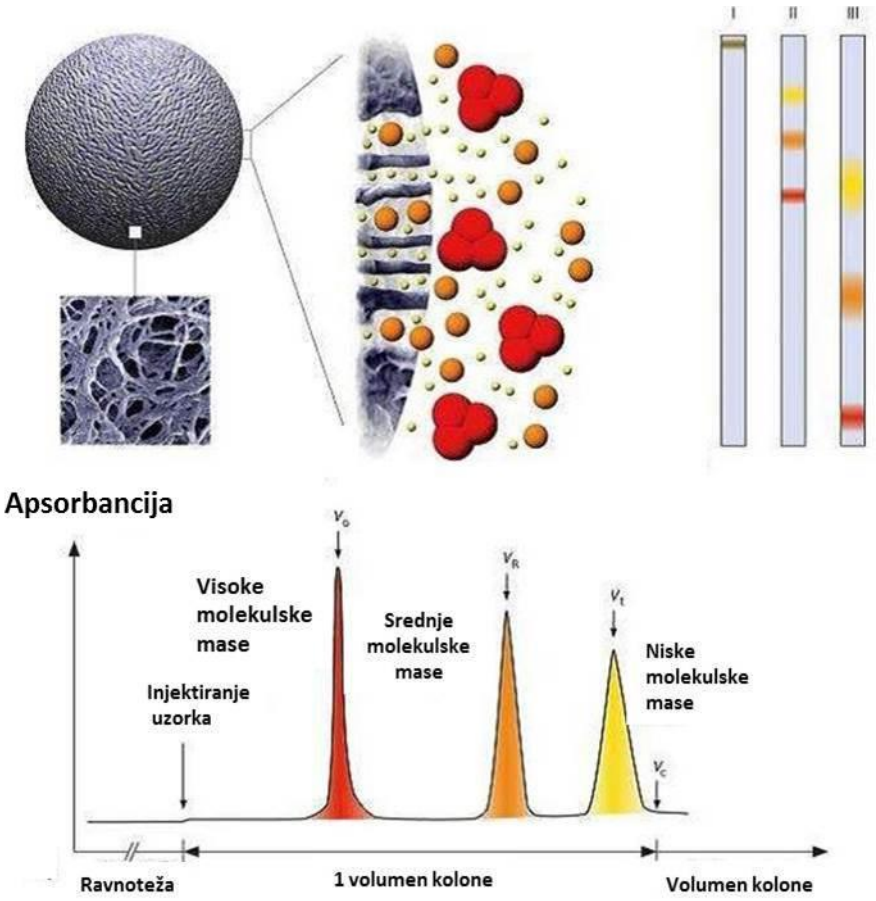
Kromatografija je jedna od analitičkih metoda koje se najčešće primjenjuju, a čiji je razvoj počeo šezdesetih godina prošlog stoljeća (Majors, 2003.). Tekućinska kromatografija osnovna je višenamjenska tehnika razdvajanja koja se primjenjuje u modernim biološkim znanostima i u komplementarnim znanstvenim područjima kao što su analitička ili preparativna kemija (Cindrić i sur., 2009.). To je tehnika koja se koristi za razdvajanje otopljenih tvari. Tvari iz otopina u različitoj mjeri stupaju u interakciju sa stacionarnom (nepokretnom) i tekućom mobilnom (pokretnom) fazom na osnovu razlika u adsorpciji, ionskoj izmjeni, razdiobi između faza, razlici u veličini čestica ili stereokemijskih interakcija. Kao posljedica navedenih razlika, različite se tvari različito dugo zadržavaju na kromatografskoj stacionarnoj fazi (Cindrić i sur., 2009.; Sattayasai, 2012.).

Za razliku od mnogih drugih tehnika razdvajanja koje imaju različita ograničenja, poput plinske kromatografije koja nije pogodna za razdvajanje toplinski nestabilnih molekula, tekućinska kromatografija može uspješno poslužiti za razdvajanje širokog raspona molekula kao što su polimeri, male molekule farmaceutika ili njihovih metabolita, kao i peptida i proteina (Cindrić i sur., 2009.). Nadalje, dodatna prednost tekućinske kromatografije je mogućnost izravnog povezivanja s drugim analitičkim tehnikama (Cindrić i sur., 2009.). Za početno pročišćavanje staničnih biomolekula koje vežu metale korisna je primjena kromatografije s isključenjem po veličini (SEC) u kombinaciji s ionsko-izmjenjivačkom kromatografijom (IEC). Primjenom ovih dviju metoda postiže se visoka osjetljivost i selektivnost (Montes-Bayón i sur., 2003.; Szpunar, 2004.) te zadovoljavajuće razlučivanje prilikom razdvajanja biomolekula koje vežu metale, odnosno izbjegava se istovremeno eluiranje više oblika istog elementa (Mounicou i sur., 2009.).

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

SEC-HPLC često se koristi kao osnovna metoda za razdvajanje metaloproteina i drugih biomolekula koje vežu metale (Slika 9.). Temelji se na učinku molekularnih sita koja omogućavaju razdvajanje molekula prema njihovoj veličini te u manjoj mjeri i prema obliku (de la Calle Guntinuas i sur., 2002.; Szpunar, 2004.). Najbolji je izbor za analize proteina i

raznih drugih biomolekula, budući da nakon pročišćavanja izvorna struktura i funkcija proteina ostaju očuvane, a može se koristiti i široki spektar pufera kako bi se ostvarili odgovarajući uvjeti za razdvajanje pojedinih proteina (Rambo, 2017.).



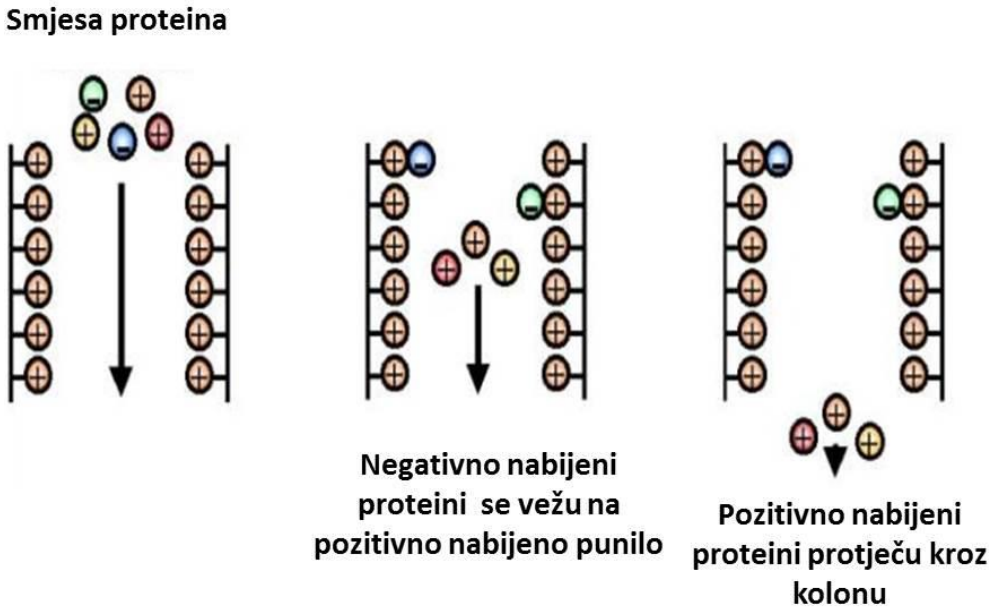
Slika 9. Kromatografija isključivanjem ili gel-filtracijska kromatografija. Metoda se temelji na difuziji molekula kroz porozne šupljine punila koje sačinjavaju kolonu. Mehanizam razdvanja se temelji na prodiranju molekula u pore čestica punila, tako da manje molekule, koje mogu ulaziti u pore, zaostaju, dok veće, koje to ne mogu, prolaze između čestica punila te prije izlaze iz kolone. S obzirom na to da pore u punilu stacionarne faze, nisu jednake veličine, samo najveće molekule bit će u potpunosti izuzete od prodiranja u pore punila, dok će sve druge ulaziti s većom ili manjom učestalosti (manje češće, veće rjeđe), te će duže zaostajati u punilu stacionarne faze. Tako će najprije izaći najveće molekule, pa onda srednje i na kraju najmanje molekule. Slika je prilagođena prema Hagel i Haneski (2010.).

Prednost ove metode je što proteini, ako se razdvajanje odvija pod blagim, fiziološkim uvjetima, ostaju gotovo netaknuti, a to je jako važno pri razdvajanju biomolekula koje su osjetljive na promjenu pH te analizama metaloproteina (De la Calle Guntiñas i sur., 2002.).

Dodatnu prednost u odnosu na ostale kromatografske metode ima zbog mogućnosti povezivanja sa spektrometrijom masa uz ionizaciju induktivno spregnutom plazmom (ICP-MS) za određivanje koncentracija metala. Odabirom odgovarajućih kromatografskih uvjeta koji ne štete analizi spektrometrijom masa (npr., hlapljivih pufera, stabilnog i niskog protoka, upotrebe polarnih organskih otapala), tekućinski se kromatograf može jednostavno povezati sa spektrometrom masa (Cindrić i sur., 2009.). U mnogim je istraživanjima tehnika SEC-HPLC u kombinaciji s ICP-MS-om korištena kao polukvantitativna tehnika za specijaciju elemenata poput Cd (Ferrarello i sur., 2000.), Se (McSheehy i sur., 2001.), ili As (McSheehy i Szpunar, 2000.).

2.4.1.2. Ionsko izmjenjivačka tekućinska kromatografija visoke djelotvornosti (IEC-HPLC)

Biološke molekule su uglavnom polarne te stoga mogu biti nabijene. Upravo je to svojstvo molekula iskorišteno u izvedbi ionsko-izmjenjivačke kromatografije (IEC), koja se zasniva na razdvajanju otopljenih molekula na osnovu razlika u naboju (Slika 10.).



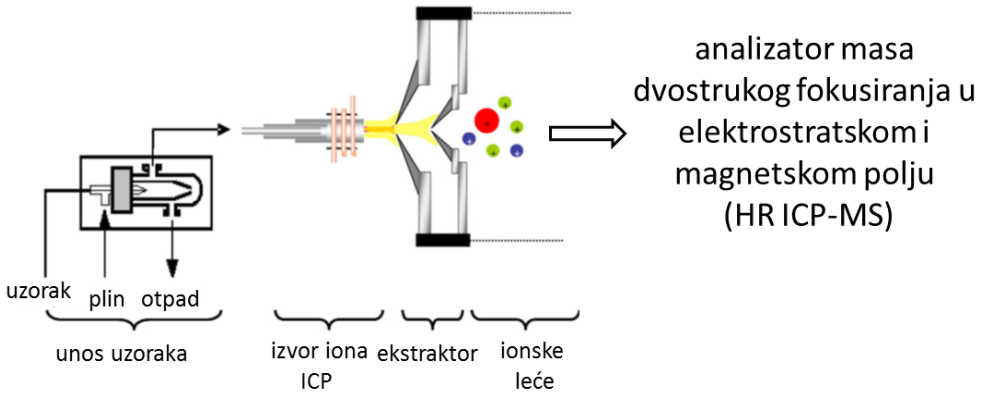
Slika 10. Princip anionsko-izmjenjivačke kromatografije. Proteini se razdvajaju na temelju svoga neto-naboja (preuzeto i prilagođeno iz Berg i sur., 2002.).

Može se primijeniti za razdvajanje gotovo bilo koje vrste nabijenih molekula uključujući velike proteine, male nukleotide i aminokiseline (Loeschner i sur., 2015.). Dvije vrste ionsko-izmjenjivačke kromatografije su anionsko-izmjenjivačka (AEC) i kationsko-

izmjenjivačka (CEC) kromatografija. Molekule koje su topljive i nabijene, kao što su proteini, aminokiseline i peptidi, vežu se na suprotno nabijene funkcionalne skupine netopljive stacionarne faze stvaranjem ionskih veza (Sattayasai, 2012.). U kationsko-izmjenjivačkoj kromatografiji primjenjuje se anionska stacionarna faza kada su željene molekule koje se razdvajaju kationi, a anionsko-izmjenjivačka kromatografija primjenjuje kationsku stacionarnu fazu za odjeljivanje aniona. Molekule vezane na stacionarnu fazu ispiru se povećavanjem ionske jakosti ili promjenom pH eluensa, odnosno mobilne faze. Kationsko i anionsko izmjenjivačka kromatografija često se koriste u istraživanjima metala u biološkim uzorcima (Sanz-Medel i sur., 2003.; Szpunar, 2000.), na primjer pri razdvajanju metalotioneina (Lehman i Klaassen, 1986.; Rodríguez-Cea i sur., 2003.; Van Campenhout i sur., 2008.) i serumskih proteina (Soldado Cabezuelo i sur., 1997.; Wrobel i sur., 1995.).

2.4.2. Analitičke metode za određivanje metala u uzorcima iz okoliša

Postoji niz različitih tehnika kojima se mogu određivati metali u uzorcima iz okoliša, kao i u kromatografski razdvojenim frakcijama tih uzoraka (Bettmer i sur., 2009.; Garcia i sur., 2006; Gómez-Ariza i sur., 2004.; 2005.; Szpunar, 2004.). Kemijske analize metala provode se korištenjem sofisticiranih metoda visoke osjetljivosti poput atomske apsorpcijske spektrometrije (AAS - plamena i grafitna tehnika), atomske emisijske spektrometrije s induktivno spregnutom plazmom (ICP-AES), spektrometrije masa s induktivno spregnutom plazmom (ICP-MS) te elektrokemijskih metoda (Csuros i Csuros, 2002.; Lobinski i Marczenko, 1997.; Michalke and Nischwitz, 2010.; Skoog i sur., 1999). U nekim slučajevima koriste se i dodatne tehnike, poput analize neutronske aktivacije (NAA) ili djelomično inducirane rendgenske emisije (PIXE).

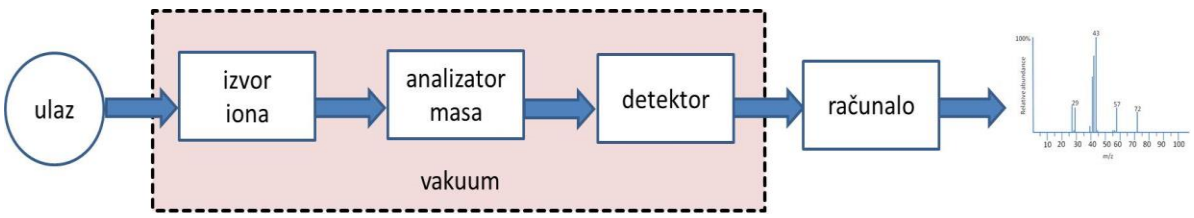


Slika 11. Shematski prikaz spektrometrije masa uz ionizaciju induktivno spregnutom plazmom (ICP-MS; preuzeto iz Bettmer i sur., 2009.)

ICP-MS (Slika 11.) je svestrana tehnika u kojoj se induktivno spregnuta plazma koristi kao ionizacijski izvor, a detekcija se vrši spektrometrijom masa, na temelju određivanja omjera mase i naboja (*m*/*z*) analiziranih iona (Montaser, 1998.; Thomas, 2013.). Od svoje prve pojave tijekom ranih 1980.-ih (Houk i sur., 1980.), ICP-MS tehnika postala je jednim od najvažnijih načina određivanja više od 70% elemenata periodnog sustava (Betmmer i sur., 2009.) te spada u najbrže razvijajuće tehnike za analizu metala u tragovima (Grochowski i sur., 2019.). Iako se njome mogu odrediti isti elementi kao i ostalim spektroskopskim tehnikama, ICP-MS ima veliku prednost zbog multielementne i brze analize te niskih granica detekcije (Al-Hakkani, 2019.; Garcia i sur., 2006.; He i sur., 2017.).

2.4.3. Primjena spektrometrije masa (MS) u analizi biomolekula koje vežu metale

U prošlom nas je desetljeću razvoj spektrometrije mase doveo u novo doba analize proteina i peptida. Spektrometrija masa radi na principu ioniziranja kemijskih komponenti promatranog uzorka stvarajući nabijene molekule ili fragmente molekula te zatim mjeri njihove omjere mase i naboja (*m*/*z*) (Banerjee i Mazumdar, 2012.; Galić, 2004.; Gross, 2004.). Metode spektrometrije masa neprekidno se razvijaju, a u današnje se vrijeme upotrebljavaju za određivanje masa čestica, sastava uzoraka ili molekula, kvalitativnog i kvantitativnog određivanja sastava smjesa te za razjašnjavanje kemijskih struktura molekula, primjerice peptida (Galić i Cindrić, 2008.; Urban, 2016.). Spektrometrija masa omogućuje određivanje masa peptida i/ili proteina, identifikaciju proteina, određivanje aminokiselinskog slijeda, identifikaciju i određivanje položaja post translacijskih modifikacija, određivanje mutacija te provjeru struktura i čistoće proteina dobivenih genetskim inženjeringom (Calderón-Celis i sur., 2018.; de Hoffman i Stroobant, 2007.; Graves i Haystead, 2002.; Rathore i sur., 2018.). Nadalje, primjenjuje se i u istraživanju različitih interakcija između proteina i iona metala, malih organskih molekula i različitih bioloških spojeva (Galić i Cindrić, 2008.; Gibson i Costello, 2000.).



Slika 12. Shematski prikaz spektrometra masa

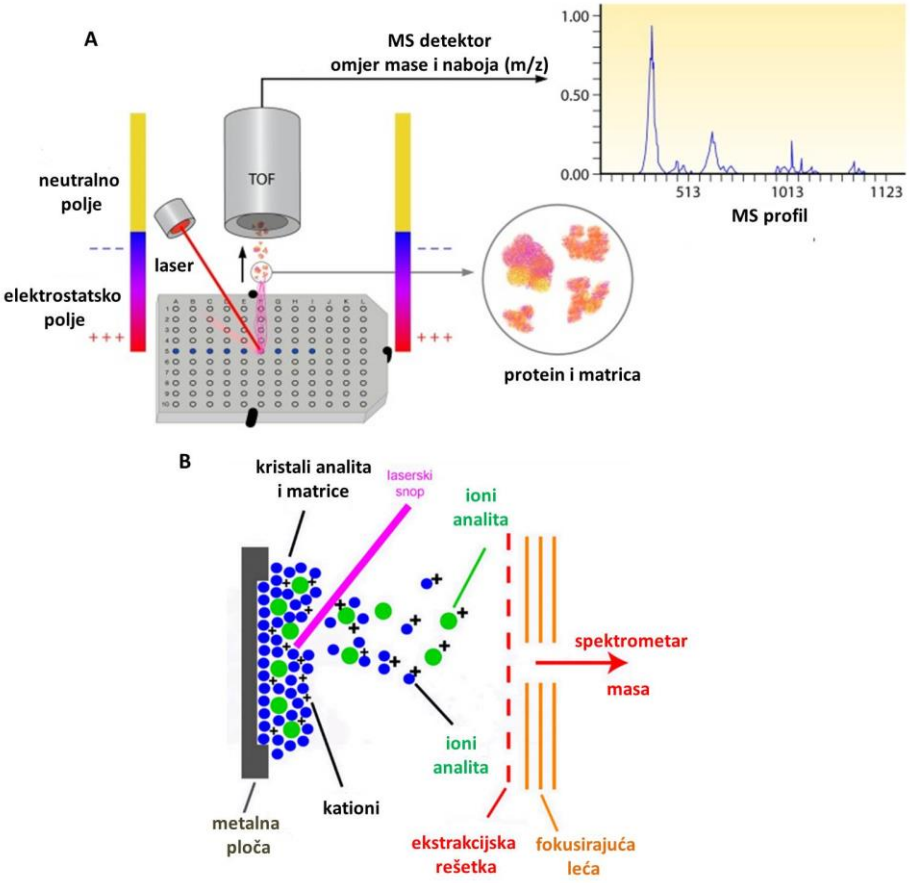
Uređaj, spektrometar masa (Slika 12.), sastoji se od ionskog izvora, analizatora masa te detektora i sustava za obradu podataka (de Hoffmann i Stroobant, 2007.; Galić i Cindrić 2008.). Nakon unosa uzorka u spektrometar masa dolazi do ionizacije i pretvorbe molekula analita u plinsku fazu što je osnovni zahtjev za daljnu analizu. Ioni nastali u plinovitoj fazi u analizatoru masa se razdvajaju na temelju omjera *m*/*z* djelovanjem magnetskog ili električnog polja (Galić, 2004.). Detekcija se vrši pomoću elektronskog pojačala ili scintilacijskog brojača (Mornar, 2013.; Sertić, 2013.; Watson, 1999.), a rezultati se prikazuju grafički u obliku spektara masa koji pokazuju relativnu zastupljenost različitih ionskih vrsta (Galić, 2004.; Nigović, 2015.).

Postoji nekoliko različitih metoda ionizacije, a za analizu peptida i proteina najčešće se koriste ESI i MALDI tehnike. Analiza proteina spektrometrijom masa obuhvaća analizu intaktnog proteina ili analizu peptida nastalih enzimskom ili kemijskom razgradnjom proteina te se može provoditi pristupima odozgo nadolje (engl. t*op-down*) i odozdo nagore (Slika 13.) (engl. b*ottom-up*) (Bogdanov i Smith, 2005.; Chait, 2006.; Kelleher, 2004.; Khalsa-Moyers i McDonald, 2006.; Monicou i sur., 2009.; Switzar i sur., 2013.).

Učestaliji način analize proteina je odozdo nagore, a uključuje cijepanje proteina i odstranjivanje posttranslacijskih modifikacija enzimskim putem (pomoću proteaza ili glikozidaza), kemijskim putem (pomoću hidrolizirajućih reagensa) ili kombinirano (Amunugama i sur., 2013.; Galić i sur., 2008.; Gillet i sur., 2016.; Manes i Nita-Lazar i sur., 2018.). Zbog visokospecifične i ponovljive reakcije cijepanja proteina najčešće korišteni proteolitički reagens je tripsin. Cijepanjem tripsinom u većini slučajeva nastaju peptidi duljine od 5-30 aminokiselina, što je optimalna duljina niza za analizu spektrometrijom masa (Corthals i sur., 1999.; Gundry i sur., 2001.). Dva osnovna pristupa u identifikaciji proteina su identifikacija proteina putem pretrage baze podataka i identifikacija proteina *de novo* sekvenciranjem (Reinders i sur., 2004). Identifikacija proteina postiže se putem pretrage baze podataka i temelji se na usporedbi eksperimentalno dobivenih podataka s teoretski izračunatim vrijednostima dobijenim iz baza podataka proteinskih sekvenci, dok se identifikacija proteina sekvenciranjem *de novo* temelji na eksperimentalnom iščitavanju slijeda aminokiselina na osnovu MS/MS spektra (Galić i Cindrić, 2008.; Nesvizhskii, 2007.; Taylor i Johnson, 2001.).

2.4.3.1. Matricom potpomognuta ionizacija uz desorpciju laserskim zračenjem (MALDI)

MALDI (engl. Matrix-Assisted Laser Desorption/Ionization) je tehnika ionizacije molekula koju su utemeljili znanstvenici Karas i Hillenkamp 1988. godine,koji su prvi snimili spektar biomolekula molekulskih masa iznad 10 kDa u smjesi uzorka s matricom, dok je Koichi Tanaka 2002. godine dobio Nobelovu nagradu za kemiju za proučavanje velikih biomolekula pomoću MALDI ionizacijske tehnike (Cho i Normile, 2002.).



Slika 13. Shematski dijagram koji prikazuje tijek rada MALDI-TOF-MS-a. Kratkim pulsevima lasera (1-20 ns) bombardiraju se kristali matrice i uzorka što dovodi do njihove desorpcije i ionizacije (preuzeto i prilagođeno iz Clark i sur., 2013.).

MALDI-TOF-MS (Slika 13.) je brza metoda, kojom se u kratko vrijeme može analizirati veliki broj uzoraka (Kuckova i sur., 2007.), te je u današnje vrijeme jedna od najpopularnijih tehnika spektrometrije masa koja se koristi za brzu i osjetljivu analizu biomolekula (Dave i sur., 2011.; Horvatić i Cindrić, 2009.; Swiatly i sur., 2017.; Webster i Oxley, 2012.). Ubraja se u blage ionizacijske tehnike koje omogućuju ionizaciju molekula

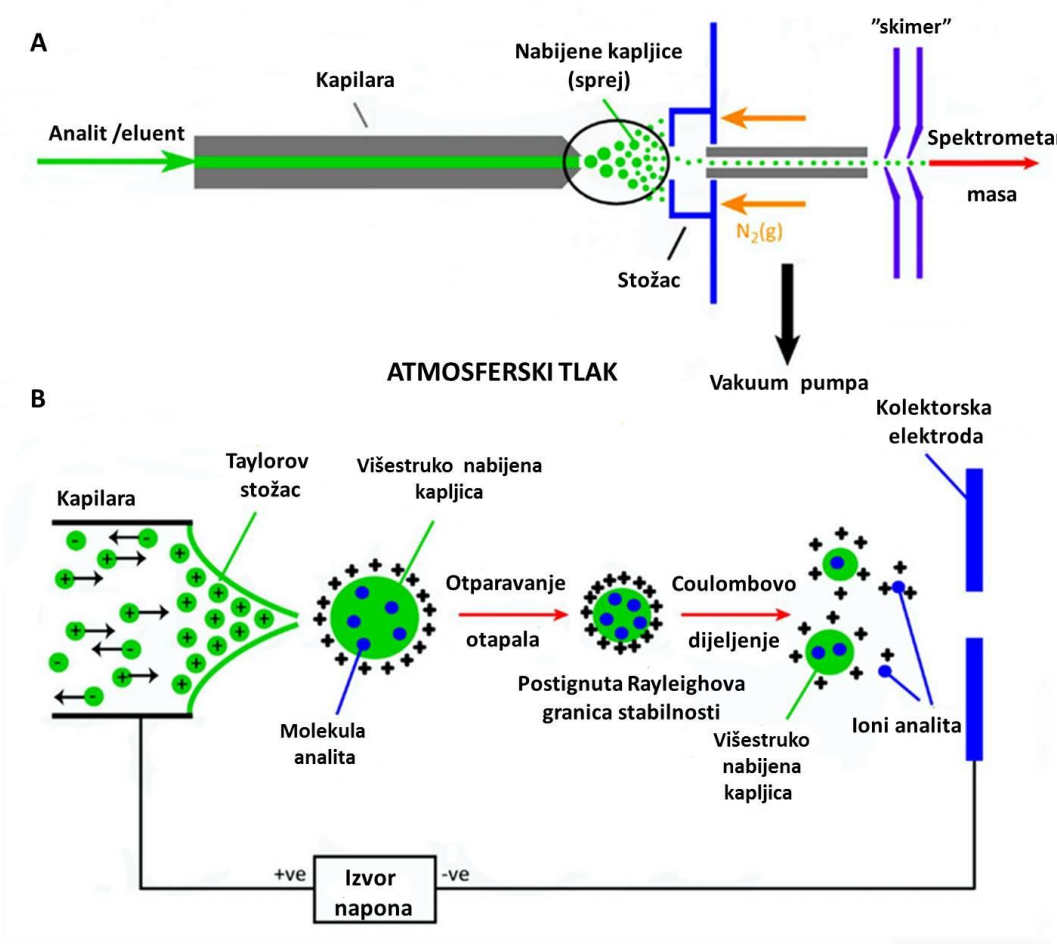
vrlo velikih molekulskih masa (do 200 kDa) te ovom tehnikom uglavnom nastaju jednostruko nabijeni ioni (Galić i Cindrić, 2008.; Horvatić i Cindrić, 2009.; Singhal i sur., 2015.). Analit se ugrađuje u kristalnu strukturu molekula matrice i kristalizira s matricom nakon isparavanja otapala (Kazazić i sur., 1999.; Chait i Kent, 1992.; Chalmers i Gaskel, 2000.; Pomastowski i sur., 2019.). Potom se kristalizirana matrica pod djelovanjem laserske zrake trenutno zagrijava (Senko i sur., 1994.) te dolazi do isparavanja i ionizacije molekula matrice i analita, pri čemu nastaju ioni protoniranih molekula, koji su najčešće jednostruko nabijeni, a javlja se i slabo izražena fragmentacija (Galić i Cindrić, 2008.). Ioni zatim ulaze u analizator masa, gdje se razdvajaju prema omjeru mase i naboja (Cindrić i sur., 2009.; Galić i Cindrić, 2008.; Kazazić i sur., 1999.; Pomastowski i sur., 2019.). Navedena se tehnika ionizacije zbog svojeg pulsnog karaktera najčešće primjenjuje u kombinaciji s analizatorom koji mjeri vrijeme leta (eng. Time Of Flight, TOF), odnosno vrijeme potrebno da ioni stignu do analizatora, što je proporcionalno njihovoj masi (Boesl, 2017.; de Hoffmann i Stroobant, 2007.; Galić i Cindrić, 2008.).

Ova je analitička metoda pogodna za analizu biomolekula i proteina koji vežu metale (Szpunar, 2004.; Szpunar i Łobiński, 2002.). MALDI-TOF-MS se odlikuje iznimnom osjetljivošću od femtomola do pikomola, a čak postoje radovi u kojima se određivanja vrše na nivou atomola (Gevaert i sur., 1997), velikom brzinom analiza (moguće je analizirati oko 100 uzoraka u 10 min) (Duncan i sur., 2008.; Galić i Cindrić, 2008.; Kuckova i sur., 2007.).

2.4.3.2. Ionizacija elektroraspršenjem (ESI)

ESI (engl. Electrospray Ionization) tehnika također spada u skupinu blagih ionizacijskih tehnika, a postupak se odvija pri atmosferskom tlaku (Slika 14.). Ioni koji nastaju mogu biti pozitivnog ili negativnog naboja te ovisno o kemijskoj strukturi analita, odnosno broju kemijskih skupina koje se mogu ionizirati, mogu biti jednostruko ili višestruko ionizirani (Cindrić i sur., 2009.; Mornar i sur., 2013.). Iako je pojava ionizacije elektroraspršenjem poznata još s početka 20. stoljeća, i prvi opis principa objavljen je 1968. (Dole i sur., 1968.), do razvoja ESI došlo je sredinom 1980.-ih nakon radova što su ih objavili John Fenn (Fenn i sur., 1989.; Yamashita i Fenn, 1984.) i grupa ruskih istraživača (Aleksandrov i sur., 1984.). Za ovaj doprinos znanosti, Fenn je dobio Nobelovu nagradu za kemiju 2002. godine. Važnost ESI-ja i njegov ogroman doprinos modernoj spektrometriji masa proizlazi iz njegovih jedinstvenih karakteristika. To je vrlo blagi način ionizacije koji uglavnom rezultira nastankom višestruko nabijenih molekulskih iona peptida/proteina te je moguće sačuvati i nekovalentno vezane biomolekularne komplekse tijekom prevođenja

molekula analita u plinsku fazu (Awad i sur., 2015.; Banerjee i Mazumdar, 2012.; Talkington i sur., 2005.).



Slika 14. Shematski dijagram koji prikazuje tijek rada u ESI-MS-u (preuzeto i prilagođeno prema Gates, 2014.). Proces elektroraspršenja prikladno je podijeliti u tri faze: nastajanje kapljica, otparavanje kapljica i nastajanje iona u plinskoj fazi (Bruins, 1998.; Cech i sur., 2001.; Galić, 2004.; Galić i Cindrić, 2009.).

Za razliku od MALDI ionizacije, primjenom ESI-ja nastaju i višestruko nabijeni ioni (Galić, 2004.; Ozeki i sur., 2019.). Kod ovog oblika ionizacije plinska faza i analit ulaze u ionizator kroz kapilaru koja predstavlja elektrodu priključenu na pozitivni kraj izvora napona (Cindrić i sur., 2009.). Pod utjecajem električnog polja razdvajaju se pozitivni i negativni naboji u otopini, pri čemu pozitivno nabijeni ioni putuju prema katodi i akumuliraju se na površini tekućine koja se nalazi na kraju kapilare (de Hoffmann i Strobant, 2007.). Pri kritičnoj jakosti električnog polja, na vršku kapilare se formira tzv. Taylorov stožac u kojem se neprestano proizvode kapljice obogaćene pozitivno nabijenim ionima (Bruins, 1998.;

Galić, 2004.; Ho i sur., 2003.;). Otapalo se zatim otparava pod utjecajem struje dušika, temperature i električnog potencijala te se smanjuje veličina kapljica (Cindrić i sur., 2009.). Kada se kapljice dovoljno smanje, tako da se svi ioni nalaze na njihovoj površini, dolazi do njihovog otparavanja ili razbijanja na manje kapljice (Coulombovo dijeljenje), nakon čega analit. Tako formirani molekulski ioni prelaze u visoki vakuum do analizatora masa (Slika 14.) (Cindrić i sur., 2009.; Crotti i sur., 2011.; Ho i sur., 2003.).

Dok je kod MALDI ionizacije neophodan kristalizirani uzorak, ESI ionizira analit u otopini što čini ovu tehniku pogodnom za povezivanje s HPLC sustavima (Glish i Vachet, 2003.). MALDI je pogodna tehnika za analizu relativno jednostavnih smjesa peptida (npr., pojedinačnih proteina), dok se HPLC-ESI-MS sustavi koriste za kompleksne uzorke (Fenn i sur., 1989.). Osjetljivost ESI tehnike dodatno je povećana smanjenjem brzine protoka analizirane otopine (npr. mikroraspršenjem ili nanoraspršenjem) pa je granica detekcije spuštena do reda veličine atomola (10^{-18} mol) (Galić, 2004.; Valaskovic i sur., 1995.). Dodatna prednost u analizi iona može se ostvariti primjenom tandemne spektrometrije masa (LC-MS/MS), s ciljem da se poboljša fragmentacija iona, kako bi se na temelju dobivenih spektara masa odredila struktura analiziranog iona (Goudog i sur., 2008.).

2.4.4. Metalomika u okolišnim istraživanjima

Od svoga su otkrića, ionizacijske metode spektrometrije masa elektroraspršenje (ESI-MS) i matricom potpomognuta ionizacija i desorpcija laserskim zračenjem (MALDI-MS) te ICP-MS postale iznimno važne tehnike u analizi bioloških makromolekula (Gómez-Ariza i sur., 2004.; Mounicou i sur., 2009.; Sanz-Medel, 2005.; Szpunar, 2004.). Iako navedene tehnike u metalomici pružaju veliki broj mogućnosti u istraživanjima (Montes-Bayón i sur., 2003.), što je prepoznato u mnogim znanstvenim područjima, primjena metalomike u okolišnim istraživanjima nije još dovoljno prisutna te se takva istraživanja uglavnom provode u strogo kontroliranim laboratorijskim uvjetima, najčešće nakon izlaganja samo jednome metalu, dok su istraživanja u stvarnim okolišnim uvjetima iznimno rijetko zastupljena. Primjer takvih laboratorijskih istraživanja su istraživanja metabolizma Fe na laboratorijskim štakorima hranjenim mlijekom bogatim željezom (Alves Peixoto i sur., 2019.; Fernández-Menéndez i sur., 2018.) te Cd i As na izlaganom domaćem mišu (*Mus musculus;* García-Sevillano i sur., 2014.).

Sustavni metalomički pristup u izučavanju biomolekula koje vežu metale u raznim vodenim organizmima još uvijek nije opsežno primijenjivan te stoga predstavlja područje širokih mogućnosti za ekotoksikološka znanstvena istraživanja. Dosad je otkriveno tek

nekoliko metaloproteina u ribama koji se primjenjuju kao biomarkeri, dok njihove funkcionalne uloge u fiziologiji riba još uvijek nisu u cijelosti poznate (Hauser-Davis i sur., 2012.). Donedavno se metalomička tehnologija na vodenim organizmima uglavnom koristila u istraživanju pojedinačnih metaloproteina, poput metalotioneina (MT), primjenom različitih tehnika tekućinske kromatografije visoke djelotovornosti (Lobinski i sur., 1998.). Razdvajanje biomolekula pomoću dvodimenzionalne kromatografije (SEC-AEC-HPLC) primijenjeno je tijekom istraživanja MT iz nekoliko ribljih vrsta, poput jegulje (*Anguilla anguilla*; Rodríguez-Cea i sur., 2003.; Van Campenhout i sur., 2008.), bisernog ciklida (*Geophagus brasiliensis*; Rodríguez-Cea i sur., 2006.), plosnatica (*Limanda limanda* i *Microstomus kitt*; Duquesne i Richard, 1994.), limande (*L. limanda*; Lacorn i sur., 2001.), kao i iz dagnji (*Mytilus edulis*; Geret i Cosson, 2002.). Nadalje, Goenaga Infante i sur. (2006.) analizirali su komplekse metala s izoformama MT primjenom AEC-HPLC-a u kombinaciji s ICP-MS-om u babuški (*Carassius auratus gibelio*), dok su Lavradas i sur. (2016.) primjenom elektroforeze (SDS-PAGE) i SEC-HPLC-a u kombinaciji s ICP-MS-om proveli istraživanje toplinski stabilnih proteina metalotioneina u školjkašima (*Perna perna*). Dvodimenzionalno razdvajanje pomoću SEC-AEC-HPLC-a nadograđeno MS analizama primijenjeno je i u identifikaciji kompleksa Hg, Cd, Cu i Zn s metalotioneinima u jetrima bjeloboke pliskavice (*Lagenorhynchus acutus*; Pedrero i sur., 2012.) te u istraživanju indukcije i vezanja Cd, Cu i Zn na MT u jetrima, bubregu i škrgama šarana (*Cyprinus carpio;* Goenaga Infante i sur., 2003.).

Nadalje, pojedini istraživači metalomički pristup koriste u istraživanju pojedinačnih metala i biomolekula koje ih vežu. Na primjer, primjenom višestrukog analitičkog pristupa koji povezuje ionsko-izmjenjivačku kromatografiju, detektore za elemente (ICP-MS) i molekularne detektore (ESI-MS, LC-MS/MS i MALDI-TOF-MS), sa svrhom olakšane identifikacije metaloproteina u vrlo niskim koncentracijama (Gómez-Ariza i sur., 2004.), omogućena je i identifikacija i karakterizacija biomolekula koje vežu Fe u kalifornijskoj pastrvi (*Oncorhynchus mykiss*; Fago i sur., 2002.), dok je karakterizacija selenoproteina u pacifičkoj plavoperajnoj tuni (*Thunnus orientalis*) provedena dvodimenzionalnim kromatografskim razdvajanjem (SEC-AEC-HPLC) u kombinaciji s LC-ESI-MS-om (Yamashita i Yamashita, 2010.).

Kanadska grupa znanstvenika tek je nedavno započela s istovremenim istraživanjem sudbine većeg broja metala u vodenim organizmima, uključujući njihovu raspodjelu unutar tkiva i u različitim unutarstaničnim odjeljcima (npr., citosolu, granulama i organelima) primjenom diferencijalnog centrifugiranja (Vijever i sur. 2004.; Campbell i sur. 2005.; Giguère i sur., 2006.) te njihovu specijaciju u citosolu primjenom različitih analitičkih tehnika

koje se primjenuju u metalomici (Caron i sur., 2018.; Urien i sur., 2018.). Primjena diferencijalnog centrifugiranja značajna je za procjenu raspodjele metala između staničnih frakcija osjetljivih na metale i detoksiciranih frakcija metala. Prema Wallace i sur. (2003.), stanične frakcije osjetljive na prisutnost metala uključuju organele (mitohondriji, lizosomi, mikrosomi) i toplinski nestabilne proteine (poput enzima), dok detoksicirane frakcije metala uključuju granule bogate metalima i toplinski stabilne proteine (poput metalotioneina). Takva je unutarstanična raspodjela metala specifična za pojedini metal, organ i organizam, ali i dinamična ovisno o uvjetima izloženosti metalima i nizu drugih okolišnih čimbenika (Wang i Rainbow, 2006.). Ista je skupina znanstvenika u novije vrijeme proširila svoja istraživanja uvođenjem metalomičkih metoda, provodeći istovremene analize cijelog niza metala u ribama s ciljem određivanja njihove raspodjele među citosolskim biomolekulama različitih molekulskih masa. Citosolske raspodjele za Ag, Cd, Cu, Co, Ni i Tl odredili su u mladim američkim žutim grgečima (*Perca flavescens*, Caron i sur., 2018.), a za Cd, Cu, As i Se u bijelim sisačima (*Catostomus commersonii*, Urien i sur., 2018.), pomoću kombinacije SEC-HPLC-a i ICP-MS-a.

Metalomičke pristupe i strategije potrebno je češće sustavno primjenjivati u okolišnim istraživanjima, sa svrhom otkrivanja mehanizama koji se nalaze u pozadini metaboličkih funkcija, mehanizama detoksikacije i toksičnih učinaka metala te naposljetku identificiranja novih biomarkera izloženosti metalima i njihovih učinaka (Gómez-Ariza i sur., 2004.; Hauser-Davis i sur., 2012.; Mounicou i sur., 2009.).

ZNANSTVENI RADOVI

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RESEARCH ARTICLE

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.)

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Abstract Association of selected essential (Co, Cu, Fe, Mn, Mo, Se, and Zn) and nonessential (Cd, Pb) trace elements with cytosolic proteins of different molecular masses was described for the liver of European chub (*Squalius cephalus*) from weakly contaminated Sutla River in Croatia. The principal aim was to establish basic trace element distributions among protein fractions characteristic for the fish living in the conditions of low metal exposure in the water. The fractionation of chub hepatic cytosols was carried out by size exclusion high performance liquid chromatography (SE-HPLC; Superdex™ 200 10/300 GL column), and measurements were performed by high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). Elution profiles of essential elements were mostly characterized by broad peaks covering wide range of molecular masses, as a sign of incorporation of essential elements in various proteins within hepatic cytosol. Exceptions were Cu and Fe, with elution profiles characterized by sharp, narrow peaks indicating their probable association with specific proteins, metallothionein (MT), and ferritin, respectively. The main feature of the elution profile of nonessential metal Cd was also single sharp, narrow peak, coinciding with MT elution time, and indicating almost complete Cd detoxification by MT under the conditions of weak metal exposure in the water (dissolved Cd concentration ≤0.3 μg L^{-1}). Contrary, nonessential metal Pb was observed to bind to wide spectrum of proteins, mostly of medium molecular masses (30–100 kDa), after exposure to dissolved Pb concentration of ~1 μg L^{-1}. The obtained information within this study presents the starting point for identification and characterization of specific metal/metalloid-binding proteins in chub hepatic cytosol, which could be further used as markers of metal/metalloid exposure or effect on fish.

Keywords European chub · Hepatic cytosol · Trace elements · Proteins · SE-HPLC · HR ICP-MS

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Introduction

Many trace elements play important biological roles, notably as integral parts of enzymes or protein structures (Smith et al. 1997). For example, metalloproteins are involved in electron transport, oxygen storage, metal transport, chemical bond hydrolysis, redox processes, and synthesis of biological compounds (Gellein et al. 2007). However, even essential metals (e.g., Cu, Fe, and Zn), and especially those that have no known physiological functions (e.g., Cd, Pb), could also be toxic. Their toxicity is often postulated to arise from reactions in the cytosol, through nonspecific binding of metals to physiologically important molecules and their consequent inactivation (Mason and Jenkins 1995). For many trace elements, biological functions and mechanisms of

toxicity in different organisms are still not thoroughly investigated, and the proteins to which they bind are only partially identified and characterized (e.g., Cd (McGeer et al. 2012), Mo (Reid 2012), and Se (Janz 2012)).

To obtain the information on the bioavailability and toxicity of metals in the aquatic environment, it is, therefore, not sufficient to determine total or cytosolic metal concentrations in the tissues of aquatic organisms (de la Calle Guntińas et al. 2002). The knowledge on metal subcellular partitioning is also needed, which can serve as a potential indicator of metal toxicity, as reported for Cd (Wang and Rainbow 2006). Fractionation and a first screening of the complex samples, such as fish tissue cytosols, which contain so far unknown element species, could be performed by the use of size exclusion chromatography in combination with measurement by inductively coupled plasma mass spectrometry (ICP-MS; Vacchina et al. 1999). By this approach, specific metal-binding proteins in fish tissue cytosols that participate in normal metabolism or in mechanisms of toxicity could be eventually identified and potentially used for detection of the consequences of metal contamination in the aquatic environment.

In our studies on metal-induced disturbances within freshwater ecosystems, we have frequently used European chub (*Squalius cephalus*) as a bioindicator organism. So far we have investigated in detail physiological variability and levels of cytosolic concentrations of several trace elements in different chub tissues (Podrug et al. 2009; Filipović Marijić and Raspor 2010; Dragun et al. 2012a, b; Filipović Marijić and Raspor 2012). However, to our best knowledge, there is no available information on metal distribution among cytosolic proteins in organs of this important bioindicator species. As a target organ in this study, we have chosen the liver because it performs many metabolic functions and serves as a detoxification center. In addition, liver is a major producer of metal-binding proteins and therefore contains high concentrations of most metals (Roesijadi and Robinson 1994). Our primary aim was to define the basic distribution profiles of seven essential (Co, Cu, Fe, Mn, Mo, Se, and Zn) and two nonessential (Cd, Pb) trace elements in the hepatic cytosol of chub living in the aquatic environment not severely contaminated with metals/metalloids, i.e., to establish to which protein group, based on their molecular masses, each trace element was associated. To achieve this aim, we have used chub from the Sutla River in Croatia, which was selected as a study area since dissolved metal concentrations in its water have been classified as either comparable to natural levels or moderately increased (Cd, 0.01–0.31 μg L^{-1}; Co, 0.06–0.42 μg L^{-1}; Cu, 0.17–3.74 μg L^{-1}; Fe, 3.1–80.5 μg L^{-1}; Mn, 0.4–261.1 μg L^{-1}; Mo, 0.5–20.1 μg L^{-1}; Pb, ≤1.18 μg L^{-1}; Zn, <5.0 μg L^{-1}; Dragun et al. 2011). The information obtained by the current study will present the first step towards defining the specific metal-binding proteins in the chub hepatic cytosol which could be eventually used as biomarkers of metal exposure and/or effects.

Materials and methods

Fish sampling

Trace element distribution among protein fractions from hepatic cytosol was studied on fish caught during the water quality survey on the Sutla River in the late summer of 2009 (September 14 to 16) at five selected locations from the river source to its mouth (Dragun et al. 2011, 2012b). The selected fish species was European chub (*S. cephalus* L.), as an omnivorous fish species, widespread in European freshwaters and therefore suitable for monitoring purposes. The sampling was performed by electrofishing. The captured fish (75 specimens) were kept alive in aerated water tank till further processing in the laboratory. After the fish were anesthetized with Clove oil (Sigma) and killed, the liver were isolated, weighed, and stored at −80 °C until further analyses. All captured fish were characterized by length of 15–35 cm, mass of 33–400 g, and age of 1–4 years. Smaller and younger chub specimens were not included in this study due to small liver mass and consequently lack of sample for analyses. As a result, HPLC-analyzed group of chub comprised 28 larger specimens with length in the range from 18 to 35 cm, mass from 54 to 400 g, and age from 2 to 4 years. Sex composition of all captured chub and of HPLC-analyzed group was comparable, with approximately 80 % of females and 20 % of males.

Isolation of cytosolic fraction from European chub liver

The samples of liver tissue were cut into small pieces, diluted six times with cooled homogenization buffer (20 mM Tris–HCl/Base, Sigma, pH 8.6 at 4 °C) supplemented with reducing agent (2 mM dithiotreitol, Sigma), and then homogenized by ten strokes of Potter–Elvehjem homogenizer (Glas–Col) in ice-cooled tube at 6,000 rpm. For better separation, the homogenates were centrifuged subsequently two times in the Avanti J-E centrifuge (Beckman Coulter) at 50,000×*g* for 2 h at 4 °C. Supernatant (S50) obtained after second centrifugation, which represents water soluble cytosolic tissue fraction containing lysosomes and microsomes (Bonneris et al. 2005), was separated. Aliquots of S50 were stored at −20 °C for metal analyses in cytosol and at −80 °C for separation by size exclusion high performance liquid chromatography (SE-HPLC).

SE-HPLC separation of chub hepatic cytosol

For the separation of chub hepatic cytosol into fractions containing different molecular mass (MM) proteins, we have used size exclusion column Tricorn™ Superdex™ 200 10/300 GL (GE Healthcare Biosciences) and HPLC system (Perkin-Elmer, series 200) equipped with high-pressure pump, online degasser, column oven, cooled auto sampler with injector

(100 μL sample loop), and a diode array UV/VIS detector. The Superdex™ 200 10/300 GL column exclusion limit was defined as MM of 1,300 kDa for globular proteins, whereas the optimal separation range was given as MM of 10–600 kDa. For determination of void volume, blue dextran was applied, with MM defined as 2,000 kDa. It was eluted from 14.5 to 18.6 min, which corresponded to MM in the range from 1,000 to 350 kDa. For column calibration, six standard proteins were used (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase—Sigma), dissolved in homogenization buffer (20 mM Tris–HCl/Base, Sigma, pH 8.1 at 22 °C), which was also used as mobile phase at a flow rate of 0.5 mL min^{-1} (isocratic mode). Non-denaturating mobile phase at physiological pH, such as the Tris buffer, stabilizes the original metalloprotein complexes and is easily tolerated by high resolution ICP-MS (HR ICP-MS) (Prange and Schaumlöffel 2002; Wang et al. 2001). For each standard protein, separate chromatographic run was performed, and chromatograms were obtained using UV detection at 280 nm (Fig. 1a). Calibration straight line was created based on known MM of standard proteins and their respective elution times (t_e, Fig. 1a, b). In addition, metallothionein (MT) standard Zn-MT95 (Ikzus) was also applied, and chromatogram was obtained using UV detection at 254 nm, characteristic for metal-thiolate bond absorption (Fig. 1a). Narrow and well-defined double peak, which was obtained for MTs at t_e from 29 to 32 min, could be a consequence of a partial overlap of MT monomer and dimer: more intense MT peak at longer retention time is characteristic for the monomers, whereas the peak at shorter retention time is characteristic for dimers or other complexes (Wang et al. 2001). The injection volume for samples (untreated hepatic cytosols) was 50 μL. The fractions were collected at 1 min intervals in the plastic tubes using a fraction collector (FC 203B, Gilson). The resolution of these fractions with respect to molecular mass is given by the equation of the calibration straight line (Fig. 1b). For each sample, four consecutive chromatographic runs were performed, i.e., collected fractions were obtained after chromatographic separation of 200 μL of hepatic cytosol.

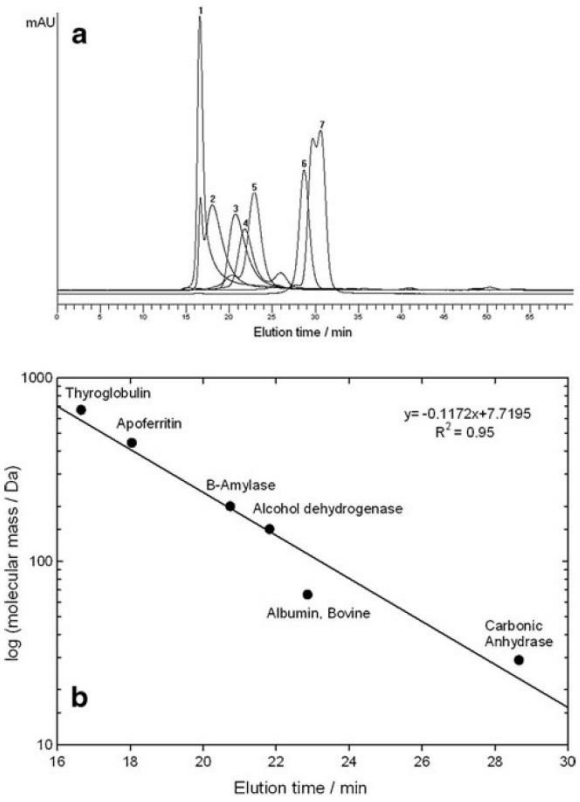

Fig. 1 **a** Separately obtained size exclusion chromatograms of six standard proteins with UV detection at λ=280 nm and of rabbit metallothionein (MT) standard (Ikzus Zn-MT95) with UV detection at λ= 254 nm: *1*—thyroglobulin (8 mg mL^{-1}; t_e, 16.65 min; MM, 669 kDa), *2*—apoferritin (10 mg mL^{-1}; t_e, 18.04 min; MM, 443 kDa), *3*—β-amylase (4 mg mL^{-1}; t_e, 20.74 min; MM, 200 kDa), *4*—alcohol dehydrogenase (5 mg mL^{-1}; t_e, 21.82 min; MM, 150 kDa), *5*—bovine serum albumin (10 mg mL^{-1}; t_e, 22.86 min; MM, 66 kDa), *6*—carbonic anhydrase (3 mg mL^{-1}; t_e, 28.66 min; MM, 29 kDa), *7*—MT (5 mg mL^{-1}; first peak: t_e, 29.85 min; MM, 16.6 kDa; second peak: t_e, 30.90 min; MM, 12.5 kDa). **b** The calibration straight line for Superdex™ 200 10/300 GL size exclusion column, with linear regression equation presented in the figure; t_e—elution time; MM—molecular mass (MM of MT was calculated from calibration equation)

Determination of trace element concentrations

Trace element concentrations were determined in hepatic cytosols and in SE-HPLC-separated cytosolic fractions. Hepatic cytosols were ten times diluted with Milli-Q water and acidified (0.65 % HNO_3, *Suprapur*, Merck) prior to measurements, whereas SE-HPLC-collected cytosolic fractions were only acidified (0.16 % HNO_3, Suprapur, Merck). Indium (Fluka) was added to all samples as an internal standard (1 μg L^{-1}). The measurements were performed on HR ICP-MS (Element 2, Thermo Finnigan), equipped with a double focusing mass analyzer using reverse Nier–Johnson geometry. An autosampler (ASX 510, Cetac Technologies) and sample introduction kit consisting of SeaSpray nebulizer and cyclonic spray chamber Twister were employed to transport the analytes into the plasma of HR ICP-MS. Measurements of ^{82}Se, ^{98}Mo, ^{111}Cd, and ^{208}Pb were operated in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium-resolution mode. External calibration was performed using standards prepared in 2 % HNO_3 (Suprapur, Merck) by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika). Quality control sample (QC for trace metals, UNEP GEMS/Water PE Study No. 7) was used for checking the accuracy of trace element measurements by HR ICP-MS. A generally good agreement was observed between

our data and the certified values. Limits of detection (LOD) were determined based on three standard deviations of ten consecutively determined trace element concentrations in the blank sample (2 mM Tris–HCl/Base, 0.2 mM dithiotreitol, and 0.65 % HNO_3). LODs for trace elements measured within this study were as follows (in micrograms per liter): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Mo, 0.004; Pb, 0.010; Se, 0.138; and Zn, 2.40.

Data processing and statistical analyses

Chromatographic results were processed using Totalchrom Version 6.3.1 software (Perkin-Elmer). Descriptive statistical analysis and graphs were created using the statistical program SigmaPlot 11.0 for Windows. Only few representative distribution profiles for each trace element are presented in the figures (Figs. 3, 4, and 5), while the remaining data are given as supplementary information.

Results and discussion

The chromatographic column applied in this study (Superdex™ 200 10/300 GL) enabled only rough categorization of proteins from chub hepatic cytosols according to their MM. We have designated four main protein categories (Table 1): HMM (high MM proteins, >100 kDa), MMM (medium MM proteins, 30–100 kDa), LMM (low MM proteins, 10–30 kDa), and VLMM (very low MM proteins, <10 kDa). Finer separation was not enabled by applied column, as can be seen from the exemplary chromatogram obtained for chub hepatic cytosol, with UV detection at two wavelengths—280 nm characteristic for aromatic amino acids (Fig. 2a) and 254 nm characteristic for metal-thiolate bond absorption (Fig. 2b). Such rough separation of protein fractions from hepatic cytosol, and subsequent estimation of protein category within which specific elements were mainly eluted, presents a first step towards defining specific metal-binding proteins in the chub hepatic cytosol.

Distribution profiles of essential elements with the narrowest cytosolic concentration range

Among the studied elements, essential elements Co, Mo, Mn, and Zn had the narrowest ranges of cytosolic concentrations in the chub liver, with maximum to minimum ratio amounting to 2–4. Their concentrations in the hepatic cytosol of the studied chub specimens (Co, 2.1–5.1 ng mL^{-1}; Mo, 8.7–38.5 ng mL^{-1}; Mn, 73.5–252.8 ng mL^{-1}; and Zn, 3.0–11.1 μg mL^{-1}) fell mainly within their previously defined basal ranges (Co, 4.1–5.1 ng mL^{-1}; Mo, 22.8–30.6 ng mL^{-1} (Dragun et al. 2012a); Mn, 110–190 ng mL^{-1}; Zn, 3.5–6.5 μg mL^{-1} (Podrug et al. 2009)). Low cytosolic Co could be explained by low Co affinity to accumulate in the liver,

Table 1 Distribution of trace elements among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by size exclusion HPLC with Superdex 200 10/300 GL column

Element		HMM peak[a] 1		HMM peak[a] 2		MMM peak[b]		LMM peak[c]		VLMM peak[d] 1		VLMM peak[d] 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
Essential elements	Co	–	–	21 (18–25)	181.3 (407.3–61.6)	–	–	–	–	35 (32–37)	4.1 (9.3–2.4)	40 (37–41)	1.1 (2.4–0.82)
	Mo	16 (15–17)	698.7 (915.2–533.5)	20 (18–25)	237.4 (407.3–61.6)	–	–	–	–	33 (31–35)	7.11 (12.2–4.1)	–	–
	Mn	–	–	22 (18–25)	138.4 (407.3–61.6)	26 (25–27)	47.0 (61.6–35.9)	29 (27–33)	20.9 (35.9–7.1)	–	–	–	–
	Zn	16 (15–18)	698.7 (915.2–407.3)	–	–	26 (16–27)	47.0 (698.7–35.9)	30 (28–32)	16.0 (27.4–9.3)	–	–	–	–
	Cu	–	–	–	–	27 (25–28)	35.9 (61.6–27.4)	30 (28–33)	16.0 (27.4–7.1)	–	–	–	–
	Fe	18 (16–21)	407.3 (698.7–181.3)	–	–	27 (25–29)	35.9 (61.6–20.9)	–	–	–	–	–	–
	Se	–	–	22 (18–25)	138.4 (407.3–61.6)	–	–	29 (25–33)	20.9 (61.6–7.1)	–	–	40 (38–42)	1.1 (1.8–0.63)
Nonessential elements	Cd	–	–	–	–	25 (23–27)	61.6 (105.6–35.9)	30 (28–32)	16.0 (27.4–9.3)	–	–	–	–
	Pb	–	–	–	–	25 (20–27)	61.6 (237.4–35.9)	30 (29–32)	16.0 (20.9–9.3)	–	–	–	–

Elution times (t_e) and molecular masses (MM) of proteins contained in the fractions in which respective elements were eluted are given in the table. Presented numbers refer to maximums of trace element peaks (i.e., the fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks

[a] HMM peak—a peak of trace element concentration in the cytosolic fractions with a maximum in high molecular mass protein region (>100 kDa)

[b] MMM peak—a peak of trace element concentration in the cytosolic fractions with a maximum in medium molecular mass protein region (30–100 kDa)

[c] LMM peak—a peak of trace element concentration in the cytosolic fractions with a maximum in low molecular mass protein region (10–30 kDa)

[d] VLMM peak—a peak of trace element concentration in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa)

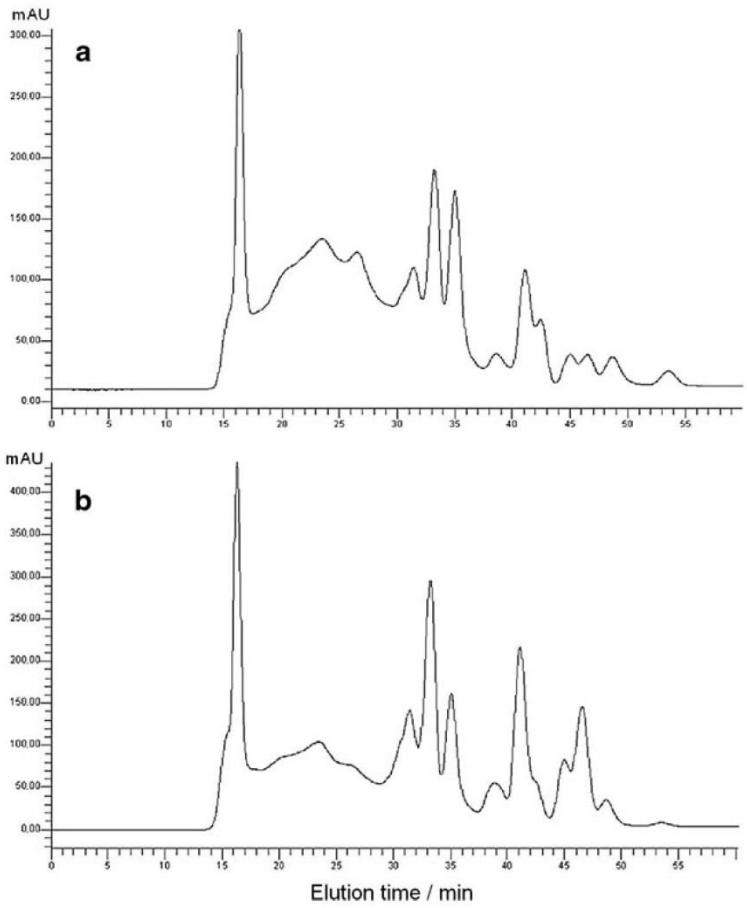

Fig. 2 An example of SE-HPLC chromatogram profile of chub hepatic cytosol (50 μL) after separation on Superdex™ 200 10/300 GL column, with UV detection at two wavelengths: **a** λ=280 nm; **b** λ=254 nm

since it was observed in different fish species that under normal Co exposure Co strongly accumulates in the gut and kidneys (Baudin and Fritsch 1989), whereas the role of the liver becomes more important only after increasing Co exposure (Mukherjee and Kaviraj 2009). Contrary to Co, Mo was shown to accumulate in the fish liver in a dose-dependent and saturable manner (Reid 2002), and despite the fact that this is an essential micronutrient, there is no known homeostatic control system for Mo in fish (Reid 2012). Low cytosolic Mo concentration therefore could be a consequence of specific Mo accumulation within the nuclei and mitochondria, as revealed by the studies on turtles (Anan et al. 2002). Similar to Mo, two out of three primary Mn metalloenzymes in mammals, Mn superoxide dismutase, which is considered to be one of the most important intracellular antioxidant enzymes (88 kDa; Fridovich and Freeman 1986), and pyruvate carboxylase (480–600 kDa; Libor et al. 1979), are localized in mitochondria, whereas only hepatic arginase is cytosolic enzyme (87 kDa; Singh and Singh 1990). And finally, fish are capable of regulating Zn accumulation over a wide range of ambient Zn levels, as shown for perch (*Perca fluviatilis*): a 100-fold increase in total water Zn concentration resulted in only a modest 1.2-fold increase of Zn concentration in the perch liver (Hogstrand et al. 1991). Dissolved Zn concentrations in the Sutla River water were lower than 5 μg L^{-1} (Dragun et al. 2011), and therefore it could not be expected to find wide range of cytosolic Zn concentrations in the liver of chub from this river. Therefore, the principal aim for Co, Mo, Mn, and Zn was to establish their basic distributions among protein fractions, characteristic either for the fish living in the conditions of low metal exposure in the water or for very good cellular regulation which was still not exceeded.

Cobalt

Co distribution profile included three separate Co-containing peaks (Fig. 3a), with the predominant peak corresponding to the HMM protein category (Table 1). The remaining two smaller peaks corresponded to VLMM protein category (Table 1). The VLMM peak with the maximum obtained at the elution time of 40 min corresponded to proteins of MM in the range from 0.8 to 2.4 kDa. Cobalt-containing compound cobalamin has an MM of 1.3 kDa (Kirschbaum 1981) and therefore could be eluted together with VLMM proteins.

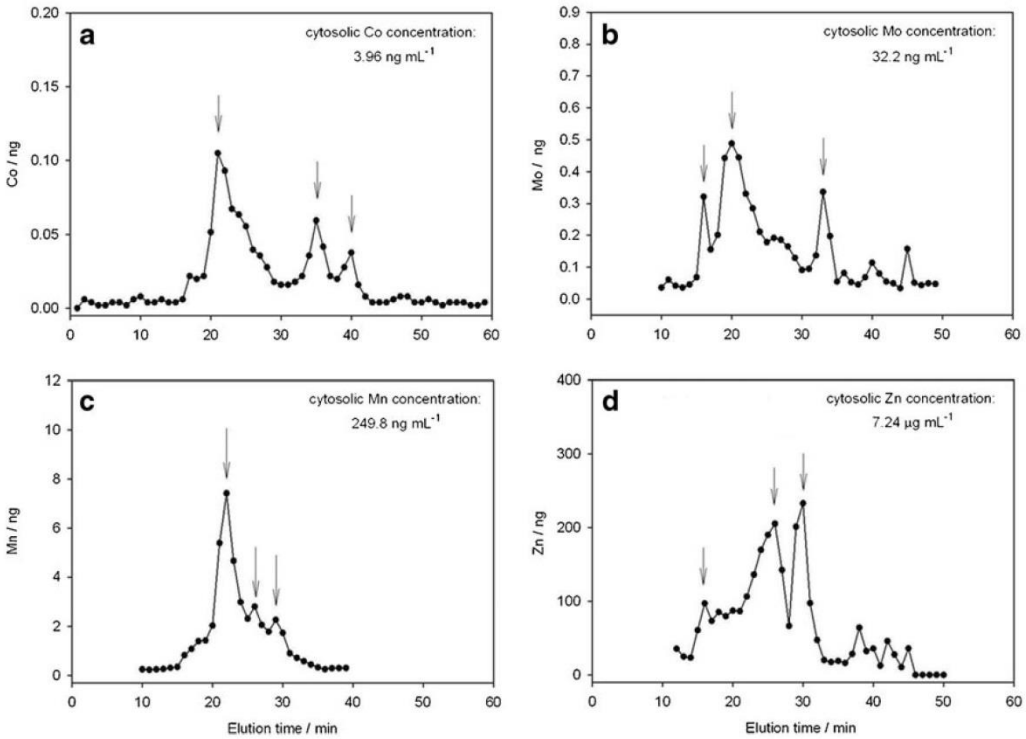

Fig. 3 Distribution profiles of essential trace elements: **a** Co; **b** Mo; **c** Mn; and **d** Zn, among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results are presented as nanograms of trace element eluted at specific elution times, after passing 200 µL of hepatic cytosol through the chromatographic column

Although the main role of Co as an essential element in the fish organism is associated with its constitutive role in cobalamin, i.e., the vitamin B12 (Blust 2012), only minor part of Co present in the chub hepatic cytosol was eluted within the fraction presumably containing cobalamin. Taking in consideration that studies concerning the molecular aspects of Co uptake, its internal processing, and mechanisms of toxicity are largely lacking (Blust 2012), it would be useful to further define the characteristics and functions of HMM cytosolic proteins that bind major proportion of cytosolic Co.

Molybdenum

Molybdenum was mainly eluted with HMM proteins (Fig. 3b). A smaller HMM peak was eluted within the void volume, corresponding to proteins with MM above 600 kDa (Table 1), which could not be distinguished by applied column. Molybdenum serves as a cofactor of at least seven enzymes (Beers and Berkow 1998), and a major HMM peak obtained in our study (Table 1) encompassed their MM (e.g., aldehyde oxidase, ~130 kDa (Uchida et al. 2003); sulfite oxidase, ~120 kDa (Johnson and Rajagopalan 1976); and Fe-Mo flavoprotein xanthine oxidase, 275 kDa (Truglio et al. 2002)). Minor part of Mo was eluted after 30 min, within VLMM fraction (Fig. 3b, Table 1). In addition, our study confirmed the absence of Mo binding to MT in the liver of chub exposed to low or moderate Mo concentrations in the river water (up to 20 µg L^{-1}), since Mo peak was not obtained at t_e of MT (Fig. 1a). This is consistent with the report of Ricketts (2009) that MT is probably not involved in the internal detoxification of Mo. After short-term exposure of rainbow trout to Mo in concentrations as high as 1,000 mg L^{-1}, Mo failed to induce the synthesis of MT in the liver, despite its obvious accumulation (Reid 2012).

Manganese

Manganese was distributed in three peaks (Fig. 3c). The predominant Mn peak was associated to HMM proteins (Table 1) and nearly coincided with the t_e of albumin (Fig. 1), which is involved in Mn transport from the intestine to the liver (Schäfer 2004). In the liver, Mn binds to transferrin (80 kDa; Martin-Antonio et al. 2009) and in that form presents a source of Mn for delivery to other tissues (Schäfer 2004). HMM peak encompassed MM of both of these transport proteins (Table 1). Smaller second and third peak could be associated to MMM and LMM proteins with MM around 50 and 20 kDa, respectively.

Zinc

Distribution profile of Zn was characterized by poorly resolved peaks covering a wide range of MM, approximately from 10 to >600 kDa (Fig. 3d). This was not surprising, since it is well known that Zn has constitutive and catalytic roles in many proteins and enzymes. More than 3,000 proteins in humans, representing 10 % of the entire human genome, as well as about 10 % of all genes in sequenced fish genomes carry the annotation of Zn binding (Andreini et al. 2005; Passerini et al. 2007). The first Zn peak appeared within t_e of void volume, same as in the case of Mo (Fig. 3b), and could be associated with HMM proteins of MM above 600 kDa (Table 1). The predominant peak was rather wide and asymmetrical covering both HMM and MMM protein categories, and with maximum in MMM area (Table 1). For example, t_e of standard protein alcohol dehydrogenase (Fig. 1), which is known as Zn-containing protein (Szpunar and Lobinski 1999), coincided with the HMM part of this peak. The third peak was narrow and sharp and appeared within LMM protein category (Table 1), with the maximum coinciding with the t_e of MT (Fig. 1a). Since MTs were reported to play important roles in detoxification of toxic metals and maintenance of homeostasis of essential metals like Zn and Cu (Huang et al. 2004), it was expected to find Zn in chub liver in the binding form such as Zn/Cu MT (Huang et al. 2007). This peak, however, also encompassed the t_e of carbonic anhydrase, which is also a Zn metallo-enzyme (Szpunar and Lobinski 1999).

Distribution profiles of essential elements with wider cytosolic concentration ranges

Further on, the distribution profiles of three additional essential trace elements are presented—Cu, Fe, and Se. Copper is an essential element for all aerobic organisms since its redox potential is utilized by mitochondrial cytochrome c oxidase; Cu also acts as a cofactor for a large number of other enzymes (Solomon and Lowery 1993). Iron is essential for life as an integral part of the oxygen binding metalloprotein hemoglobin and of cytochrome c oxidases in respiratory chain acting as an electron donor or acceptor (Bury et al. 2012). It also plays a role in DNA synthesis (Bury et al. 2012) and in the defense against bacterial infection (Vidal et al. 1993). Selenium is an essential element to living organisms, with a very narrow range between essentiality and toxicity (Jukola et al. 1996). Despite their essentiality, Cu, Fe, and Se occurred in somewhat wider concentration ranges in the chub hepatic cytosol compared to Co, Mo, Mn, and Zn (maximum to minimum ratio, 7–9) indicating enhanced trace element accumulation in the liver of some chub specimens possibly due to increased exposure and less strict cellular regulation. Cytosolic concentrations of Cu, Fe, and Se in the liver of the studied chub specimens were the following, respectively: 0.4–3.9 and 2.3–16.8 μg mL^{-1} and 25.6–229.2 ng mL^{-1}. Copper and especially Fe levels somewhat exceeded previously defined basal ranges (Cu, 0.7–2.3 μg mL^{-1}; Fe, 3.4–6.6 μg mL^{-1} (Podrug et al. 2009)). For Cu, Fe, and Se, therefore, not only the basic distribution profiles could be described but also the possible changes in their distribution within chub hepatic cytosol due to increased accumulation.

Copper

Copper was mainly eluted within the LMM protein category (Fig. 4a) with a maximum at elution time of 30 min (Table 1), coinciding with the t_e of MT (Fig. 1a). It was an indication that the major part of Cu was probably associated with MT fraction. Apart from MT, many proteins are known to contain Cu (Szpunar and Lobinski 1999), such as transcuprein (270 kDa; Liu et al. 2007), β-amylase (200 kDa; Fig. 1), ceruloplasmin (132 kDa; Boivin et al. 2001), albumin (66 kDa; Fig. 1), superoxide dismutase (32 kDa; Richardson et al. 1975), and carbonic anhydrase (29 kDa; Fig. 1). However, additional smaller Cu peak which appeared within MMM protein region implicated Cu binding only to proteins of MM from 7 to 60 kDa (Table 1), such as carbonic anhydrase (LMM peak) or superoxide dismutase (SOD; MMM peak). Cu-SOD association, for example, could point both to Cu's essential role in the SOD activity as a protection against oxidative stress (Sanchez et al. 2005) or to risk of Cu inhibitory effect on this antioxidant enzyme (Vutukuru et al. 2006).

Increase of cytosolic Cu concentrations was principally reflected as the increase of Cu peak height in the LMM region as much as ten times in the specimens with the highest cytosolic Cu (Fig. 4a). MT contribution to binding of total cell Cu content is relatively minor (Hogstrand et al. 1991) and can account for no more than 30–40 % of the total cellular Cu pool. However, our results indicate that in the hepatic cytosol MT has predominant role in the binding of Cu.

Iron

In chub hepatic cytosol, the basic distribution profile of Fe was characterized by two clear Fe-containing peaks of comparable height (Fig. 4b). The first peak was eluted within HMM region with maximum associated to proteins of MM around 400 kDa (Table 1) and corresponded well with the t_e and MM of apoferritin (Fig. 1). Ferritin (450 kDa) is a protein mainly present in the liver tissue which serves as Fe storage protein and keeps Fe in a soluble bioavailable nontoxic form in the cytoplasm (Szpunar and Lobinski 1999; Martin-Antonio et al. 2009; Bury et al. 2012). The second Fe peak appeared within MMM region with maximum corresponding to proteins with MM of 36 kDa. It, however, covered the range

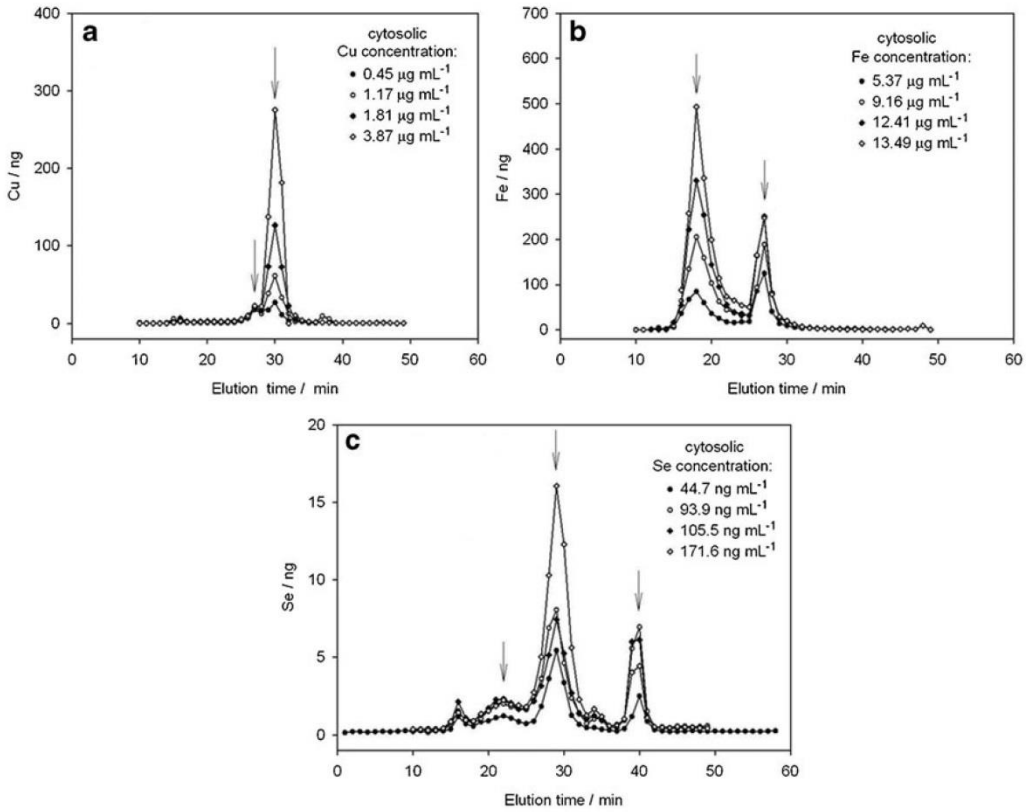

Fig. 4 Distribution profiles of essential trace elements: **a** Cu; **b** Fe; and **c** Se, among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results are presented in the same way as described in the caption of Fig. 3

of MM from 20 to 60 kDa (Table 1), which could possibly involve minor binding to known Fe-containing proteins of different functions, such as catalase (60 kDa) or myoglobin (17 kDa, Martin-Antonio et al. 2009). Similar to absence of Cu HMM peak which could be associated to ceruloplasmin, Fe peak was not registered within the region which would correspond to hemoglobin (65 kDa; Martin-Antonio et al. 2009), indicating the absence of blood proteins in the samples of hepatic cytosol (Martin-Antonio et al. 2009).

Slight Fe cytosolic concentration increase was reflected in the proportional double increase of both peaks (Fig. 4b). Additional increase of cytosolic Fe concentration above 10 µg mL^{-1}, however, resulted almost completely in binding to storage protein ferritin. It could be presumed based on clear increase of the height of HMM peak, up to five times in the specimens with the highest cytosolic Fe (Fig. 4b).

Selenium

In chub liver, basic Se distribution among cytosolic protein fractions included three peaks (Fig. 4c). The first peak was the smallest with a maximum in the HMM region, whereas the second peak, connected to the first one, was predominant and had a maximum within the LMM protein category (Table 1). Both peaks together covered the wide range of MM from approximately 10 to 400 kDa, encompassing MM of several well-characterized fish selenoproteins (Janz 2012), such as enzymes involved in antioxidant defense (glutathione peroxidase, 85 kDa (Shulgin et al. 2008); thioredoxin reductase, 66 kDa (Larsson 1973)), in thyroid hormone metabolism (iodothyronine deiodinase, 65 kDa (Fekkes et al. 1980)), as well as MTs (12.5–16.6 kDa, Fig. 1). Paliwal et al. (1986) established that a particular isoform of MT showed a unique binding of Se, whereas Ferrarello et al. (2002) suggested that the association of Se with MT plays a synergistic protective role against heavy metal toxicity. Iwai et al. (1988), however, established that radiolabeled Se was mostly eluted in the fraction corresponding to MM larger than that of MT. In our study, although the t_e of MT was encompassed within the second Se peak, it was placed at the peak's right tail, thus implying only the possibility of minor Se binding to MT. The majority of Se was eluted with proteins

of higher MM, same as described by Iwai et al. (1988). The third peak was eluted within the VLMM category, and its maximum corresponded to MM of 1.1 kDa, pointing to the presence of some small Se-containing compound.

Slight increase of Se cytosolic concentrations in chub liver was reflected in Se sequestration within the VLMM category (Fig. 4c). More substantial Se excess in the hepatic cytosol, however, resulted in its association with LMM proteins (Fig. 4c).

Distribution profiles of nonessential elements

Nonessential elements Cd and Pb had the widest cytosolic concentration ranges in the chub liver of all studied elements (maximum to minimum ratio, ~20), confirming a well-known fact that regulation of nonessential elements is not characteristic for fish (Heath 1987). Cytosolic concentrations in the chub liver in this study were 3.4–59.4 ng mL^{-1} for Cd and from LOD to 44.2 ng mL^{-1} for Pb. Since both Cd and Pb are known to be toxic in very low concentrations, it is very important to establish to which proteins in the soluble tissue fraction is excess quantity of these metals bound. Potential toxicity will relate to metal proportion bound to detoxifying versus non-detoxifying cellular constituents, and metal-binding proteins should be therefore well characterized (Mager 2012).

Cadmium

Basic Cd distribution among cytosolic protein fractions of chub liver included narrow Cd peak within the LMM protein region with maximum obtained at t_e of 30 min (Table 1 and Fig. 5a), corresponding to MT peak (Fig. 1a). After prominent increase in cytosolic Cd concentrations, the majority of Cd was still eluted within the same protein fraction, indicating predominant association of Cd with MTs. The height of presumable Cd-MT peak increased eight times in the samples with the highest compared to the samples with the lowest cytosolic Cd concentrations (Fig. 5a). Furthermore, we have observed the additional small Cd peak within the MMM protein category (approximately 35 to 100 kDa; Table 1, Fig. 5a). It became more obvious in the samples with higher cytosolic Cd concentrations, above the range defined for the liver of chub (3–13 ng mL^{-1}; Podrug et al. 2009) living in relatively non-contaminated river water. Cadmium elution in MMM protein category, as low as it was, still was an indication that increased Cd accumulation in the hepatic cytosol could result with incomplete Cd detoxification and binding to proteins of higher MM than MTs.

The pathways of Cd metabolism in different fish organs are complex, and many of Cd-responsive proteins are yet to be identified (McGeer et al. 2012). The intracellular detoxification of Cd is primarily mediated by GSH and MTs. Insufficient binding to these ligands may lead to potential Cd competition

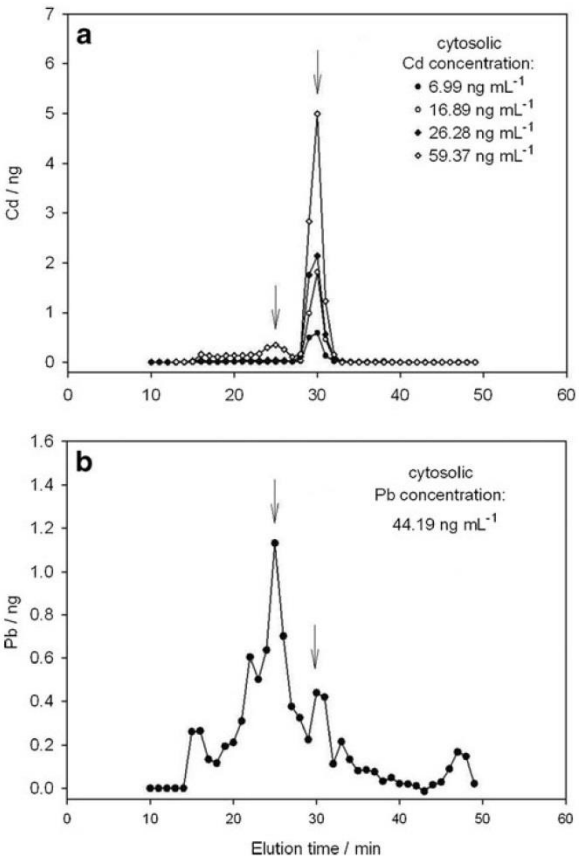

Fig 5 Distribution profiles of nonessential trace elements: **a** Cd and **b** Pb, among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results are presented in the same way as described in the caption of Fig. 3

with the essential metals for binding sites on non-MT proteins, which could induce cellular damage (McGeer et al. 2012). However, in addition to GSH and MTs, recent studies suggest that the induction of heat shock proteins (e.g., HSP70, HSP90) expression also plays an important role in the physiological changes related to metabolism and cell protection that occur in Cd-exposed aquatic animals including fish (Kwong et al. 2011; Matz and Krone 2007). As seen from Table 1 and Fig. 5a, MM of HSPs was encompassed by Cd-MMM peak. Further studies, preferably with higher Cd exposure, are therefore needed, to determine more precisely to which non-MT proteins excessive quantity of cytosolic Cd binds: does the observed MMM peak reflect an additional mode of detoxification or a potential for toxic effects.

Lead

Although dissolved Pb concentrations in the Sutla River water were within recommended levels for natural waters,

they were noticeably increased at one sampling site (~1 μg L^{-1}; Dragun et al. 2011), which was reflected in the increased Pb concentrations in the chub hepatic cytosols (11.8–44.2 ng mL^{-1}; Dragun et al. 2012b). At four "uncontaminated" sites with dissolved Pb in river water ≤0.1 μg L^{-1} (Dragun et al. 2011), Pb cytosolic concentrations were rather low (below 5 μg L^{-1}, with average value amounting to 1.9 μg L^{-1}; Dragun et al. 2012b) and comparable with previously reported cytosolic Pb concentrations (0.97–5.93 ng mL^{-1}) in the liver of chub from weakly contaminated Sava River water (Dragun et al. 2012a). Due to exceeding dilution during the HPLC separation, it was not possible to measure Pb in SE-HPLC-separated fractions from uncontaminated sites. Lead distribution was, therefore, presented only for the one "contaminated" site, with the aim to define the protein category within which surplus Pb was sequestered.

As shown in Fig. 5b, Pb was eluted mainly in the fractions corresponding to MMM proteins, with maximal quantity at elution time of 25 min which could be associated to proteins with MM of about 60 kDa. The remaining Pb was distributed among several LMM and VLMM fractions, among which the most recognizable was the fraction corresponding to MT peak (Fig. 1a and Table 1). Pavičić et al. (1993) reported the appearance of small, but well-defined Pb maximum at MT position on the elution profile obtained from mussels exposed to metal mixture (Cd, Cu, and Pb). On the other hand, several authors reported a lack of evidence on the induction of Pb-binding proteins related to MTs in fish tissues (Reichert et al. 1979; Roesijadi and Robinson 1994). However, a classical characteristic feature of Pb exposure in fish is Pb sequestration within the metal-rich granules and cellular debris (e.g., in the whole body of killifish; Goto and Wallace 2010). Similarly, in mammals, Pb could be found within insoluble Pb protein aggregates known as inclusion bodies (Goyer 1983). Evidence has indicated that MT, α-synuclein (Zuo et al. 2009), and a cleavage product of α_2-microglobulin (Fowler 1998) are critical to inclusion body formation. Since Pb is a poor inducer of MT compared to other metals such as Cd and Zn (Waalkes and Klaassen 1985), the role of MT, and possible Pb-MT association observed in this study, may be also associated to inclusion body formation (Mager 2012).

Conclusions

Basic distributions of essential elements Co, Cu, Fe, Mn, Mo, Se, and Zn and nonessential elements Cd and Pb among cytosolic proteins of different molecular masses in the liver of European chub were defined for the first time based on the study performed on feral chub specimens from aquatic environment weakly contaminated by metals/metalloids (Cd, 0.01–0.31 μg L^{-1}; Co, 0.06–0.42 μg L^{-1}; Cu, 0.17–3.74 μg L^{-1}; Fe, 3.1–80.5 μg L^{-1} Mn, 0.4–261.1 μg L^{-1}; Mo, 0.5–20.1 μg L^{-1}; Pb, ≤1.18 μg L^{-1}; Zn, <5.0 μg L^{-1}; Dragun et al. 2011). Several essential elements (Cu, Fe, and Se) and nonessentail elements Cd and Pb were present in the hepatic cytosol in wide concentration ranges (maximum to minimum ratio, 7–20), thus allowing additional observation of the changes of their distributions among protein fractions as a consequence of increased accumulation of these elements in the chub liver. Increased quantity of cytosolic Cu and Cd was almost completely sequestered by MT, whereas Fe was associated to Fe storage protein ferritin. In the case of Se and Pb, it was not possible to define single protein fraction to which their additional amounts in the hepatic cytosol associate, but rather a spectrum of proteins, mostly of low (10–60 kDa) or medium molecular masses (30–100 kDa), respectively. Based on our results, the changes of cytosolic trace element distributions could serve as a sensitive tool for identification of metal/metalloid-induced stress in chronically exposed fish.

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RESEARCH ARTICLE

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.)

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Abstract The distribution of essential elements Co, Cu, Fe, Mn, Se, and Zn, and nonessential element Cd among cytosolic proteins of different molecular masses in the gills of European chub (*Squalius cephalus*) sampled in the moderately contaminated Sutla River in September of 2009, was studied after the protein separation by size exclusion high-performance liquid chromatography (SEC-HPLC), and the metal determination in the obtained fractions by high-resolution inductively coupled plasma mass spectrometry (HR ICP-MS). The aims of the study were to characterize the distribution profiles of metals within different protein categories in gills in the conditions of low metal exposure in the river water, and to compare them with the previously published hepatic profiles. The distribution profiles of analyzed metals were mainly characterized with several peaks. However, some observations could be emphasized: both Cu and Cd were eluted near metallothionein elution time; elution time of one of Co peaks could be associated with Co-containing compound cobalamin; increasing cytosolic Fe concentrations resulted in possible Fe binding to storage protein ferritin; both Mn and Zn had poorly resolved peaks covering wide ranges of molecular masses and indicating their binding to various proteins; both Zn and Se increased in protein fractions of molecular masses <5 kDa following their concentration increase in the gill cytosol; expected clear metallothionein peak was not observed for Zn. Comparison of gill profiles with previously published hepatic profiles revealed similar and in case of some elements (e.g., Co, Fe, Mn, and Se) almost identical distributions in both organs regarding elution times. On the contrary, heights of obtained peaks were different, indicating possible metal binding to the same proteins in the gills and liver, but in different proportions. The results obtained in this study can be used as a basis for comparison in monitoring studies, for identification of changes that would occur after exposure of chub to increased metal concentrations.

Keywords Cytosolic proteins · European chub · Gills · HR ICP-MS · Metals · SEC-HPLC

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Introduction

In an aquatic environment, the degree of metal pollution is often evaluated by establishing the effects of increased metal exposure on aquatic organisms, such as fish, specifically by measuring metal concentrations in the liver and gills (Kamaruzzaman et al. 2010). Gills have a large surface area that is continuously in contact with the external medium, and thus present the main uptake route of contaminants from aqueous phase (Playle 1998). In addition, through blood circulation, gills can also accumulate chemicals that were taken up by other exposure routes (Levine and Oris 1999). The study of metal effects on gills is important because gills play a key role in fish physiology, for example, in respiration, osmotic and ionic regulation, and acid–base balance (Ahmad et al. 2008). Metal ions can interfere with these gill functions by causing cellular damage to gill cells (Evans 1987; De Boeck et al. 2001). Although some metals, such as Cu, Co, Fe, Mn, Se, and Zn are essential micronutrients which are required for numerous physiological processes, they can also be toxic. The ability to induce toxic effects is not only a feature of metals with no known functions in the organism (e.g., Cd),

but also of the essential elements, when they are present in organisms in concentrations above their threshold. They can all induce toxic effects by different modes of action, for example, some of them can generate reactive oxygen species (ROS); as a result of an effort to maintain ROS levels within physiological limits, the activity of biotransformation and antioxidant enzymes, such as glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD), increases (Formigari et al. 2007). Therefore, to assess the biological effects of metals, it is insufficient to only measure metal concentrations in the gills and other tissues (Lehtonen and Schiedek 2006) but also, it is necessary to detect and characterize protein molecules which bind metals, as well. Next to the identification of metalloproteins, for the understanding of protein processes, it is also necessary to identify nonproteinaceous molecules of a relatively small size, which deliver metals to metalloproteins (Outten and O'Halloran 2001). Separation of proteins by size exclusion high-performance liquid chromatography (SEC-HPLC) combined with metal detection techniques such as inductively coupled plasma mass spectrometry (ICP-MS) has been previously described as a valuable tool for accomplishing such a goal (Prange and Schaumlöffel 2002; Krasnići et al. 2013; Stržak et al. 2014).

The focus of this study was the investigation of distribution of essential elements Cu, Co, Fe, Mn, Se, and Zn, and nonessential metal Cd among SEC-HPLC separated cytosolic proteins of different molecular masses from the gills of European chub (*Squalius cephalus*) sampled in the moderately contaminated Sutla River (Dragun et al. 2011). A similar study was previously performed on the liver of the same chub specimens (Krasnići et al. 2013), but to our knowledge there is no such information available for gills either of chub or other freshwater fish. Therefore, the main aim of the current study was to define the basal metal distributions of seven selected elements among different protein categories, i.e., the distributions characteristic for the conditions of low metal exposure in the water. An additional aim was to compare metal distribution profiles in gills with previously published profiles in liver (Krasnići et al. 2013), and to define the similarities and differences of cytosolic metal allocation within these two functionally different organs.

Materials and methods

Fish selected for analyses

For this study, we have selected seven specimens out of 75 European chub (*S. cephalus*) caught in the Sutla River in September of 2009. The fish were caught by electrofishing and then dissected, as previously described in detail by Dragun et al. (2011; 2012; 2013) and Krasnići et al. (2013). They were 20.1 to 29.7 cm long, with masses ranging from 94.5 to 260.6 g, and age from 2 to 4 years (Table 1). Sex composition of chub specimens selected for analyses was 86 % females (Table 1). The selection of the samples for analyses was based on two criteria: the sample availability and cytosolic metal concentrations in the chub gills. For smaller chub specimens, the gills were not large enough to obtain sufficient volume of cytosol for HPLC separation. Among the remaining samples, the basic criteria for selection were the cytosolic metal concentrations, which were mainly rather low in the gills, for example, much lower than in the liver. Therefore, we have chosen for this study gill cytosols with the highest metal concentrations, to ensure the best possible resolution of obtained peaks. Consequently, number of fish selected for this study (n=7) was smaller compared to study performed on liver (n=28, Krasnići et al. 2013).

Isolation of cytosolic fraction from European chub gills

The isolation of cytosol from chub gill tissue was previously described (Dragun et al. 2012, 2013; Krasnići et al. 2013). In brief, gill tissues were homogenized by Potter–Elvehjem homogenizer (Glas-Col), using 20 mM Tris–HCl/Base (Sigma, pH 8.6 at 4 °C) supplemented with reducing agent 2 mM dithiotreitol (Sigma) as homogenization buffer, and then centrifuged subsequently two times in the Avanti J-E centrifuge (Beckman Coulter) at 50,000×g for 2 h at 4 °C. Supernatant (S50) obtained after second centrifugation, which represents water-soluble cytosolic tissue fraction containing lysosomes and microsomes (Bonneris et al. 2005), was separated for further analyses.

SEC-HPLC fractionation of chub gill cytosol

For the fractionation of chub gill cytosol, we have used size exclusion column Tricorn™ Superdex™ 200 10/300 GL (GE Healthcare Biosciences) and PerkinElmer HPLC system (series 200), as previously described in detail by Krasnići et al. (2013). Column exclusion limit was defined as molecular mass (MM) of 1,300 kDa for globular proteins, whereas the optimal separation range was given as 10–600 kDa. The void volume was determined by use of blue dextran (defined MM: 2,000 kDa), which was eluted at 16.31 min. For column calibration, six protein standards were used (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase, Sigma), dissolved in 20 mM Tris–HCl/Base (Sigma, pH 8.1 at 22 °C), which was also used as mobile phase at a flow rate of 0.5 mL min^{-1} (isocratic mode). Calibration straight line was created based on known MM of protein standards and their respective elution times (t_e; Table 2; presented in detail by Krasnići et al. 2013). Metallothionein (MT) standard Zn-MT95 (Ikzus) was also run through the column and narrow and well-defined double peak

Table 1 Basic biometric characteristics and total cytosolic protein concentrations in the gills of seven European chub (*Squalius cephalus*) specimens caught in the Sutla River in September of 2009, which were used for this study (legend for sex: F - female; M - male)

Fish no.	Length cm	Mass g	Age years	Sex	Total cytosolic proteins mg mL^{-1}
1	29.7	260.6	4	F	14.4
2	25.3	140.0	3	F	14.7
3	23.1	120.4	3	F	16.1
4	20.1	94.5	2	F	13.6
5	24.3	153.5	3	F	16.8
6	26.5	174.9	3	M	15.7
7	25.0	156.5	4	F	16.1

was obtained with maxima at t_e 29.85 and 30.90 min. For MTs, more intense peak at longer retention time is characteristic for the monomers, whereas the peak at shorter retention time is characteristic for dimers or other complexes (Wang et al. 2001). The injection volume for gill cytosol samples was 100 μL (except for fish no. 1=50 μL), and for each sample, four consecutive chromatographic runs were performed (total sample volume=400 μL; fish no. 1=200 μL). The fractions were collected at 1-min intervals in the plastic tubes using a fraction collector (FC 203B, Gilson). The resolution of these fractions with respect to molecular mass is given by the equation of the calibration straight line (y=0.1172x+7.7195; y=MM; x=t_e; Krasnići et al. 2013).

Determination of trace element concentrations

Trace element concentrations were determined in ten times diluted gill cytosols and in SEC-HPLC-separated cytosolic fractions, both acidified by HNO_3 (Suprapur, Merck; final acid concentrations: 0.65 % and 0.16 %, respectively). Indium (Fluka) was added to all samples as an internal standard (1 μg L^{-1}). The measurements were performed on high resolution ICP-MS (Element 2, Thermo Finnigan), using an autosampler (ASX 510, Cetac Technologies) and sample introduction kit consisting of SeaSpray nebulizer and cyclonic spray chamber Twister. Due to low cytosolic metal concentrations in the gills, the analyses were performed for only six essential (Co, Cu, Fe, Mn, Se, and Zn) and one nonessential metal (Cd) and did not include Mo and Pb, which were previously analyzed in the liver. Measurements of ^{82}Se and ^{111}Cd were operated in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium resolution mode. External calibration was performed using standards prepared in 2 % HNO_3 (Suprapur, Merck) by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika). For quality control, QC sample for trace elements was used (UNEP GEMS/Water PE Study No. 7), and generally good agreement was observed between our data and the certified values. Limits of detection were as follows (in μg L^{-1}): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Se, 0.138; and Zn, 2.40 (Krasnići et al. 2013).

Table 2 Elution times (t_e) and molecular masses (MM) of six protein standards for Superdex™ 200 10/300 GL size exclusion column calibration, and of rabbit metallothionein standard

Protein	t_e min	MM kDa	Concentration mg mL^{-1}
Thyroglobulin	16.7	669	8
Apoferritin	18.0	443	10
β-amylase	20.7	200	4
Alcohol dehydrogenase	21.0	150	5
Bovine albumin	22.9	66	10
Carbonic anhydrase	28.7	29	3
Metallothionein (1st peak)	29.9	16.6[a]	5
Metallothionein (2nd peak)	30.9	12.5[a]	5

[a] MM of metallothionein was calculated from calibration equation

Determination of total cytosolic protein concentrations

The concentrations of total proteins in the gill cytosol were measured according to Lowry et al. (1951). The Bio-Rad DC Protein Assay was applied according to manufacturer's instructions. The measurements were performed on the spectrophotometer/fluorometer (Tecan, Infinite M200) at 750-nm wavelength. Calibration curve was constructed with five different concentrations (0.25–2.0 mg mL^{-1}) of bovine serum albumin (Serva, Germany) dissolved in the homogenization buffer. Total protein concentrations are presented in Table 1, separately for each of the analyzed samples of chub gill cytosol.

Data processing and statistical analyses

Chromatographic results were processed using TotalChrom Version 6.3.1 software (PerkinElmer). Graphs were created using the statistical program SigmaPlot 11.0 for Windows.

Results and discussion

In living organisms, most metal ions are bound to specific proteins or enzymes, and could act as active or structural centers of proteins (Garcia et al. 2006). Therefore, it is essential to expand the knowledge on specific proteins to which trace metals are associated in different fish tissues, such as gills and liver, to be able to understand their essential functions, their potential role in detoxification processes, as well as

possible undesirable impacts on fish. The analysis of distribution of trace elements in the gill cytosol of European chub (*S. cephalus*) presents a supplementation of the similar study recently performed on the hepatic cytosol of the same fish species (Krasnići et al. 2013). The distributions of selected metals among four protein categories, as previously defined by Krasnići et al. (2013) (high molecular mass proteins, HMM=>100 kDa; medium molecular mass proteins, MMM=30–100 kDa; low molecular mass proteins, LMM=10–30 kDa; and very low molecular mass proteins, VLMM=<10 kDa), which were established in this study, represent the first step towards identification of specific metal binding proteins in the chub gills. Protein separation of better quality could not be obtained in this phase of the study due to the limitation imposed by the applied column (Superdex™ 200 10/300 GL), as seen from a chromatogram presented in Fig. 1. However, when comparison was made with hepatic chromatogram (Krasnići et al. 2013), it can be seen that sharper and better distinguished protein peaks were obtained in the gills (Fig. 1), possibly due to approximately 25 % lower total cytosolic protein concentrations in the gills of all sampled chub (n=75; median=14.5 mg mL^{-1}; range=6.5–17.8 mg mL^{-1}) compared to liver (n=75; median=19.1 mg mL^{-1}; range=12.0–24.7 mg mL^{-1}) (Dragun et al. 2013).

In addition, the variations in metal allocation in different fish organs were established by comparison between gill profiles presented in this paper (Figs. 2 and 3) and previously published hepatic profiles (Krasnići et al. 2013). In general, the cytosolic concentrations of majority of metals in gills of all sampled chub (e.g., Cd=0.68±0.36 ng mL^{-1}, Cu=42.6±10.4 ng mL^{-1}, Dragun et al. 2013; Pb=5.3±9.3 ng mL^{-1}, Dragun et al. 2012) were much lower compared to the liver (e.g., Cd=19.4±11.6 ng mL^{-1}, Cu=1.5±0.7 μg mL^{-1}, Dragun et al. 2013; Pb=6.6±16.1 ng mL^{-1}, Dragun et al. 2012), which could be expected considering that gills can transfer absorbed metals by blood to the liver as a main detoxification organ (Souza et al. 2013). However, low metal concentrations in the gills were, in many cases, the cause of rather undefined metal distribution profiles in this tissue. Therefore, only several representative distribution profiles, with clear and distinguishable peaks, are presented in Figs. 2 and 3, whereas the profiles for all seven chub specimens are presented as supplementary information (Figs. SI-1–SI-7). Since the chub specimens analyzed in this study originated from moderately contaminated Sutla River (dissolved metal concentrations in the river water: Cd, 0.01–0.31 μg L^{-1}; Co, 0.06–0.42 μg L^{-1}; Cu, 0.17–3.74 μg L^{-1}; Fe, 3.1–80.5 μg L^{-1}; Mn, 0.4–261.1 μg L^{-1}; Zn, <5.0 μg L^{-1}; Dragun et al. 2011), the profiles presented in this paper could be regarded as characteristic for fish non-exposed to metals and can serve as a basis for comparison in the future studies of metal distribution in the gills with higher cytosolic metal concentrations.

Distribution profiles of essential elements

Cobalt

Cobalt was found in protein fractions covering wide range of MMs, with three distinguished peaks (Fig. 2a). The first one occurred within HMM protein category, with maximum corresponding to protein MM of 80 kDa (Table 3). The other two smaller peaks occurred within VLMM protein category, with maxima corresponding to protein MMs of 4 and 2 kDa, respectively (Table 3). Cobalt profiles obtained for six chub with cytosolic Co concentrations in the gills ranging from 1.02 to 1.60 ng mL^{-1} were comparable. Only in one fish the first VLMM peak (4 kDa) was approximately 10 times higher, which could not be explained by higher cytosolic Co concentration in the gills of that chub (cytosolic Co concentration=1.06 ng mL^{-1}; Fig. SI-1). The distribution profile of gill Co was almost identical to previously published hepatic profile (Krasnići et al. 2013), with the exception that Co peaks in the hepatic cytosol were higher, narrower and sharp, which could be explained by 2.5 times higher cytosolic Co concentration in presented hepatic sample (3.96 ng mL^{-1}) compared to the gills (1.60 ng mL^{-1}). The association of Co with VLMM protein fraction in gills can be explained as possible binding to known Co-containing compound, cobalamin (1.3 kDa; Kirschbaum 1981), as already observed in liver (Krasnići et al. 2013). However, in the liver, HMM peak was considerably higher than VLMM peak, indicating almost negligible portion of Co possibly associated to cobalamin. Contrary, in the gills all three peaks were nearly equal. It can be hypothesized that Co starts to bind to HMM proteins when present in cytosol in higher concentrations. As already pointed out for the liver (Krasnići et al. 2013), it would be beneficial to identify these Co-binding proteins in the gills, too, because waterborne metal cations, like Co^{2+}, can interfere with normal function of gills in ionic regulation, acid base balance, gas transfer, and nitrogenous waste excretion (Richards and Playle 1998), for example, by disrupting Ca transport (Hille 1992).

Copper

The distribution profile of Cu in chub gills is presented for two samples with different cytosolic Cu concentrations (Fig. 2b). The sample with lower Cu concentration (42.1 ng mL^{-1}) was characterized with two Cu-peaks (Fig. 2b, Table 3). The first peak occurred within HMM region with maximum associated to protein MM of about 500 kDa (Table 3). The second peak appeared in MMM region and had a maximum at t_e of 27 min,

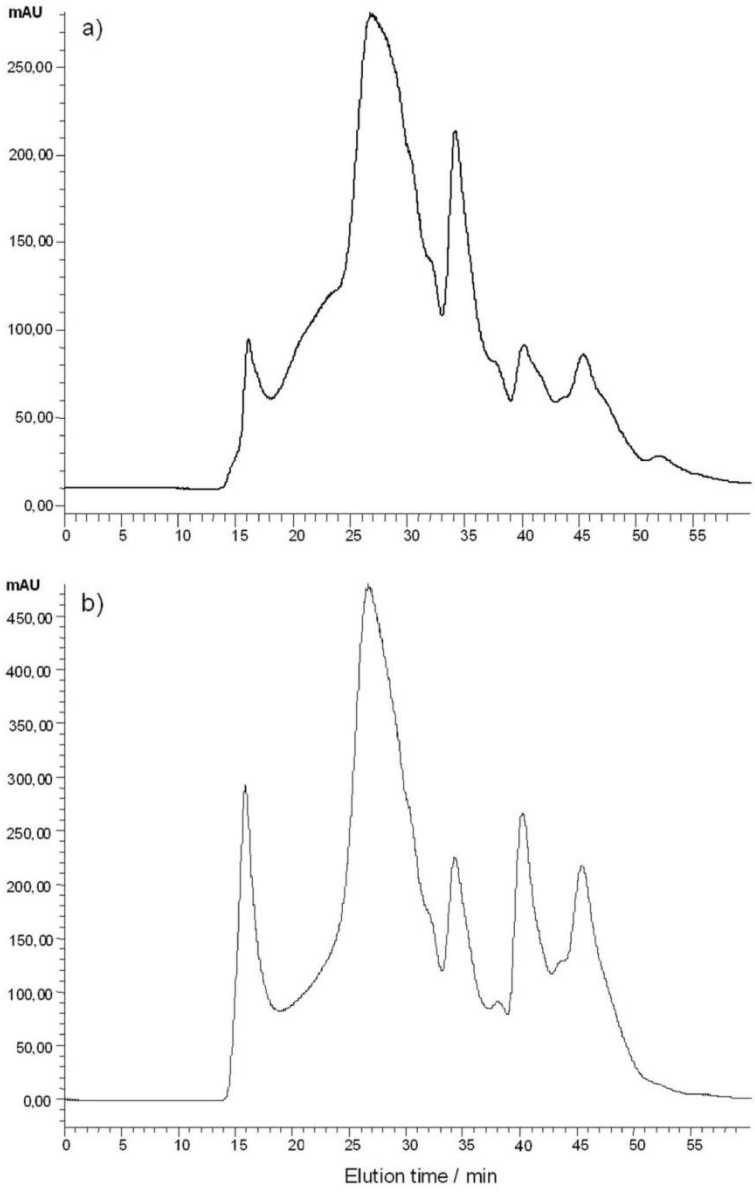

Fig. 1 An example of SEC-HPLC chromatogram profile of chub gill cytosol (100 μL) after separation on Superdex™ 200 10/300 GL column, with UV detection at two wavelengths: **a** λ=280 nm (characteristic for peptide bond); **b** λ=254 nm (characteristic for Cd–mercaptide bond)

which could be associated to protein MM of 35 kDa (Table 3). The range of molecular masses covered by this peak also included MTs, with t_e of 30.9 min (Table 2). Such profile was obtained for six chub specimens, with cytosolic Cu concentrations ranging from 40.7 to 49.9 ng mL^{-1} (Fig. SI-2). In the other profile presented in Fig. 2b, originated from the sample with almost twice higher cytosolic Cu concentration (76.0 ng mL^{-1}), both of these peaks were higher. Also, an additional HMM peak which was not observed at lower Cu concentrations was present in that profile. It had a maximum at t_e of 22 min, and covered the range of protein MMs from 60 to 310 kDa, which could possibly indicate Cu binding to several well-known Cu-containing proteins, such as albumin (66 kDa; Table 2), ceruloplasmin (151 kDa; Boivin et al. 2001), β-amylase (200 kDa; Table 2), or transcuprein (270 kDa; Liu et al. 2007). Similar feature of Cu profiles in gills and liver was Cu elution within MT peak which increased with increasing cytosolic Cu concentration (Fig. 2b; Krasnići et al. 2013). However, this association was more evident in the hepatic cytosol, probably due to significantly higher Cu concentrations (0.45–3.87 μg L^{-1}) compared to gill cytosol. On the other hand, in gill cytosol, Cu was found in HMM region indicating possible presence of blood protein ceruloplasmin in the sample, whereas in the hepatic cytosol, blood proteins

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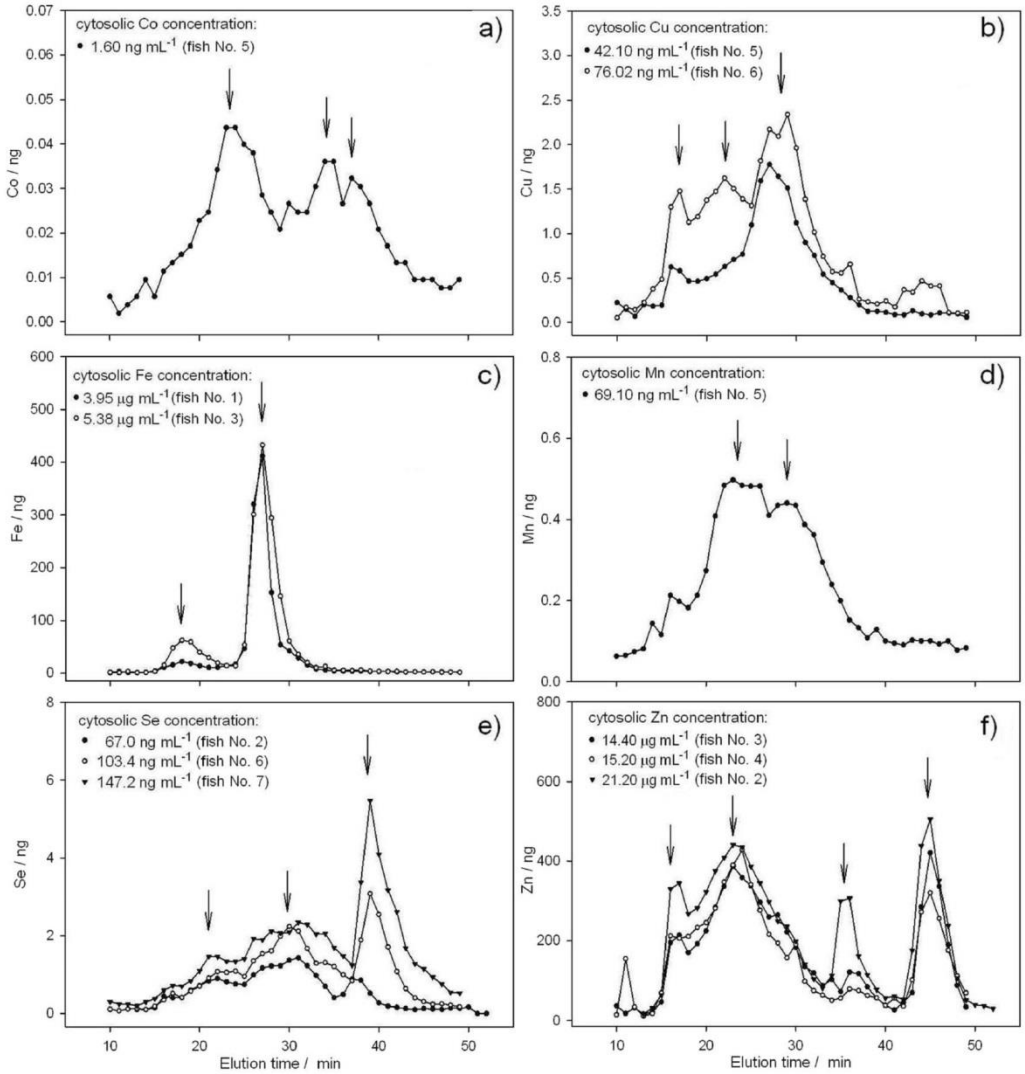

Fig. 2 Distribution profiles of essential trace elements among cytosolic proteins of different molecular masses from European chub gills, separated by SEC-HPLC with Superdex™ 200 10/300 GL column **a** Co; **b** Cu; **c** Fe; **d** Mn; **e** Se; and **f** Zn; the results are presented as nanograms of trace element eluted at specific elution times, after passing 400 µL of gill cytosol through the chromatographic column; the results obtained for fish no. 1 were multiplied by 2, because they were obtained by passing 200 µL of gill cytosol through the chromatographic column

were not recorded (Krasnići et al. 2013), or Cu association with them was negligible compared to its association with MTs.

Iron

The distribution profile of Fe in chub gills is presented, same as for Cu, for two samples with different Fe concentrations, and in both samples, it was characterized with two Fe-containing peaks (Fig. 2c). The predominant peak was found in the MMM region and covered the range of molecular masses from 10–80 kDa. The smaller peak was found in the HMM region with maximum corresponding to protein MM of 405 kDa, and it became more evident in the sample with higher Fe concentration (Table 3). The MMM peak was observed in all seven analyzed chub specimens, whereas HMM peak was more evident in gills of two specimens with cytosolic Fe concentrations above 5 µg mL^{-1} (Fig. SI-3). The position of Fe peaks in gill Fe profile was identical as in the hepatic profile (Krasnići et al. 2013). The predominant MMM peak was explained as a possible binding of Fe to certain Fe-containing proteins (Krasnići et al. 2013), like enzyme catalase (60 kDa) or transport protein myoglobin (17 kDa) (Wolf et al. 2007). The HMM peak, on the other hand, was attributed

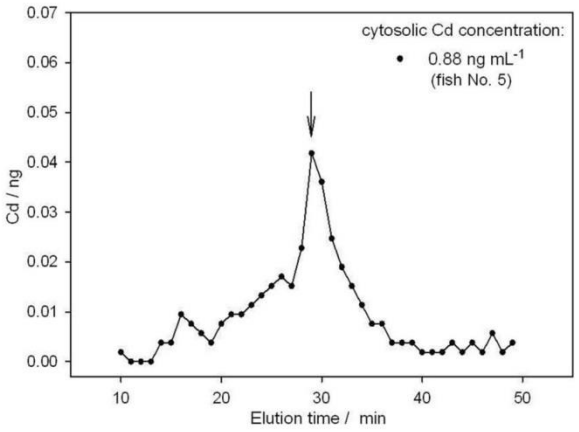

Fig. 3 Distribution profile of nonessential trace element Cd among cytosolic proteins of different molecular masses from European chub gills, separated by SEC-HPLC with Superdex™ 200 10/300 GL column; the results are presented as described in the caption of Fig. 2

to possible Fe storage in a form of ferritin (450 kDa; Szpunar and Lobinski 1999) (Krasnići et al. 2013). Assumed binding to ferritin was easier to observe in liver than in the gills due to higher cytosolic Fe concentrations in the liver, indicating more pronounced role of the liver than gills in Fe storage.

Manganese

Manganese distribution profile in chub gills included two poorly resolved peaks (Fig. 2d) covering a wide range of MMs from HMM to LMM region (310–2 kDa; Table 3). The first maximum corresponded to protein MM of about 105 kDa which could involve binding to one of known Mn containing proteins, such as superoxide dismutase or arginase, both having MM about 100 kDa (Wolf et al. 2007), or even transferrin (80 kDa; Martin-Antonio et al. 2009). The range of cytosolic Mn concentrations in chub gills was rather narrow (33.7–69.1 ng mL^{-1}), and therefore almost identical distribution profiles were obtained in the gills of all seven chub (Fig. SI-4). In hepatic cytosol, sharper Mn peaks were observed, with the predominant peak within HMM region (Krasnići et al. 2013). However, Mn concentration in the gill cytosol was 3.5–8 times lower compared to hepatic Mn concentration (250 ng mL^{-1}; Krasnići et al. 2013). Therefore, gill distribution profile actually corresponded only to the wide baseline of the hepatic profile, without clear peaks which were found in liver at higher Mn concentrations.

Selenium

Selenium profile was characterized by three peaks (Fig. 2e). The first two were joined and poorly resolved, and covered a wide range of molecular masses from 2 to 310 kDa, with maxima in the HMM region (180 kDa) and LMM region

Table 3 Distribution of trace elements among cytosolic fractions of chub gill containing proteins of different molecular masses, separated by size exclusion HPLC with Superdex™ 200 10/300 GL column

Element		HMM peak[a] 1		HMM peak[a] 2		MMM peak[b]		LMM peak[c]		VLMM peak[d] 1		VLMM peak[d] 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
Essential elements	Co	–	–	24 (19–29)	80 (310–20)	–	–	–	–	34 (32–36)	4 (9–2)	37 (36–40)	2 (2–1)
	Cu	17 (15–18)	530 (915–410)	22 (19–25)	140 (310–60)	27 (25–34)	35 (60–5)	–	–	–	–	–	–
	Fe	18 (16–22)	405 (700–140)	–	–	27 (24–31)	35 (80–10)	–	–	–	–	–	–
	Mn	–	–	23 (19–27)	105 (310–35)	–	–	29 (27–38)	20 (35–2)	–	–	–	–
	Se	–	–	21 (19–24)	180 (310–80)	–	–	30 (25–37)	15 (60–2)	–	–	39 (37–44)	1 (≤2)
	Zn	17 (15–18)	530 (915–405)	23 (19–31)	105 (310–10)	–	–	–	–	36 (34–40)	3 (5–1)	45 (43–48)	<1 (<1)
Non-essential element	Cd	–	–	–	–	–	–	29 (27–35)	20 (35–4)	–	–	–	–

Elution times (t_e) and molecular masses (MM) of proteins contained in the fractions in which respective elements were eluted are given in the table. Presented numbers refer to maxima of trace element peaks (i.e., the fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks. The results presented in this table are based on analyses of gill cytosol of seven chub specimens

[a] HMM peak—a peak of trace element quantity in the cytosolic fractions with a maximum in high molecular mass protein region (>100 kDa)

[b] MMM peak—a peak of trace element quantity in the cytosolic fractions with a maximum in medium molecular mass protein region (30–100 kDa)

[c] LMM peak—a peak of trace element quantity in the cytosolic fractions with a maximum in low molecular mass protein region (10–30 kDa)

[d] VLMM peak—a peak of trace element quantity in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa)

(15 kDa), respectively (Table 3). The third peak was detached and sharp. It became evident in the sample with Se concentration higher than 100 ng mL^{-1}, and further increased with increasing cytosolic Se concentration. In two samples with cytosolic Se concentrations lower than 100 ng mL^{-1}, that peak was still rather indistinct and had a maximum at t_e of 37 min (Fig. SI-5a). However, in the other five samples with cytosolic Se concentrations in the range from 103.4 to 147.2 ng mL^{-1}, the peak height increased 4–7 times and the maximum shifted to lower molecular masses (t_e of 39 min; Fig. SI-5b, c). It corresponded to VLMM proteins in the range of molecular masses below 2 kDa (Table 3). Selenium cytosolic concentrations in the gills (67–147 ng mL^{-1}) and the liver (45–171 ng mL^{-1}; Krasnići et al. 2013) were similar, which enabled objective profile comparison. Both Se profiles in the gills and in the liver had three peaks at the same locations. However, Se increase in the gill cytosol was mainly reflected in the sharp increase of VLMM peak (Fig. 2e), which could be associated to low molecular mass selenocompounds effective in the defense against oxidative stress, for example, by acting as a strong free radical scavenger, such as newly identified organic Se species in bluefin tuna (*Thunnus orientalis*), selenoneine (~0.5 kDa; Yamashita and Yamashita 2010; Yamashita et al. 2012) or selenomethionine (~0.2 kDa; Klotz et al. 2003). On the contrary, increase of cytosolic Se concentrations in the liver resulted with sharp increase of LMM peak (Krasnići et al. 2013), which could be associated to several selenoproteins catalytically active in redox processes, such as glutathione peroxidases, iodothyronine deiodinases, thioredoxin reductases (Hauser-Davis et al. 2012), whereas in the gills, HMM and LMM peaks increased only slightly.

Zinc

Zinc profile in chub gills was characterized by several peaks covering wide range of protein MMs from HMM to VLMM region (Fig. 2f). The first Zn peak was the widest and had two maxima within HMM protein region, but extended from ~10–900 kDa. The first maximum was within t_e of void volume and could be associated with protein MM of ~500 kDa, whereas the second one could be associated with a protein MM of ~100 kDa (Table 3). The second and third peak were better resolved and appeared within VLMM protein category, the second one with maximum at 3 kDa, and the third one below 1 kDa (Table 3). However, the second peak (maximum at 3 kDa) was distinctly observed only in two gill samples with cytosolic Zn concentrations above 21 μg mL^{-1}, whereas it could not be clearly distinguished in five samples with cytosolic Zn in the range from 9.6–15.2 μg mL^{-1} (Fig. SI-6). Similarly to gills, the hepatic Zn profile was also characterized by wide and poorly resolved peaks covering broad range of MMs (Krasnići et al. 2013). This was an indication of Zn binding to large number of proteins both in the liver and in the gills, for example transport protein albumin (66 kDa, Table 2), and enzymes alcohol dehydrogenase (150 kDa, Table 2), Cu–Zn superoxide dismutase (32.5 kDa) or carbonic anhydrase (29 kDa, Table 2) (Sanz-Medel et al. 2003). In the gills, MM of MTs (16.6 and 12.5 kDa, Table 2) was also encompassed by the right tail of the first wide peak, but clear Zn–MT peak was not observed. On the contrary, hepatic Zn peak which presumably indicated association to MTs was sharp and could be clearly differentiated from the other Zn peaks (Krasnići et al. 2013). However, it should be emphasized, that contrary to other metals, Zn concentrations in the gills (14.4–21.2 μg mL^{-1}) were two to three times higher than in the liver (7.2 μg mL^{-1}, Krasnići et al. 2013), and possibly, Zn binding to various HMM and MMM proteins masked Zn-MT association. On the other hand, the gill Zn profile was distinguished by two high VLMM peaks, which increased following the increase of Zn cytosolic concentration (Fig. 2f), whereas hepatic Zn profile had only a few small, barely visible Zn peaks in the VLMM region (Krasnići et al. 2013).

Distribution profile of nonessential metal cadmium

The most prominent peak of nonessential element Cd in the gills was found in the LMM protein region (Fig. 3), with maximum at elution time of MTs (t_e ~29 min; Tables 2 and 3), same as previously described for chub hepatic cytosol (Krasnići et al. 2013). It was observed in all seven analyzed chub specimens, but somewhat higher in the gills of two specimens with cytosolic Cd concentrations above 1 ng mL^{-1} (Fig. SI-7b) compared to five specimens with cytosolic Cd concentration in the range from 0.48–0.88 ng mL^{-1} (Fig. SI-7a). Some indication of Cd distribution within HMM and MMM proteins was also observed (Fig. 3), especially in two samples with cytosolic Cd concentrations above 1 ng mL^{-1} (Fig. SI-7b), which could point to association to various proteins, such as, for example, transferrin (801 kDa), which is recently recognized as a major Cd binding protein in fish blood plasma (De Smet et al. 2001). Next to association with LMM fractions, a small portion of cytosolic Cd was found associated with MMM fractions (35–105 kDa) even in the hepatic cytosol of chub (Krasnići et al. 2013), whereas in the hepatic cytosol of squid (*Todarodes pacificus*) a large portion was bound to species with MM >70 kDa (Tanaka et al. 1983). However, at low cytosolic Cd concentration, as found in the presented gill sample (0.88 ng mL^{-1}; Fig. 3), such association could not be clearly established. Prevailing Cd allocation within MT peak was indicated both by Cd distribution profile in the gills at low cytosolic Cd concentration (Fig. 3, Tables 2 and 3) and in the liver at 8–67 times higher cytosolic Cd concentrations (7–59 ng mL^{-1}; Krasnići et al. 2013). It was a confirmation of known high affinity of Cd for MTs, as a mechanism of protection against toxicity (Roesijadi 1992; Park et al. 2001) .

Conclusions

Based on metal determination by HR ICP-MS after fractionation of chub gill cytosol by SEC-HPLC, distribution profiles of several essential and nonessential trace elements (Co, Cu, Fe, Mn, Se, Zn, and Cd) among cytosolic proteins of different molecular masses were determined. Comparison of gill profiles with previously published hepatic profiles (Krasnići et al. 2013) revealed almost identical distributions of Co, Fe, Mn, and Se in both organs. The obtained peaks had similar or identical t_e, but different heights, indicating possible binding to same proteins in the gills and liver, but in different proportions. For example, with increasing cytosolic Fe concentration, a peak appeared at t_e of Fe storage protein ferritin (t_e 18 min; MM ~400 kDa), but much smaller compared to hepatic Fe profile, indicating more important function of liver in Fe storage. Selenium, on the other hand, increased in the VLMM region in the range of MM below 2 kDa following the increase of cytosolic Se in the gills, contrary to hepatic Se which was allocated mainly with LMM or MMM proteins (10–60 kDa). Furthermore, for both Cu and Cd, a peak was obtained near t_e of MTs (27 and 29 min, respectively), same as in the hepatic cytosol. However, an additional Cu peak in HMM region (>100 kDa) was obtained in the gills, which was not previously observed in the chub liver. Zinc had wide and poorly resolved peaks in both organs, but unlike hepatic cytosol, expected clear MT peak was not observed in the gills, possibly due to binding of Zn in higher proportion to other proteins of higher molecular masses. Similar to Se, as a result of increase of cytosolic Zn concentration in the gills, Zn increase was observed in the VLMM region at MM <5 kDa, which was not registered in the chub liver. The obtained profiles were mainly characteristic for fish non-exposed to metals, i.e., for low total cytosolic metal concentrations, and thus could be used as a basis for comparison in monitoring studies, as well as for detection of changes in the profiles of the fish exposed to increased metal concentrations.

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Size-exclusion HPLC analysis of trace element distributions in hepatic and gill cytosol of Vardar chub (*Squalius vardarensis* Karaman) from mining impacted rivers in north-eastern Macedonia

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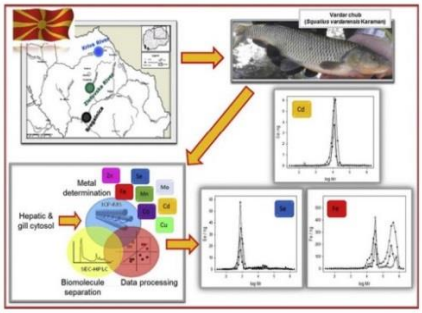
HIGHLIGHTS

- Metallomics approach in monitoring effects of metal pollution on Vardar chub
- Use of SEC-HPLC/HR ICP-MS for determination of metal distributions within cytosol
- Distribution profiles of Cd, Co, Cu, Fe, Mn, Mo, Se and Zn in chub liver and gills
- Changes in the distribution profiles due to increased metal exposure level in water
- Comparison of metal profiles in Vardar and European chub, two related fish species

GRAPHICAL ABSTRACT



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ABSTRACT

Many bioindicators have not yet been well characterized regarding their tendency to bind trace elements by different cytosolic biomolecules in response to trace element exposure. Accordingly, our principal aim was to define the cytosolic distributions of Cd, Co, Cu, Fe, Mn, Mo, Se, and Zn among the biomolecules of different molecular masses in liver and gills of Vardar chub (*Squalius vardarensis* Karaman), a representative fish species of Macedonian rivers, and to determine distribution changes which occur as a consequence of increased exposure to specific trace elements. Additionally, we aimed to confirm the presence of heat-stable biomolecules in chub hepatic and gill cytosols. Distribution profiles were obtained by separation of cytosols and heat-treated cytosols using size-exclusion high performance-liquid chromatography, and by offline determination of trace element concentrations using high resolution inductively coupled plasma-mass spectrometry. Distribution profiles of trace elements were mainly characterized by several peaks encompassing different ranges of molecular masses, as a sign of incorporation of trace elements in various biomolecules within hepatic and gill cytosols. Especially interesting finding was probable binding of Fe to ferritin, which was especially pronounced in the liver, as a sign of important liver function in Fe storage. Furthermore, association with heat-stable proteins, metallothioneins (MT), was indicated for Cd, Cu, and Zn in the hepatic cytosol, as well as for Cd in the gill cytosol, whereas a sign of Zn-MT association was not observed in the gills. The presence of Mo- and Se-binding heat-stable compounds of very low molecular masses (<10 kDa) in the cytosol was determined for both liver and the gills. Trace elements under

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all studied conditions were found associated to the same biomolecules, and only their proportions associated to specific cytosolic compounds have changed as a consequence of their increased bioaccumulation in the liver and gills of Vardar chub.

1. Introduction

Trace element contamination which originates from anthropogenic sources, such as the mining activities (Ramani et al., 2014), presents one of the major concerns for the preservation of high ecological status of aquatic ecosystems. Some trace elements are essential in the development and functions of living organisms (e.g. Zn, Cu, Fe and Se), and they play an important role as cofactors of a number of metalloproteins and enzymes (García-Sevillano et al., 2012). However, in high concentrations they can also compete with the other elements for protein binding sites, and thus may cause toxic effects. A number of the other trace elements (e.g. Ag, Cd, Hg, Pb) are considered non-essential, and are associated with harmful effects in the organisms even in very low concentrations. Therefore, a long term exposure to high levels of trace elements in the water, which can result in the increased bioaccumulation of trace elements in organs of aquatic organisms, can consequently cause various toxic effects, starting with reactions in the cytosol, such as nonspecific binding of trace elements to physiologically important molecules and their consequent inactivation (Mason and Jenkins, 1995).

In the pollution assessment, it is important to analyze not only the concentrations of accumulated trace elements in the organs of aquatic organisms, but also their fate within the cells by studying the biomolecules which bind trace elements, as potential biomarkers of trace element exposure and their possible toxicity. Particularly useful organisms for the assessment of the effect of waterborne and sediment-deposited trace elements are fish, due to their high sensitivity and readily measured responses to trace elements (Goenaga Infante et al., 2003). Analysis of different biomolecules that bind trace elements in fish, known as metalloproteins, can provide insight in the impact of contaminants on fish cellular metabolism and global homeostasis (García-Sevillano et al., 2014). So far only a few fish metalloproteins have been discovered and applied as successful biomarkers of trace element exposure, such as metallothioneins (MTs), but their exact functional roles in fish physiology are not yet well understood (Hu et al., 2013; Lavradas et al., 2016). Therefore, it is important to study metalloproteins in fish in more detail, and the development of metallomic techniques in the recent years offers the possibility to perform such comprehensive studies (Hauser-Davis et al., 2012).

We have applied metallomic techniques in the study of biomolecules that bind trace elements in Vardar chub (*Squalius vardarensis* Karaman) from three mining and agriculturally impacted Macedonian rivers. In Macedonia, mining is still one of the most important industries, with Pb/Zn ores in the north-eastern part of the country being the most significant mineral deposits for exploitation (Midžić and Silajdžić, 2005; Barišić et al., 2015). As a result, many natural freshwater ecosystems, especially in the north-eastern Macedonia, are contaminated with trace elements. Selected fish species, Vardar chub (*S. vardarensis*), is a representative fish in rivers of Vardar basin, as well as a member of genus *Squalius*, wide spread in European rivers, thus providing the possibility for comparison among distant geographical regions (Barišić et al., 2015). The exposure of fish to trace elements can result in their accumulation in different organs, and in this study we have chosen two of them: liver as a metabolic and detoxification centre of an organism (Krasnići et al., 2013; Van Campenhout et al., 2004; Dragun et al., 2012, 2015), as well as a major producer of trace element-binding proteins (Roesijadi and Robinson, 1994); and gills, as a good indicator of current environmental conditions, due to their direct and permanent contact with contaminants in the water (Bernet et al., 1999; Amiri et al., 2011), and their fast response and high sensitivity even to low concentrations of contaminants (Monteiro et al., 2008). To our knowledge, so far there is no information available concerning the accumulation of trace elements and their cytosolic distribution in organs of Vardar chub.

The relationship between environmental exposure, bioaccumulation and distribution among cytosolic biomolecules of seven essential elements (Co, Cu, Fe, Mo, Mn, Se, Zn) and one non-essential element (Cd) in Vardar chub liver and gills was studied applying the separation of cytosolic biomolecules by size-exclusion high performance liquid chromatography (SEC-HPLC) combined with offline determination of trace elements by high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). Our main aim was to define, for the first time, the distribution profiles of studied elements among cytosolic biomolecules of different molecular masses, and to determine changes which occur as a consequence of increased exposure to specific trace elements. Having in mind that some heat-stable biomolecules, such as MT, have important role in trace element detoxification, our additional objective was to analyze cytosolic distribution of trace elements after cytosol heat-treatment, according to the procedure applied for MT analysis (Erk et al., 2002). Similar study was previously performed on mussels by Lavradas et al. (2016), but, to the best of our knowledge, this is the first attempt to determine distribution of selected elements (Cd, Cu, Mo, Se, Zn) among various cytosolic heat-stable biomolecules in organs of fish.

2. Materials and methods

2.1. Fish sampling and organ dissection

Selected bioindicator species, Vardar chub (*S. vardarensis*), was collected in spring (May/June) of 2012 from three rivers in north-eastern Macedonia: the moderately agriculturally contaminated Bregalnica River and two rivers, Zletovska and Kriva, impacted by active Pb/Zn mines Zletovo and Toranica, respectively. In total, 90 fish was sampled, 30 from each river. The map and detailed description of the sampling area were already published (Ramani et al., 2014). The fish were caught by electro fishing, using electrofisher Samus 725G, according to relevant standard (CEN EN 14011:2003). Captured fish were kept alive in a tank with aerated river water until further processing in the laboratory. Fish capture and their handling complied with the current laws of the Republic of Macedonia. Individual fish were anesthetized with Clove Oil (Sigma Aldrich, USA). First, the biometric data were recorded (total length, whole mass), whereas Fulton condition indices (FCI) were calculated according to Rätz and Lloret (2003). After the fish were anesthetized and sacrificed, the liver, gills and gonads were removed. Liver and gills were weighed and stored in liquid nitrogen immediately after sampling and then held at −80 °C until further analyses. Gonads were used for histological examination of sex.

Since previous studies have demonstrated high reliability and repeatability of trace element distribution profiles obtained after the repeated measurements in the same cytosolic samples, or in the samples of the same fish species and target organ, by the same approach as applied in this study (same chromatographic column, HPLC and HR ICP-MS system) (Krasnići et al., 2013, 2014), we have decided to design this study based on the analyses of only three fish per each river, due to the time restrictions and limited resources. The selection of fish for analyses was based on two criteria: 1) the availability of the sample for analysis, which depended on the fish size and 2) cytosolic trace element concentrations in liver and gills. We wanted to study samples with a

wide range of different cytosolic trace element concentrations, from low to high, to be able to define what happens with the additional quantity of trace elements accumulated in Vardar chub liver and gills as a consequence of higher environmental exposure. Since some of the fish selected for hepatic analysis had too low gill cytosolic concentrations, or too small volume of gill cytosol available for analyses, we were forced to select additional fish for gill analyses. Also, due to smaller fish size in the Zletovska River, and thus also smaller volume of gill cytosol, we have analyzed only two gill samples from the Zletovska River, and instead we have analyzed four gill samples from the Kriva River. Therefore, we have analyzed in total 13 specimens of Vardar chub, and their biometric characteristics and total cytosolic protein concentrations are given in Table 1. Hepatic analyses were performed on the nine fish specimens marked with the following ordinal numbers: 1, 2, 4, 5, 6, 7, 8, 10, and 12 (Table 1, Figs. 1–2). Gill analyses were performed on the nine fish specimens marked with the following ordinal numbers: 2, 3, 4, 5, 7, 9, 11, 12, and 13 (Table 1, Fig. 3). Analyses of heat-stable biomolecules were performed on one hepatic sample (No. 8, Table 1, Fig. 4), and on two gill samples (No. 11 and 13, Table 1, Fig. 5). Few profiles were excluded from presentation, due to analytical problems, such as contamination with certain elements during the chromatographic separations or measurement, namely Mo in hepatic samples 4, 7, and 12 (Fig. 2a); Se in hepatic sample 4 (Fig. 2b); Fe in gill sample 11 (Fig. 3a); Zn in gill sample 13 (Fig. 3c); and Cd in gill sample 12 (Fig. 3d).

2.2. Isolation of cytosolic fractions from Vardar chub liver and gills

Whole frozen livers and gills of Vardar chub (0.84–5.46 g and 0.29–2.31 g, respectively) were cut into small pieces. Then cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4 °C) supplemented with reducing agent (1 mM dithiothreitol, Sigma)] was added (*w*/*v* 1:5), which was followed by homogenization in an ice cooled Potter-Elvehjem homogenizer (Glas-Col, USA) applying 10 strokes of motor-driven PTFE-pestle at 6000 rpm. The homogenates were centrifuged (Avanti J-E centrifuge, Beckman Coulter) at 50,000 ×*g* for 2 h at 4 °C. The obtained supernatants, which correspond to water-soluble cytosolic tissue fractions (Van Campenhout et al., 2010) containing lysosomes and microsomes (Bonneris et al., 2005), were aliquoted and stored at −80 °C for separation by size exclusion high performance liquid chromatography (SEC-HPLC) and for analyses of trace elements (Krasnići et al., 2013, 2014).

Table 1
Basic biometric characteristics and total cytosolic proteins in livers and gills of 13 specimens of Vardar chub (*Squalius vardarensis*) used in this study, which were caught in moderately contaminated Bregalnica River and two mining impacted rivers (Zletovska and Kriva) in north-eastern Macedonia in spring (May/June) of 2012.

Fish no.	River	Length/cm	Mass/g	Sex	FCI/%	Total proteins/mg mL^{-1}	
						Liver	Gills
1	Bregalnica	21.1	108.1	F	1.15	23.6	17.6
2	Bregalnica	22.0	137.3	F	1.29	22.4	20.1
3	Bregalnica	20.4	105.8	F	1.25	24.7	21.2
4	Bregalnica	17.5	58.6	F	1.09	21.4	16.3
5	Zletovska	21.3	106.7	F	1.10	16.1	15.1
6	Zletovska	15.2	37.4	F	1.06	16.1	–
7	Zletovska	19.4	69.3	F	0.95	19.7	16.0
8	Kriva	30.2	350.0	F	1.27	17.3	17.3
9	Kriva	20.1	99.9	F	1.23	19.3	17.5
10	Kriva	27.5	231.5	F	1.11	21.1	15.5
11	Kriva	22.5	138.3	F	1.21	19.3	22.1
12	Kriva	26.0	210.6	M	1.20	24.1	24.2
13	Kriva	21.4	125.1	F	1.28	20.2	22.8

2.3. Heat-treatment of hepatic and gill cytosols

The heat-treatment of hepatic and gill cytosols was performed according to Erk et al. (2002), with slight modifications. The cytosols were heat-treated at 70 °C for 10 min using The Dri Block (Techne) (Bibby Scientific Limited, Staffordshire, UK). After the heat-treatment, cytosols were placed on ice and kept for 30 min at 4 °C, and subsequently centrifuged at 10,000 ×*g* for 15 min at 4 °C (Heraeus Biofuge Fresco, Kendro, USA). Supernatants obtained after this step were stored at −80 °C until further analysis. This heat-stable fraction is expected to show lower protein content than cytosolic fraction, due to denaturation of thermo-labile and high molecular mass proteins from cytosol samples at temperatures above 60–70 °C (Krizkova et al., 2011). Heat-treated cytosols, for example, contain heat-stable cytosolic proteins MTs (Yang et al., 1995).

2.4. SEC-HPLC analysis of cytosols and heat-treated cytosols from chub liver and gills

Distribution of trace elements among biomolecules of different molecular masses in the cytosols and heat-treated cytosols from Vardar chub livers and gills was studied by SEC-HPLC (Perkin Elmer HPLC system, series 200, USA), as previously described in Krasnići et al. (2013, 2014). Hepatic and gill cytosols and heat-treated cytosols were separated into fractions containing biomolecules of different molecular masses (MM) using two types of size exclusion columns (SEC): prepacked Tricorn™ Superdex 200 10/300 GL (GE Healthcare Biosciences, USA) with a separation range of 10 kDa–600 kDa and Tricorn™ Superdex 75 10/300 GL column (GE Healthcare Biosciences, USA) with a separation range of 3 kDa–70 kDa, for globular proteins. Immediately before application on the column, cytosol samples were centrifuged at 10,000 ×*g* for 10 min at 4 °C (Heraeus Biofuge Fresco, Kendro, USA). The sample volumes of 100 μL were applied on the column. For each sample, two consecutive chromatographic runs were performed, i.e. 200 μL in total of each cytosol sample was run through the column. The separation was achieved using 20 mM Tris-HCl/Base (Sigma, pH 8.1 at 22 °C, flow 0.5 mL min^{-1}) as a mobile phase (isocratic mode). The fractions were collected at 1 min intervals in the plastic tubes using a fraction collector (FC 203B, Gilson, USA). In total 40 fractions were collected for each sample, starting with 13th minute to 52nd minute. For column calibration, several protein standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, conalbumin, bovine albumin, ovalbumin, carbonic anhydrase, cytochrome C, vitamin B12, Sigma, USA) were run through the column under the same conditions as the samples (Table 2). The

Table 2
Elution times (t_e) and molecular masses (MM) of seven proteins used as standards for calibration of Superdex™ 200 10/300 GL and Superdex™ 75 10/300 GL size exclusion columns, as well as of rabbit metallothionein standard (Enzo Metallothionein-1, Enzo Metallothionein-2).

	Protein	t_e/min	MM/kDa	Concentration/ mg mL^{-1}
Superdex™ 200 10/300 GL	Thyroglobulin	16.08	669	8
	Apo-ferritin	17.80	443	10
	β-amylase	20.36	200	4
	Alcohol dehydrogenase	21.72	150	5
	Bovine albumin	23.13	66	10
	Superoxide dismutase	27.87	32	1.25
	Carbonic anhydrase	29.66	29	3
	Metallothionein - 2	31.22	6.1	1
	Metallothionein - 1	32.32	6.1	1
Superdex™ 75 10/300 GL	Conalbumin	18.57	75	3
	Ovalbumin	17.74	43	4
	Superoxide dismutase	19.97	32	1.25
	Carbonic anhydrase	21.26	29	5
	Cytochrome C	24.80	12	3
	Metallothionein-2	23.02	6.1	1
	Metallothionein-1	24.06	6.1	1
	Vitamin B12	36.14	1.35	3

equation of the calibration straight line for Superdex™ 200 was: y = −0.2808× + 1.6469; and for Superdex™ 75: y = − 0.3343× + 1.6664; y = Kav; x = log MM. In addition, elution times were also determined for superoxide dismutase (Sigma, USA), MT-1 and MT-2 (Enzo Life Sciences, USA). The void volume was determined by use of blue dextran (defined MM: 2000 kDa), which was eluted in columns Superdex™ 200 and Superdex™ 75 at 16.3 min and 15.6 min, respectively.

2.5. Determination of trace element concentrations in chub hepatic and gill cytosols and in the fractions obtained by SEC-HPLC separation

The concentrations of eight trace elements (Co, Cu, Fe, Mo, Mn, Se, Zn and Cd) were determined within this study. For determination of cytosolic trace element concentrations in the liver and gills of Vardar chub (Table 3), cytosols were ten times diluted with Milli-Q water and acidified with HNO_3 (*Suprapur*, Merck, Germany; final acid concentration in the samples: 0.65%). Fractions of liver and gill cytosols obtained after SEC-HPLC separation were only acidified with HNO_3 (*Suprapur*, Merck, Germany, final acid concentration in the samples 0.16%) prior to offline measurement of trace elements. Indium (Fluka, Germany) was added to all samples as an internal standard (1 μg L^{-1}). The measurements were performed using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister. Typical instrumental conditions and measurement parameters were reported previously (Fiket et al., 2007). Measurements of ^{82}Se, ^{98}Mo, ^{111}Cd were operated in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium resolution mode. External calibrations were performed using multielement standard solution for trace elements (Analitika, Czech Republic). All standards were prepared in 1.3% HNO_3 (*Suprapur*, Merck, Germany) and supplemented with In (1 μg L^{-1}; Fluka, Germany). The accuracy of trace element measurements by HR ICP-MS was checked by analysis of quality control sample (QC for trace elements, catalog no. 8072, lot no. 146142-146143, Burlington, Canada). A generally good agreement was observed between our data and the certified values, with the following recoveries (%) (based on 23 measurements in control sample for trace elements): Cd (99.2 ± 3.7), Co (100.0 ± 4.9), Cu (100.7 ± 11.2), Fe (92.3 ± 7.3), Mn (102.3 ± 13.2), Mo (95.9 ± 2.4), Se (95.4 ± 3.9), and Zn (97.7 ± 27.7). Somewhat higher deviation of Zn recoveries in comparison to other analyzed elements was due to much lower Zn concentrations in the control sample than in the gill and hepatic cytosols. Limits of detection (LODs) were determined based on three standard deviations of ten consecutively determined trace element concentrations in the blank sample (Tris-HCl/Base, dithiothreitol, HNO_3). LODs for trace elements measured within this study were as follows (in μg L^{-1}): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Mo, 0.004; Se, 0.138; and Zn, 2.40 (Krasnići et al., 2013, 2014).

Table 3
Total trace element concentrations (ng mL^{-1} or μg mL^{-1}) in analyzed hepatic and gill cytosols of Vardar chub (*Squalius vardarensis*) which were caught in moderately contaminated Bregalnica River and two mining impacted rivers (Zletovska and Kriva) in north-eastern Macedonia in spring (May/June) of 2012.

Fish no.	Organ	Co	Cu	Fe	Mn	Mo	Se	Zn	Cd
		ng mL^{-1}	μg mL^{-1}	μg mL^{-1}	ng mL^{-1}	ng mL^{-1}	ng mL^{-1}	μg mL^{-1}	ng mL^{-1}
1	Liver	4.77	1.67	13.5	322.4	26.5	228.0	5.17	2.69
2	Liver	5.54	2.87	8.47	288.2	19.5	254.0	6.31	2.38
	Gills	–	–	18.3	–	–	88.5	4.28	0.34
3	Gills	–	–	17.3	–	–	93.4	5.28	0.41
4	Liver	5.21	3.66	6.61	408.6	–	–	6.93	6.40
	Gills	–	–	11.1	–	–	89.1	5.61	0.57
5	Liver	3.34	3.10	7.06	231.7	13.2	85.8	3.51	36.9
	Gills	–	–	5.48	–	–	57.9	14.4	33.8
6	Liver	2.56	2.35	6.40	214.5	12.6	115.1	5.21	18.0
7	Liver	3.39	4.19	12.7	233.2	–	124.6	5.51	33.8
	Gills	–	–	8.57	–	–	70.3	11.7	22.6
8	Liver	4.22	6.54	7.44	237.1	26.6	402.7	7.61	40.5
9	Gills	–	–	9.70	–	–	178.9	12.0	10.5
10	Liver	5.27	3.70	7.45	250.4	21.2	289.8	8.37	28.7
11	Gills	–	–	–	–	3.05	300.8	5.76	22.9
12	Liver	4.43	4.86	14.7	257.6	–	631.1	7.87	68.2
	Gills	–	–	28.5	–	–	469.0	6.23	–
13	Gills	–	–	22.6	–	2.55	449.7	6.23	30.0

2.6. Data processing

Chromatographic results were processed using TotalChrom Version 6.3.1 software (Perkin Elmer, USA). All basic calculations were done in Microsoft Excel 2007, whereas graphs were created using the statistical program SigmaPlot 11.0.

3. Results and discussion

This research presents the first attempt to gain information on the influence of water contamination and of the subsequent trace element bioaccumulation on the distribution of trace elements among unknown cytosolic biomolecules in two organs, gills and liver, of Vardar chub (*S. vardarensis*). Information on cytosolic trace element distribution of this type was previously reported for gills and liver of European chub (*Squalius cephalus*) from moderately contaminated Sutla River in Croatia (Krasnići et al., 2013, 2014), but still does not exist for Vardar chub. Thirteen selected specimens of Vardar chub, analyzed in this study, whose biometric characteristics and total cytosolic protein concentrations are presented in Table 1, were caught in three rivers in north-eastern Macedonia, displaying different degrees and types of environmental contamination: three specimens were caught in the Zletovska River and six specimens in the Kriva River, both being contaminated by waste of active Pb/Zn mines, and four specimens in the Bregalnica River, as river less contaminated with trace elements and impacted by agricultural runoff. Severe trace element contamination of the Zletovska River and slighter contamination of the Kriva River were confirmed by water analysis performed in parallel with fish sampling (Ramani et al., 2014). Consequent harmful impact on the fish was also reported, which was reflected in observable histopathological damages on gills, liver and spleen of Vardar chub (Barišić et al., 2015; Jordanova et al., 2016, 2017).

We have defined the basic cytosolic distributions of trace elements among biomolecules of different molecular masses in the liver and gills of Vardar chub, which are characteristic for low exposure to trace elements. According to Langston et al. (2002), increased environmental concentrations of trace elements may cause shifts in their distribution profiles among cytosolic ligands, and thus the distribution changes as a consequence of increased accumulation of trace elements in the selected chub organs were also determined. The technology applied for that purpose consisted of SEC-HPLC, using Superdex™ 200 column with a linear separation range between 10 and 600 kDa, and HR ICP-MS, coupled offline. Several studies have demonstrated the potential of SEC-HPLC/ICP-MS coupling as a sensitive multi-elemental approach for the quantitative analysis of metalloproteins (Mason and Storms, 1993; Ferrarello et al., 2000; de la Calle Guntiñas et al., 2002; Montes-Bayón et al., 2003). The elution times of analyzed trace elements were associated to specific protein molecular masses by use of calibration straight line, obtained by chromatographic analysis of standard proteins. For the easier handling of the obtained results, cytosolic biomolecules were categorized in four groups, according to their molecular masses (MM), as previously defined by Krasnići et al. (2013, 2014): high MM (HMM), >100 kDa; medium MM (MMM), 30–100 kDa; low MM (LMM), 10–30 kDa; and very low MM (VLMM), <10 kDa.

3.1. Distributions of trace elements in hepatic and gill cytosols of Vardar chub

As defined by Bonneris et al. (2005), tissue cytosols obtained after centrifugation of tissue homogenates at 50,000 ×*g* (a protocol applied in our study) contain both heat-sensitive and heat-stable biomolecules that bind trace elements, with latter being considered as a detoxified trace element forms (Giguère et al., 2006; Rosabal et al., 2015). Analysis of trace element distributions among cytosolic biomolecules therefore gave insight into both potentially toxic and detoxified cellular pools of trace elements.

It is well known that bioaccumulation of trace elements depends on different factors, such as fish species, nature of trace elements, as well as the organ in which accumulation occurs. The cytosolic concentrations of many trace elements in Vardar chub gills were much lower compared to the liver, as can be seen from cytosolic concentrations listed in Table 3. This could be explained by the ability of gills to transfer absorbed trace elements by blood to the liver as a main detoxification and storage organ in fish (Souza et al., 2013; Amiri et al., 2011). Much higher hepatic than gill cytosolic concentrations of several elements were the reason why we were able to describe cytosolic distributions for as much as eight elements in the liver (Co, Cu, Fe, Mn, Mo, Se, Zn, and Cd), whereas in the gills the same thing was done for only four elements (Fe, Se, Zn, and Cd). Cytosolic distributions of trace elements in the Vardar chub liver are presented in the Figs. 1 and 2, and in the gills in the Fig. 3, whereas their elution times and molecular masses of corresponding biomolecules are given in Table 4. Each studied element will be separately discussed further in the text.

3.1.1. Cobalt

Cobalt distribution profiles were determined only for the hepatic cytosol (Fig. 1a). In general, the toxicity of Co to fish seems to be quite low compared with the effects of the other metal ions, especially during in situ environmental exposures (Marr et al., 1998; Kubrak et al., 2011). Nevertheless, Co has also been recognized as a stress inducing factor, which can participate in free radical processes, resulting in reactive oxygen species (ROS) production (Wang et al., 1993; Olivieri et al., 2001; Battaglia et al., 2009). Cobalt was eluted in four peaks, and the major proportion of the cytosolic Co in liver was eluted within the HMM peak (~110–380 kDa), with a maximum corresponding to biomolecules of MM ~230 kDa (Table 4). It is consistent with previous reports that ions of Co, as an essential element, have a high affinity for binding to high MM enzymatic proteins (Paustenbach et al., 2013; Wojcieszek and Ruzik, 2016). Much smaller amounts of Co were present in three remaining peaks, associated with MMM biomolecules (~30–80 kDa, with a maximum at 66 kDa), and with VLMM biomolecules distributed in two peaks (2–5 kDa, with a maximum at 4 kDa; and 0.7–2 kDa, with a maximum at 1.2 kDa) (Table 4). This last peak, with a maximum at 1.2 kDa, could possibly correspond to Co-containing compound cobalamin, which has a MM of 1.3 kDa (Kirschbaum, 1981). The studies on mussels also showed that a considerable proportion of Co was associated to biomolecules of MM below 4 kDa (Ferrarello et al., 2000). Although the main described role of Co in the fish organism is associated with its constitutive role in cobalamin, i.e. the vitamin B12 (Blust, 2012; Krasnići et al., 2013), and although the liver plays a major role in the vitamin B12 metabolism (Wang et al., 2001), only minor part of Co present in the Vardar chub hepatic cytosol was eluted within the fraction presumably containing cobalamin. Distribution profile of Co in Vardar chub liver was further compared to previously published Co hepatic profile of European chub (*S. cephalus*), and they were almost identical, with the only exception that Co HMM and MMM peaks in the hepatic cytosol of European chub were merged together and were not clearly resolved (Krasnići et al., 2013).

Nine studied specimens of Vardar chub from three rivers had quite narrow range of cytosolic Co concentrations (2.6–5.5 ng mL^{-1}, Table 3), and therefore the differences between the profiles were hardly even notable. Cobalt elution associated to HMM biomolecules was found to increase only slightly with increasing cytosolic Co

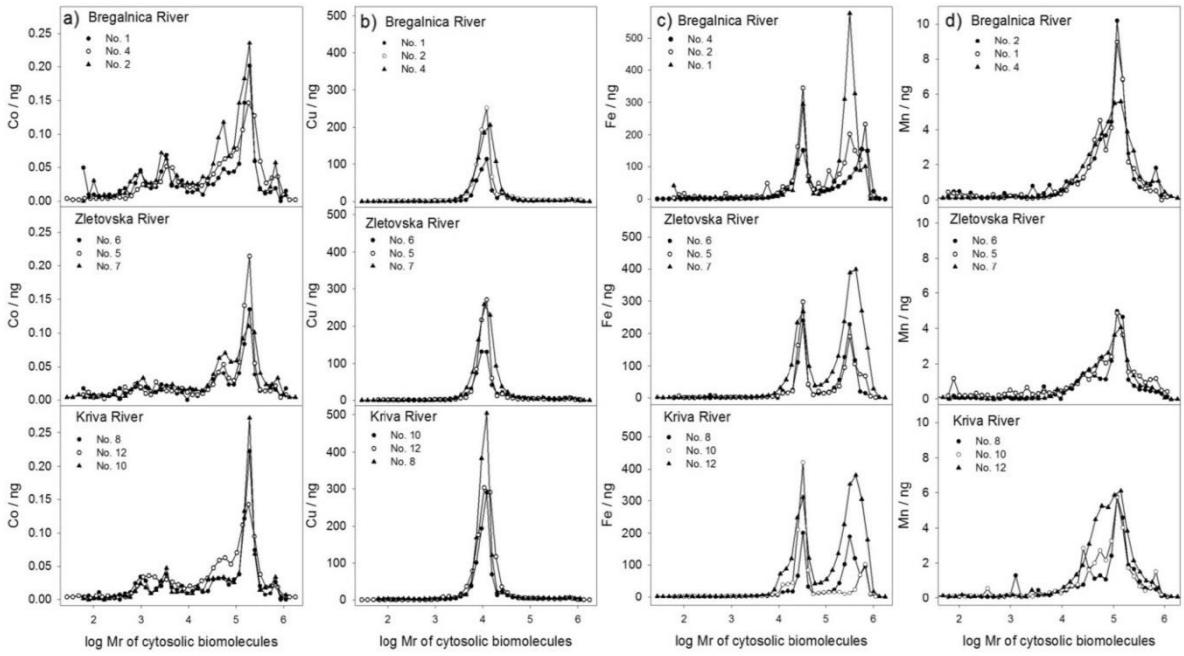

Fig. 1. Hepatic distribution profiles of four selected trace elements (a - Co, b - Cu, c - Fe, and d - Mn) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*) caught in a moderately contaminated Bregalnica River and two mining impacted rivers, Zletovska and Kriva. The profiles were obtained by separation of hepatic cytosols on SEC-HPLC with Superdex™ 200 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Nine fish were used for these analyses (No.1, 2, 4, 5, 6, 7, 8, 10, and 12, Table 1), and their total trace element concentrations in hepatic cytosols are presented in Table 3.

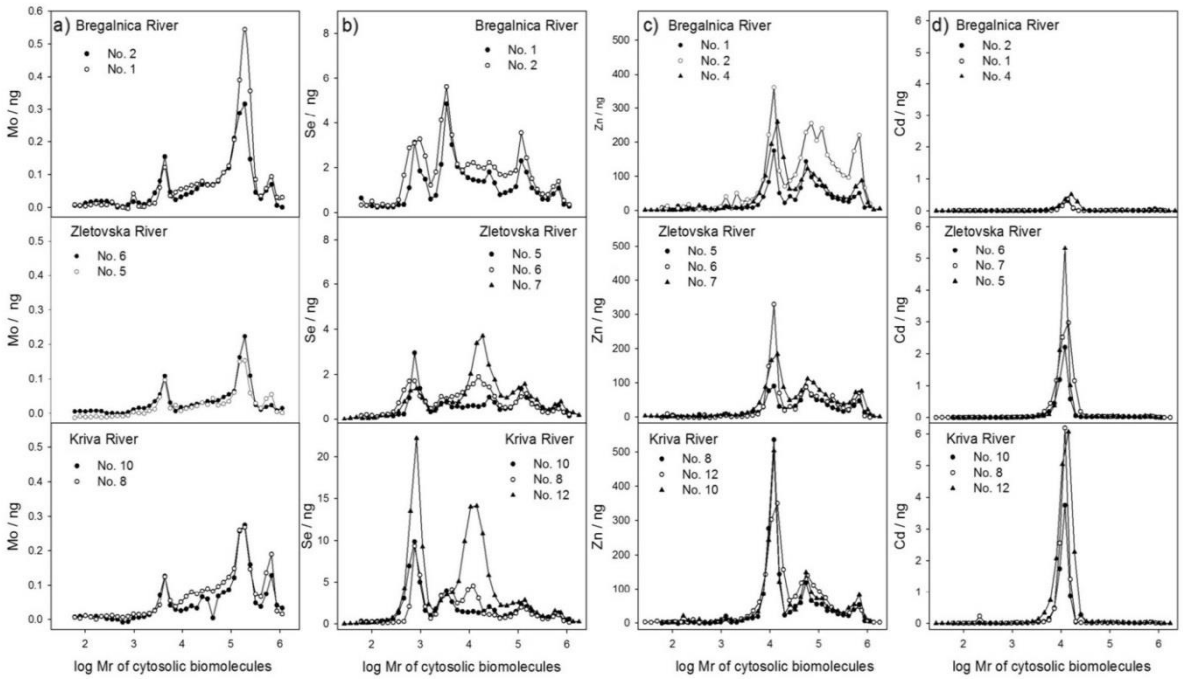

Fig. 2. Hepatic distribution profiles of four selected trace elements (a - Mo, b - Se, c - Zn, and d - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*) caught in a moderately contaminated Bregalnica River and two mining impacted rivers, Zletovska and Kriva. The profiles were obtained by separation of hepatic cytosols on SEC-HPLC with Superdex™ 200 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Nine fish were used for these analyses (No.1, 2, 4, 5, 6, 7, 8, 10, and 12, Table 1), and their total trace element concentrations in hepatic cytosols are presented in Table 3.

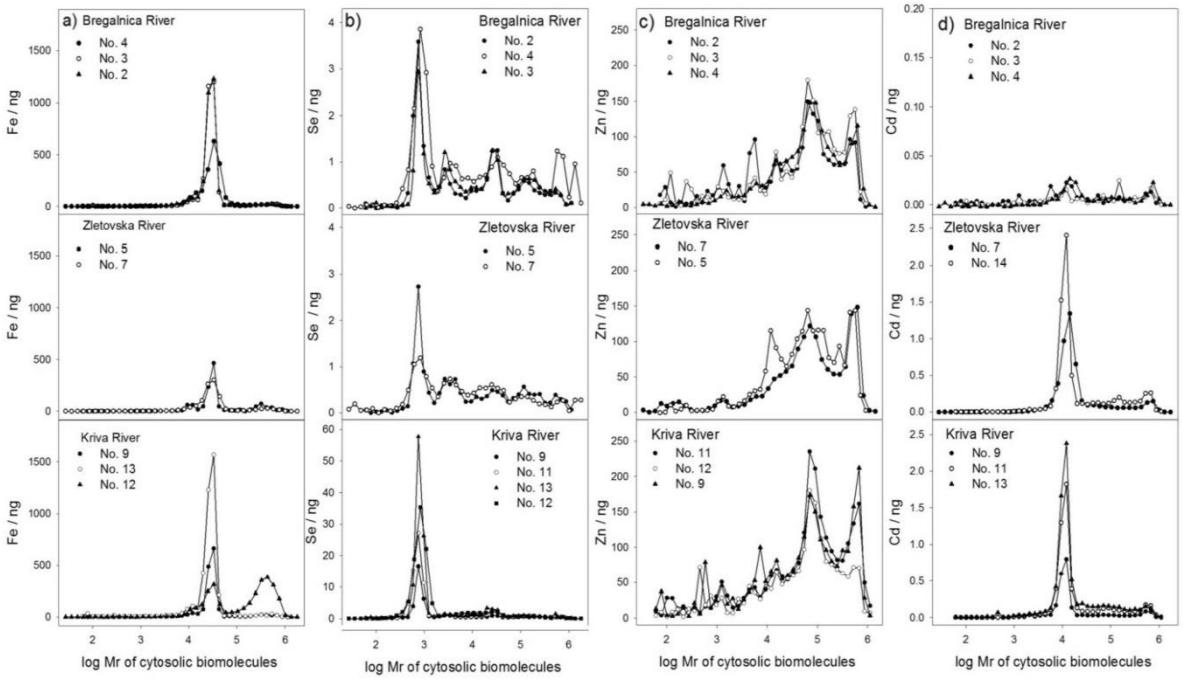

Fig. 3. Gill distribution profiles of four selected trace elements (a - Fe, b - Se, c - Zn, and d - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*) caught in a moderately contaminated Bregalnica River and two mining impacted rivers, Zletovska and Kriva. The profiles were obtained by separation of gill cytosols on SEC-HPLC with Superdex™ 200 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Nine fish were used for these analyses (No. 2, 3, 4, 5, 7, 9, 11, 12 and 13, Table 1), and their total trace element concentrations in gill cytosols are presented in Table 3.

Table 4
Elution times (t_e) and molecular masses (MM) of cytosolic proteins from liver and gills of Vardar chub (*Squalius vardarensis*) contained within the fractions in which respective elements were eluted, after separation of cytosols by size exclusion HPLC with Superdex™ 200 10/300 GL column. Presented data refer to maximums 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			VLMM peak 1[a]		VLMM peak 2[a]		LMM peak[b]		MMM peak[c]		HMM peak 1[d]		HMM peak 2[d]	
		Organ	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
Essential elements	Co	Liver	41 (43–40)	1.2 (0.7–2)	36 (38–35)	4 (2–5)			25 (28–24)	66 (31–85)	20 (23–18)	231 (109–383)		
	Cu	Liver					31 (34–27)	15 (7–40)						
	Fe	Liver							27 (29–26)	40 (24–51)			18 (20–16)	383 (231–633)
		Gills							27 (29–26)	40 (24–51)			18 (20–16)	383 (231–633)
	Mn	Liver							25 (29–24)	66 (24–85)	22 (24–20)	140 (85–382)		
	Mo	Liver			35 (37–34)	5 (3–7)					20 (24–18)	231 (85–382)	15 (17–14)	814 (492–1047)
	Se	Liver	42 (44–39)	1 (0.6–2)	36 (38–35)	4 (2–5)	28 (29–26)	31 (24–51)			22 (23–19)	140 (109–298)		
		Gills	42 (44–40)	1 (0.6–2)	37 (38–35)	3 (2–5)	28 (30–26)	31 (19–51)						
	Zn	Liver					31 (34–29)	15 (7–24)	25 (28–18)	66 (31–383)			15 (17–14)	814 (492–1047)
		Gills							24 (32–19)	85 (11–298)			15 (18–14)	814 (383–1047)
Non-essential element	Cd	Liver					31 (34–29)	15 (7–24)						
		Gills					31 (34–29)	15 (7–24)						

[a] VLMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa).
[b] LMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in low molecular mass protein region (10–30 kDa).
[c] MMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in medium molecular mass protein region (30–100 kDa).
[d] HMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in high molecular mass protein region (>100 kDa).

concentrations, and in general this increase showed a good relationship with cytosolic Co levels (Fig. 1a), indicating that additional attention should be dedicated to study of HMM Co-binding proteins.

3.1.2. Copper

Same as for Co, Cu distribution profiles were also determined only for the hepatic cytosol (Fig. 1b). Copper is an essential trace element for the most living organisms, necessary for certain metabolic processes (e.g., formation of connective tissue, formation and maintenance of myelin, cellular respiration, scavenging of the free radical superoxide; Gaetke et al., 2014), because it plays an important role as a cofactor in a number of enzymes and metalloproteins (Hauser-Davis et al., 2012). Accordingly, Cu was eluted mainly within one single LMM peak (7–40 kDa), with a maximum corresponding to biomolecules of MM of 15 kDa (Table 4), coinciding with the elution time of MT (Table 2), but also encompassing the MM of the other known Cu-containing biomolecules, such as superoxide dismutase (SOD; MM 32 kDa) (Table 2), which indicates Cu essential role in the protection against oxidative stress (Sanchez et al., 2005; Krasnići et al., 2013). Our results, however, indicate that the major part of Cu was probably associated with MTs. MTs constitute a family of low MM, cysteine-rich proteins involved in the binding, regulation and storage of essential metals such as Cu and Zn, and the detoxification of non-essential metals such as Cd (Coyle et al., 2002; Mason et al., 2004). The capacity for MT induction is the greatest in tissues that are active in uptake, storage and excretion of trace elements, such as gills, intestine, liver and kidney (Roesijadi and Robinson, 1994). Cu distribution profile in liver of Vardar chub was in agreement with the previous results of the investigation on Cu-binding biomolecules in hepatic cytosol of European chub (Krasnići et al., 2013), where the most of Cu was detected in LMM protein region (~7–27 kDa), and had the same elution time as MT standard (Krasnići et al., 2013). Copper association with MTs was also confirmed in the liver of eel (*Anguilla anguilla*; Rodríguez-Cea et al., 2003).

In the liver of nine studied specimens of Vardar chub from three rivers, Cu cytosolic concentrations were in rather narrow range, from 1.7 to 6.5 ng mL^{-1} (Table 3). Positions of Cu peaks within the distribution profiles were comparable in Vardar chub from all three rivers (Fig. 1b), whereas increasing cytosolic concentrations resulted with increased elution of Cu in MT region, which was especially observable at the Kriva River. Peak widening towards the region of higher MM was also observed, which could possibly indicate that Cu was also associated to the other cytosolic biomolecules, when present in the cell in higher concentrations.

3.1.3. Iron

Many proteins contain essential metal Fe in ionic form, either within their own structures or bound to their active sites (del Castillo Busto et al., 2010). Thus, under physiological conditions, the majority of Fe is bound to proteins and, to a lower extent, to the other small biomolecules (e.g., citrates), to decrease the free metal ion toxicity (del Castillo Busto et al., 2010). Iron distribution profiles were recorded for both hepatic and gill cytosols of Vardar chub (Figs. 1c and 3a), and were characterized with two Fe containing peaks. Positions of the peaks were comparable in both organs. The first peak was positioned in the MMM region and covered the range of MM from ~25–50 kDa, with a maximum at the MM of 40 kDa (Table 4), encompassing with its borders MM of known Fe-containing biomolecules, such as enzyme catalase (60 kDa) and transport protein myoglobin (17 kDa) (Wolf et al., 2007). The second peak was located in the HMM region and covered the range of MM from ~230–630 kDa, with a maximum corresponding to biomolecules of MM amounting to ~380 kDa (Table 4), which approximately corresponded to ferritin, the major Fe storage protein in almost all living organisms (450 kDa; Szpunar and Lobinski, 1999).

The difference between two organs was in the fact that the majority of Fe in the gill cytosol was eluted within the first MMM peak (Fig. 3a), whereas in the liver the quantity of Fe eluted within the second HMM

peak was either comparable or even higher than in the first MMM peak (Fig. 1c). In the gills, clear presence of Fe in the HMM region was recorded in only one sample with the highest gill cytosolic Fe concentration (28.5 μg mL^{-1}; Fig. 3a). The lack of HMM-Fe peak in the gill samples with lower Fe cytosolic concentrations lead to a conclusion that Fe association with storage protein ferritin was more strongly expressed in the liver than in the gills, even though cytosolic Fe concentrations were lower in the liver (6.4–14.7 μg mL^{-1}, Table 3) compared to gills (5.5–28.5 μg mL^{-1}, Table 3). This was a confirmation of more pronounced role of liver than gills in Fe storage. Distribution profiles of Fe in Vardar chub liver and gills were comparable to previously reported Fe profiles in the liver and gills of European chub, where stronger Fe association with HMM proteins, probably ferritin, was also observed in the liver (Krasnići et al., 2013, 2014).

Positions of Fe peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 1c and 3a). The increase of hepatic Fe concentrations resulted with the increased Fe elution in HMM peak. This was an indication that in the case of increasing Fe concentration in the liver, the part of Fe probably bounds to ferritin. Synthesis of ferritin is known to be induced by increased Fe availability, whereas it is repressed under iron deprivation conditions (Torti and Torti, 2002). Contrary, in the gills, increase of cytosolic Fe mainly resulted with the increased Fe elution in MMM peak.

3.1.4. Manganese

Distribution profiles for essential element Mn were determined only for the hepatic cytosol (Fig. 1d). Manganese was eluted within two poorly resolved peaks covering MMM and HMM regions. Manganese in MMM peak was associated to biomolecules of MM in the range from 24 to 85 kDa, with a maximum at 66 kDa, whereas HMM peak encompassed biomolecules of MM from ~85–230 kDa, with a maximum at 140 kDa (Table 4). These peaks comprised MM of albumin (66 kDa, Table 2) and transferrin (80 kDa; Martin-Antonio et al., 2009). Albumin is a protein involved in the Mn transport from intestine to liver, whereas in the liver, Mn binds to transferrin, and in that form presents a source of Mn for delivery to the other tissues (Krasnići et al., 2013; Schäfer, 2004). Obtained MMM and HMM peaks in the distribution profiles of Mn in the liver of Vardar chub were consistent with Mn association with MMM biomolecules of ~35–60 kDa and HMM biomolecules of ~60–400 kDa in hepatic cytosol of European chub (Krasnići et al., 2013). The only difference between two species was lack of clear Mn binding to LMM biomolecules (<20 kDa) in Vardar chub, which was observed in European chub (Krasnići et al., 2013).

Positions of Mn peaks within the hepatic distribution profiles were comparable in Vardar chub from all three rivers (Fig. 1d). The cytosolic Mn concentrations in liver of all nine analyzed Vardar chub were within the following range: 215–409 ng mL^{-1} (Table 3). The differences associated to concentration changes could not be clearly determined, since in some cases there was an increase in MMM peak, in the others in HMM peak, whereas mostly the peaks were nearly identical (Fig. 1d).

3.1.5. Molybdenum

Distribution profiles for essential element Mo were also determined only for the hepatic cytosol (Fig. 2a). Molybdenum was eluted within three peaks, one VLMM peak and two HMM peaks. Minor part of Mo, shown as a first peak, was eluted within VLMM biomolecule region (Fig. 2a; 3–7 kDa), with a maximum at 5 kDa (Table 4). The major part of Mo, shown as a second peak, was eluted within HMM biomolecule region (Fig. 2a; ~85–380 kDa), with a maximum at ~230 kDa (Table 4). According to Beers and Berkow (1998), Mo serves as a cofactor of different enzymes, such as aldehyde oxidase (~130 kDa; Uchida et al., 2003), sulphide oxidase (~120 kDa; Johnson and Rajagopalan, 1976), and Fe-Mo flavoprotein xanthine oxidase (275 kDa, Truglio et al., 2002) (Krasnići et al., 2013), which were all encompassed by the second Mo peak. A small amount of Mo was eluted within the void volume of the column (Fig. 2a), as a sign of Mo association to HMM biomolecules above ~500 kDa (Table 4), possibly containing protein complexes and aggregates. Molybdenum distribution profiles in the liver of Vardar chub were similar to those previously reported for the liver of European chub, where Mo was also mostly bound to biomolecules of HMM (~60–400 kDa), whereas only minor Mo part was associated to VLMM biomolecules (4–12 kDa) (Krasnići et al., 2013).

Positions of Mo peaks within the hepatic distribution profiles were comparable in Vardar chub from all three rivers (Fig. 2a). The range of cytosolic Mo concentrations in liver of six analyzed Vardar chub was the following: 12.6–26.6 ng mL^{-1} (Table 3) and the increase of cytosolic Mo concentrations was reflected in the increased Mo elution within HMM peaks, confirming the predominant Mo association to HMM biomolecules.

3.1.6. Selenium

Selenium is a nonmetal, an essential micronutrient for the synthesis of selenoproteins, which plays an important role in the overall metabolism (Rayman, 2012; Braga et al., 2017). However, very little is known about how Se is metabolized in fish, and for the most fish selenoproteins functions are yet not known (Hauser-Davis et al., 2012). Selenium distribution profiles were determined for both hepatic and gill cytosols of Vardar chub (Figs. 2b and 3b). Hepatic profiles of Se were characterized with four Se containing peaks. The first two peaks were located within the VLMM region, and the first one corresponded to biomolecules of MM in the range from 0.6–2 kDa, with a maximum at 1 kDa, whereas the second VLMM peak corresponded to biomolecules of MM from 2 to 5 kDa, with a maximum at 4 kDa (Table 4, Fig. 2b). The other two Se hepatic peaks were eluted in LMM and HMM protein regions (~25–50 kDa, with a maximum at ~30 kDa, and ~110–300 kDa, with a maximum at 140 kDa, respectively; Table 4, Fig. 2b). Gill Se distribution profiles were almost identical to hepatic profiles, with the only exception that they were lacking clear HMM peak (Fig. 3b). The main characteristic of gill profiles was that majority of Se was eluted in detached and sharp peak within the first VLMM region (0.6–2 kDa). The association of Se with VLMM biomolecules was already reported by many other authors. For example, Se presence in the cell in the form of selenomethionine (~0.2 kDa) was reported by Klotz et al. (2003), whereas very low MM organic selenocompound selenoneine (~0.5 kDa), effective in the defense against oxidative stress by acting as a strong free radical scavenger, was identified in bluefin tuna (*Thunnus orientalis*) by Yamashita and Yamashita (2010) and Yamashita et al. (2012) (Krasnići et al., 2014). On the other hand, the higher proportion of Se eluted in LMM region in the liver compared to the gills, could be associated to several selenoproteins catalytically active in redox processes, such as glutathione peroxidase, iodothyronine deiodinase, and thioredoxin reductase (Hauser-Davis et al., 2012). Hepatic and gill Se profiles obtained for Vardar chub were mainly comparable with those previously reported for European chub (Krasnići et al., 2013, 2014).

Positions of Se peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 2b and 3b). However, notable quantitative difference was observed in the hepatic profiles of Vardar chub from the Bregalnica River in comparison to the Zletovska and the Kriva rivers. In the liver of Vardar chub from the Bregalnica River pronounced Se elution was observed within the second VLMM peak (2–5 kDa) whereas much lower Se proportion was eluted within the LMM region. In the chub from the other two rivers situation was opposite: the second VLMM peak was negligible, and Se was eluted in high quantity in LMM region (~25–50 kDa). This finding could be possibly associated to the type of pollution, since Bregalnica is contaminated with agricultural runoff, whereas the Zletovska and the Kriva rivers are contaminated with mining waste. The influence of Se environmental speciation on its fate in the fish organism should be further explored. The range of cytosolic Se concentrations in the liver of eight analyzed Vardar chub (Fig. 2b) was the following: 85.8–631.1 ng mL^{-1} (Table 3), whereas Se concentrations in the gills of

nine Vardar chub (Fig. 3b) ranged from 57.9–469.0 ng mL^{-1} (Table 3). Increasing cytosolic concentrations of Se in the liver of chub from mining impacted rivers have accordingly resulted with increased Se elution in LMM region and within the first VLMM peak (<2 kDa), whereas in the case of the gills increased Se bioaccumulation resulted exclusively with obvious sharp increase of the first VLMM peak (<2 kDa). The same was found for the liver and gills of European chub, with reported increases in LMM and VLMM regions, respectively (Krasnići et al., 2013, 2014).

3.1.7. Zinc

Zinc is an essential metal which has constitutive and catalytic roles in many proteins and enzymes (de la Calle Guntiñas et al., 2002). Zinc distribution profiles were determined for both hepatic and gill cytosols of Vardar chub (Fig. 2c and 3c). Zinc distribution profiles in liver were characterized by three peaks. The majority of Zn was eluted in narrow, sharp first peak associated to LMM biomolecules (7–24 kDa, with a maximum at 15 kDa) (Table 4, Fig. 2c), which coincided with the elution time of known Zn-binding protein, MT (Table 2). Furthermore, a considerable amount of hepatic Zn was found in MMM biomolecules region (~30–400 kDa, with a maximum at 66 kDa; Table 4, Fig. 2c), coinciding, for example, with elution time of standard protein alcohol dehydrogenase (Table 2), which is well known Zn-containing protein (Krasnići et al., 2013; Szpunar and Lobinski, 1999). The third Zn peak appeared within the void volume, and could be associated with HMM biomolecules (above ~500 kDa; Table 4, Fig. 2c). The comparison of hepatic and gill Zn profiles of Vardar chub (Figs. 2c and 3c) showed that Zn was much better resolved in the liver than in the gills. In the gills, it was eluted within two major peaks, mostly within one broad peak covering both LMM and MMM region (~10–300 kDa, with a maximum at 85 kDa, Table 4, Fig. 3c). Unlike in the liver, the clear peak which would indicate Zn binding to MTs was not observed in the gills, but MT elution time was still encompassed by the edge of this wide LMM-MMM peak. A portion of Zn was eluted in HMM region (above 400 kDa), same as observed in the hepatic Zn profile (Figs. 2c and 3c). Similar results as for Vardar chub were previously obtained for the liver and gills of European chub, where clear MT peak was observed only in Zn hepatic profiles (9–27 kDa), whereas in the gills MT peak was missing (Krasnići et al., 2013, 2014). Only difference between Zn profiles in Vardar and European chub was the presence of two high VLMM peaks in the gills (<5 kDa) only in European chub, which increased following the increase of Zn cytosolic concentrations (Krasnići et al., 2014).

Positions of Zn peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 2c and 3c). In the liver of nine analyzed Vardar chub (Fig. 2c) cytosolic Zn concentrations were within rather narrow range (3.5–8.4 μg mL^{-1}, Table 3), similar as in the case of gills of eight analyzed Vardar chub (Fig. 3c) (4.3–14.4 μg mL^{-1}, Table 3), and therefore it was not possible to comment on the changes which would occur in the case of increased Zn bioaccumulation.

3.1.8. Cadmium

Cadmium is an element without known functions in the organisms, and therefore possibly toxic already in very low concentrations. Cadmium distribution profiles were determined for both hepatic and gill cytosols of Vardar chub (Figs. 2d and 3d). Similarly to Cu, Cd was eluted within single clear and sharp peak in the LMM region (7–24 kDa, with a maximum at 15 kDa) in both organs of Vardar chub (Figs. 2d and 3d; Table 4). This peak had maximum at elution time of 31 min, which corresponded to elution time of standard MT-2 (Table 2). Obtained results confirmed the known high affinity of MTs for Cd binding, as a mechanism of protection against Cd toxicity (Roesijadi, 1992; Park et al., 2001). The same observations as made for Vardar chub liver and gills were previously made for European chub, with the major part of Cd bound to biomolecules of molecular masses and elution times which corresponded to MTs, both in the liver (9–27 kDa) and in the gills (4–35 kDa) (Krasnići et al., 2013, 2014).

Positions of Cd peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 2d and 3d). In the liver of nine analyzed Vardar chub (Fig. 2d), cytosolic Cd concentrations amounted to 2.4–68.2 ng mL^{-1} (Table 3), whereas in the gills of eight Vardar chub (Fig. 3d) they amounted to 0.3–33.8 ng mL^{-1} (Table 3). The range of cytosolic Cd concentrations was rather wide, thus enabling determination of the Cd distribution changes caused by prominent increase of Cd bioaccumulation. Increase of cytosolic Cd in both liver and gills of Vardar chub from mining impacted Zletovska and Kriva rivers resulted only with the increase of Cd elution within the probable Cd-MT peak, which was well coordinated with the cytosolic concentration changes. The presence of Cd-MT peak at all studied concentrations, from low in the Bregalnica River, to rather high in the Zletovska and Kriva rivers, indicated almost complete Cd detoxification in both organs of Vardar chub under the studied conditions, as previously observed by Lavradas et al. (2016) in the study on mussels. According to Goenaga Infante et al. (2003), MTs were found to be the most important Cd, Cu, and, to a lesser extent, Zn-binding compounds, and, therefore, MT induction can serve as a biological marker of metal exposure in fish.

3.2. Changes in the cytosolic distribution profiles of Cu, Zn, Cd, Mo, and Se after the heat-treatment

Heat-stable proteins and peptides are considered as detoxified fractions of trace elements in the cell, whereas heat-denaturated biomolecules are defined as fraction sensitive to trace elements, and a possible target of their toxicity (Giguère et al., 2006; Goto and Wallace, 2010; Rosabal et al., 2015). Applying the heat-treatment procedure used for analysis of MTs, which is based on cytosol heating at 70 °C for 10 min (Erk et al., 2002), we have removed the heat-sensitive proteins from the cytosol, and further on analyzed only trace element distribution among heat-stable biomolecules of different molecular masses. For that purpose, we have used SEC-HPLC with Superdex™ 75 column (linear separation range between 3 and 75 kDa), coupled offline with HR ICP-MS detection. In the Figs. 4 and 5, and Tables 5 and 6, we have presented only the information on the distribution profiles of those elements for which the clear presence of the heat-stable biomolecules was confirmed in the cytosols of liver (Cu, Zn, Cd, Mo, and Se) and of gills (Zn, Cd, Mo, and Se). The peaks obtained before and after the heat-treatment of the same samples were at first visually compared (Figs. 4 and 5). We have further calculated the percentage decreases or increases of eluted quantities of specific trace elements at specific elution times, which have occurred after the heat-treatment (Table 6), to determine if analyzed trace elements were bound to heat-stable biomolecules (unaltered peaks) or heat-sensitive biomolecules (decreased peaks). With the exception of the detailed studies of the heat-stable protein metallothionein (e.g., Rodríguez-Cea et al., 2003; Mason et al., 2004; Goenaga Infante et al., 2006; Hauser-Davis et al., 2012), to our knowledge the information on heat-stable proteins and peptides that bind specific trace elements in fish organs, and therefore possibly can serve in the process of detoxification, is yet not available in the scientific literature.

3.2.1. Distribution of Cu, Zn, and Cd in the heat-treated hepatic and gill cytosols

In the region of the studied molecular masses (approximately from 0.5 to 140 kDa, Table 5), Cu (Fig. 4a), Zn (Fig. 4d), and Cd (Fig. 4e) in the untreated hepatic cytosol were all eluted within the same LMM peak (~10–30 kDa, with the maximum at 20 kDa, Table 5), having the same elution time as MT-2 standard (Table 2). Only Zn was eluted within the additional MMM peak (~40–110 kDa, with the maximum at ~70 kDa, Table 5). After the heat-treatment, Zn-MMM peak was almost completely removed (Zn quantity decreased for 76%, Table 6), pointing to heat-sensitivity of those biomolecules, whereas metal-binding biomolecules within LMM peak were confirmed as heat-stable, based on

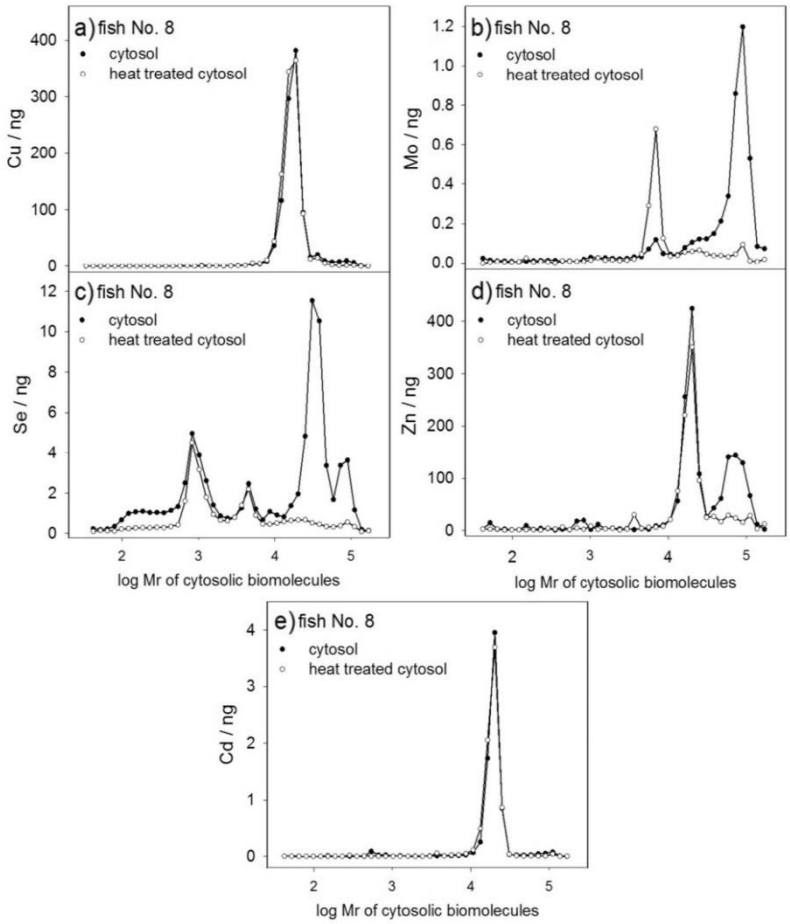

Fig. 4. Influence of heat-treatment on hepatic distribution profiles of five selected trace elements (a - Cu, b - Mo, c - Se, d - Zn, and e - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*). The profiles were obtained by separation of hepatic cytosol and heat-treated cytosol on SEC-HPLC with Superdex™ 75 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. One fish was used for these analyses (No. 8, Table 1), and its total trace element concentrations in hepatic cytosol are presented in Table 3.

the changes of metal quantity eluted within that peak which generally have not surpassed 10% (Table 6), and which further corroborated above suggested binding of Cu, Zn and Cd to MTs in the liver of Vardar chub.

In the gills, Cu profiles were not determined, due to rather low cytosolic Cu concentrations in the gills, both before and after the heat-treatment. In both gill samples, all of Zn (Fig. 5e, f) in untreated cytosols was eluted within the MMM peaks (~30–140 kDa, with the maximum at ~90 kDa, Table 5), which were almost completely removed after the heat-treatment (Zn quantity decreased for 76 and 86%, Table 6), and which coeluted with MMM peak of the hepatic cytosol. On the other hand, in each untreated gill sample Cd was eluted within two peaks (Fig. 5g, h). LMM peaks (~10–30 kDa, with the maximum at 20 kDa, Table 5), as already observed in the liver, indicated Cd binding to MTs, whereas the additional MMM peaks (~60–110 kDa, with the maximum at ~90 kDa, Table 5) indicated that part of Cd, which was accumulated in the gills, was not detoxified. Cadmium-LMM peaks were confirmed to contain heat-stable proteins, most probably MTs, since the decrease of Cd quantity eluted within that region after the heat-treatment amounted to ~10% (Table 6), whereas Cd-MMM peaks obviously comprised heat-sensitive proteins, since decrease of Cd quantity eluted within MMM region after the heat-treatment amounted to 65–75%.

Binding to heat-stable proteins, likely MTs, was thus demonstrated for Cu, Zn, and Cd in the liver, as well as for Cd in the gills of Vardar chub, whereas previously observed absence of Zn binding to MTs in the gills of Vardar chub in this study (Fig. 3c), and the European chub from the Sutla River (Krasnići et al., 2014), was further confirmed. Various authors have reported Cu, Zn and Cd association to MT proteins. In the liver of eel (*A. anguilla*) MTs were proven as the most important Cu-binding compounds and, to a lesser extent, Zn-binding compounds (Goenaga Infante et al., 2003). Lavradas et al. (2016), in the investigation of heat-stable metalloproteins in *Perna perna* mussels, also found that Cu was mostly bound to the proteins with lower MM (13 kDa).

3.2.2. Distribution of Mo in the heat-treated hepatic and gill cytosols

After the separation of untreated hepatic cytosol of Vardar chub on Superdex™ 75 column, Mo was eluted within two peaks (Fig. 4b). The first one was barely visible VLMM peak (5–9 kDa, with the maximum at 7 kDa, Table 5), whereas the second one was high and sharp MMM peak (~50–110 kDa, with the maximum at ~90 kDa, Table 5). After the cytosol heat-treatment, however, MMM peak was almost completely removed (decrease of 92%, Table 6), whereas VLMM peak has significantly increased (almost 300%, Table 6), as an indication that possibly due to heat-treatment, heat-sensitive MMM proteins or peptides have decomposed to small biomolecules of very low molecular masses, which are heat-stable. Contrary, in the gills, cytosolic Mo was eluted within single VLMM peaks (5–11 kDa, with the maximum at 7 kDa, Table 5) in both analyzed samples, even before the heat-treatment.

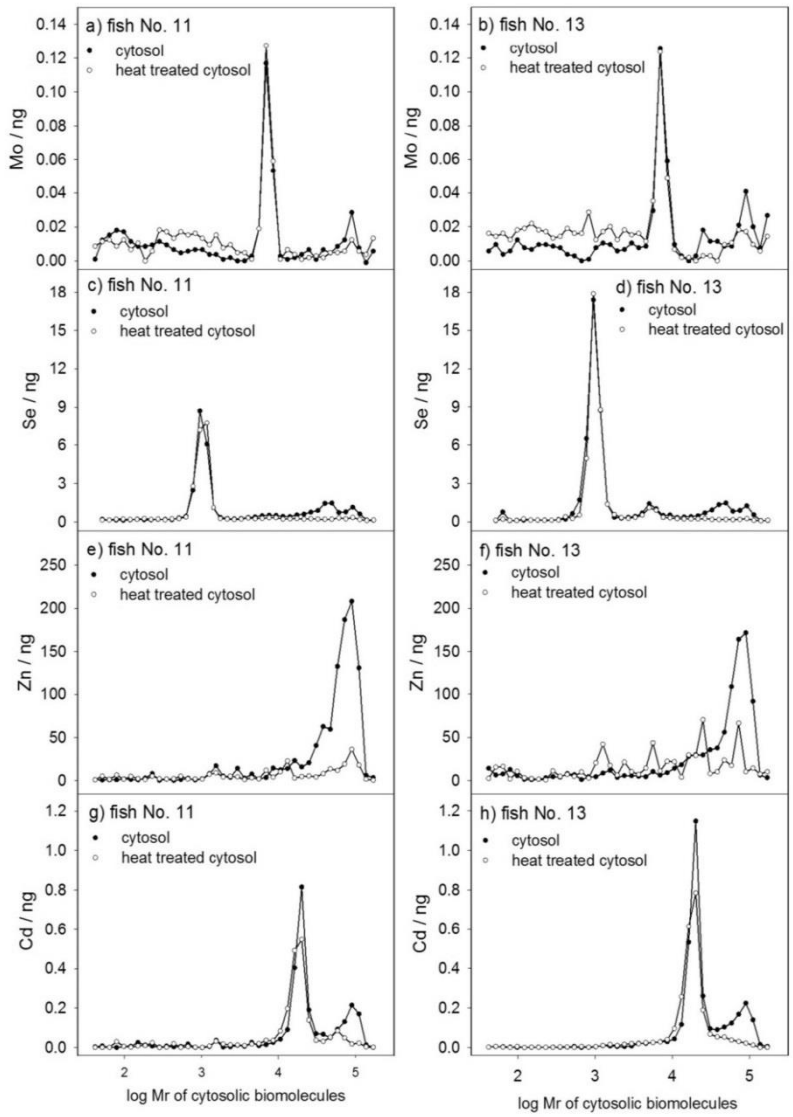

Fig. 5. Influence of heat-treatment on gill distribution profiles of four selected trace elements (a, b - Mo, c, d - Se, e, f - Zn, and g, h - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*). The profiles were obtained by separation of gill cytosol and heat-treated cytosol on SEC-HPLC with Superdex™ 75 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Two fish were used for these analyses (No. 11 and 13, Table 1), and their total trace element concentrations in gill cytosols are presented in Table 3.

After the heat-treatment, these VLMM peaks remained practically unchanged (7–11% peak increase) (Table 6, Figs. 5a, b), thus proving the heat-stability of the compounds comprised within them. Since there is no information available about heat-stable biomolecules that bind Mo, it would be very interesting to further investigate the fate and character of Mo-binding compounds in the hepatic and gill cytosol of the fish.

3.2.3. Distribution of Se in the heat-treated hepatic and gill cytosols

Separation of untreated hepatic cytosol of Vardar chub on Superdex™ 75 column resulted with four Se peaks (Fig. 4c). The first two peaks were eluted within VLMM region (0.5–1.6 kDa, with the maximum at 0.8 kDa; and 3–6 kDa, with the maximum at 5 kDa; Table 5). The most of Se was eluted within the third, high and sharp, MMM peak (~20–50 kDa, with the maximum at ~30 kDa, Table 5). The fourth peak was also eluted within the MMM region (~60–110 kDa, with a maximum at ~90 kDa), but it was much smaller and corresponded to void volume of the column. After the cytosol heat-treatment, both MMM peaks were almost completely removed (decreases of approximately 90%, Table 6), whereas VLMM peaks were only slightly decreased (10–26%, Table 6), indicating that a portion of hepatic Se binds to small, heat-stable compounds within the cytosol. In both untreated gill samples, cytosolic Se was mostly eluted within the single, sharp and narrow VLMM peaks (0.5–1.3 kDa, with a maximum at 0.8 kDa; Fig. 5c, d), which coincided with the first peak in the hepatic cytosol (Fig. 4c; Table 5), and corresponded well with the results obtained for the gills after the cytosol separation on Superdex™ 200 column (Fig. 3b). These VLMM peaks were not affected by the heat-treatment (peak variations within 10%, Table 6). Selenium binding to VLMM compounds in both liver and gills of Vardar chub might correspond to some Se-containing peptides or selenoamino acids, and based on the analyses performed after the cytosol heat-treatment, it can be concluded that those compounds are heat-stable.

Table 5
Elution times (t_e) and molecular masses (MM) of cytosolic proteins from liver and gills of Vardar chub (*Squalius vardarensis*) contained within the fractions in which respective elements were eluted, after separation of cytosols and heat-treated cytosols by size exclusion HPLC with Superdex™ 75 10/300 GL column. Presented data refer to maximums 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		Organ	VLMM peak 1[a]		VLMM peak 2[a]		LMM peak[b]		MMM peak 1[c]		MMM peak 2[c]	
			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
Essential elements	Cu	Liver					23 (26–21)	20 (11 − 31)				
	Mo	Liver			28 (30–27)	7 (5–9)					16 (19–15)	89 (47–111)
		Gills			28 (30–26)	7 (5–11)						
	Se	Liver	38 (40–35)	0.8 (0.5–1.6)	30 (32–29)	5 (3–6)			21 (23–19)	31 (20–47)	16 (18–15)	89 (58–111)
		Gills	38 (40–36)	0.8 (0.5–1.3)								
	Zn	Liver					23 (26–21)	20 (11–31)			17 (20–15)	72 (38–111)
		Gills									16 (14–21)	89 (31–137)
Non-essential element	Cd	Liver					23 (25–21)	20 (13 − 31)				
		Gills					23 (25–21)	20 (13–31)			16 (18–15)	89 (58–111)

[a] VLMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa).
[b] LMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in low molecular mass protein region (10–30 kDa).
[c] MMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in medium molecular mass protein region (30–100 kDa).

4. Conclusions

This comprehensive study of cytosolic distributions of eight trace elements (Co, Cu, Fe, Mo, Mn, Se, Zn, and Cd) in the liver and gills of Vardar chub (*S. vardarensis*) from three differently contaminated Macedonian rivers by use of metallomics approach, based on SEC-HPLC cytosol separation and HR ICP-MS detection, has enabled determination of the ranges of molecular masses of biomolecules that bind trace elements under different conditions of environmental exposure. Especially interesting findings were: (i) probable binding of Fe to storage protein ferritin observed in much higher proportion in the liver than in the gills; (ii) association with metallothioneins observed for Cd, Cu and Zn in the liver, and Cd in the gills; (iii) Se binding to compounds of very low molecular masses, below 5 kDa, observed in both organs, but in higher proportion in the gills. Additional study of distributions of trace elements in the hepatic and gill cytosols after their heat-treatment further confirmed binding of Cd, Cu and Zn to heat-stable proteins, probably MTs, as well as existence of the heat-stable cytosolic compounds of very low molecular masses (<10 kDa) that bind Mo and Se. Analysis of the influence of increased environmental exposure to trace elements on their cytosolic distributions in liver and gills of Vardar chub revealed that the changes were generally of quantitative and not qualitative nature. In other words, trace elements under all studied conditions were found associated to the same biomolecules, and only their proportions associated to specific cytosolic compounds have changed as a consequence of their increased bioaccumulation in the liver and gills of Vardar chub. Comparison of the results obtained for the liver and gills of Vardar chub from the Macedonian rivers with previously published information on the same organs of European chub (*S. cephalus*) from the Sutla River (Croatia) indicated that distributions of trace elements are generally comparable in these two species from the same genus, *Squalius*, which enables their comparative use in the monitoring programmes, even in the distant parts of the world.

Table 6
Heat-treatment induced changes in distribution profiles of Cu, Mo, Se, Zn and Cd in cytosol of chub liver and gills, after separation by SEC-HPLC with Superdex™ 75 10/300 GL column. The results are presented as percentage decrease (−) or increase (+) of metal quantity in certain peaks which occurred after heat treatment of the cytosol.

	Protein MM	Liver	Gills	
		Fish No. 8	Fish No. 11	Fish No. 13
Cu	11–31 kDa	+8%		
Mo	5–9 kDa	+272%	+11%	+7%
	47–111 kDa	−92%		
Se	0.5–1.6 kDa	−26%	+2%	−6%
	3–6 kDa	−10%		
	20–47 kDa	−89%		
	58–111 kDa	−92%		
Zn	11–31 kDa	−11%		
	38–111 kDa	−76%	−86%	−76%
Cd	13–31 kDa	+5%	−10%	−11%
	58–111 kDa		−65%	−75%

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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†

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Metals play crucial physiological roles, but they can also cause irreparable toxic effects through binding to important cellular biomolecules in aquatic organisms. The aim of this study was to determine the exact molecular masses and to identify several selected metal-binding biomolecules in hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928). Methods applied for the achievement of this goal were SEC-AEC-HPLC for two-dimensional separation of cytosolic biomolecules, HR ICP-MS for metal measurements, and mass spectrometry (MALDI-TOF-MS and LC-MS/MS) for biomolecule mass determination and identification. The analyzed biomolecules included: Fe-binding biomolecules, which were identified as hemoglobin subunit β in the liver (molecular masses of ~15 kDa), and hemoglobin subunits α and β in the gills (molecular masses of ~11 kDa, ~13 kDa and ~15 kDa); heat-stable Cd-binding biomolecules, which were identified as MT isoforms MT-I and MT-II (molecular mass of ~6.0 kDa in both liver and gills, and an additional 4.9 kDa isoform in the gills); and heat-stable Mo-binding biomolecules of molecular masses equal to 3.3 kDa (in the gills) and 8.5 kDa (in the liver). An important finding of this study was the obvious presence of different isoforms of the same biomolecules in the liver and gills. This was, among others, manifested through the absence of Zn binding to MTs only in the gills, indicating that the same type of biomolecule can be responsible for different functions in different organs. Thus, for better understanding of metal behaviour in aquatic organisms, it is crucial to identify cellular metal-binding biomolecules and their functions.

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Significance to metallomics

This study has brought us one step closer to the understanding of the fate of metals bioaccumulated in the organs of an important aquatic bioindicator species, namely Vardar chub. Application of two-dimensional chromatographic separation of cytosolic biomolecules according to their sizes and charges, and of subsequent metal and biomolecule analyses using various mass spectrometry techniques (HR ICP-MS, MALDI-TOF-MS, and LC-MS/MS) enabled the recognition of important Fe, Cd, and Mo-binding biomolecules in Vardar chub liver and gills. Differences in biomolecule characteristics observed between the two organs have confirmed the importance of studies of organ-specific isoforms of various proteins within cells.

Introduction

As a consequence of human activities (such as mining, industry, traffic, and agriculture), environmental contamination with metals, especially contamination of aquatic systems, is steadily increasing and affecting living organisms.[1] For the evaluation of the degree of metal contamination and possible pollution of aquatic ecosystems, fish play an important role due to their key position in many food webs, thus being useful for the assessment of the bioaccumulation

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and effects of metals originating from water, food and sediments.[2–5] Metals bioaccumulated in fish organs bind to or interact with cytosolic molecules and can interfere with cell functions, leading to various deleterious effects.[6,7] On the other hand, metals also play an important physiological role in living organisms, since many biological functions critically depend on interactions with certain metals within the cell; for example, several proteins require a metal ion to be able to perform their catalytic activities, to stabilize their structures and to properly perform their functions.[8–10] Therefore, metal physiological functions, as well as their possible toxic effects on metabolism and homeostasis of fish organisms can be assessed by analyses of various metal binding biomolecules within the cells, such as various metalloenzymes and other metalloproteins.[11–14] A large number of studies on metal-binding biomolecules were carried out in the frame of various metal speciation projects, but only a few fish metalloproteins have so far been identified and their functions clarified, allowing better insight into the intracellular fate of bioaccumulated metals, as well as toxic and defensive mechanisms that are triggered by metal exposure.[15–17]

To obtain valuable information on the identity and functions of metal-binding biomolecules in living systems it is necessary to apply adequate state of the art analytical equipment.[18] The researchers in this field generally use a combination of high-sensitivity atomic detectors, such as inductively coupled plasma mass spectrometry (ICP-MS), different techniques of high performance liquid chromatography (HPLC), and various types of mass spectrometry (MS), to enable metal/nonmetal analyses within the cells, as well as characterization and possible identification of specific metal-binding biomolecules.[19–24] Association of multidimensional separation approaches (*e.g.* size exclusion (SEC)-HPLC and anion exchange (AEC)-HPLC) with various mass spectrometry techniques (*e.g.* matrix assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS, or liquid chromatography (LC) MS) further improves the reliable identification of so far unknown and undescribed metal binding biomolecules, that will in turn provide a possibility of precise assessment of hazardous environmental situations.[25,26]

In our previous work we have implemented offline coupling of SEC-HPLC and high resolution (HR) ICP-MS to determine distributions of several elements (Cd, Co, Cu, Fe, Mn, Mo, Pb, Se, Tl and Zn) among cytosolic biomolecules of the gills and/or liver of three fish species, European chub (*Squalius cephalus*),[27,28] Vardar chub (*Squalius vardarensis*)[29] and brown trout (*Salmo trutta*).[30] In our studies, the liver was chosen as a key metabolic and detoxification organ of fish, containing a high amount of various metalloproteins which are characterized by a wide range of molecular masses.[7,31] The gills were chosen as an important entry route of contaminants, such as metals; they are known for their good reflection of the current environmental situations, through metal bioaccumulation and oxidative stress responses.[32] However, the complete separation and a precise determination of molecular masses of all metal-binding biomolecules contained within hepatic or gill cytosols of selected fish species would be rather difficult, and probably not even possible, based solely on the SEC-HPLC-HR-ICP-MS measurements. The resolution and sensitivity of the SEC-HPLC method is such that it offers a rough separation of the studied metal-binding biomolecules, and the obtained elution peaks of each studied element generally cover rather wide ranges of molecular masses, which can include several metalloproteins. Furthermore, elution peaks of different metals can partially or completely overlap, thus needing a further separation before any kind of additional analyses.[27–30]

Hence, in this study, which presents the continuation of our previous study on the liver and the gills of Vardar chub,[29] our first goal was to implement anion-exchange chromatography as an additional separation technique following size-exclusion chromatography, to isolate purified fractions that contain, whenever possible, individual metal-binding biomolecules. Our second goal was to implement two mass spectrometry techniques into our research (MALDI-TOF-MS and LC-MS/MS), to more precisely determine the molecular masses of several metal-binding biomolecules from hepatic and gill cytosols of Vardar chub, and, when possible, to identify them. Our attention was directed towards analyses of several hepatic and gill metal-binding biomolecules, which were roughly determined in our previous study on Vardar chub,[29] including: (1) medium molecular mass (~25–50 kDa) Fe-binding biomolecules; (2) heat-stable low molecular mass (~7–25 kDa) Cd-, Cu- and Zn-binding biomolecules, presumably metallothioneins (MTs); and (3) heat-stable very low molecular mass (~5–10 kDa) Mo-binding biomolecules. By the application of two-dimensional HPLC separation followed by HR ICP-MS analyses of metals and MS analyses of the above listed metal-binding biomolecules, our general aim in this study was to extend the knowledge and understanding of metal handling strategies in Vardar chub, as an important bioindicator species.

Experimental data

Fish sampling and organ dissection

As a bioindicator species in this study we have used Vardar chub (*Squalius vardarensis* Karaman, 1928), a member of the Cyprinidae family, because it is a widespread fish species in the Macedonian rivers, and closely related to European chub (*Squalius cephalus*), fish species abundant in European freshwaters. Fish sampling was performed in the spring period (June 2015) in two rivers in north-eastern Macedonia, Bregalnica (N 41°43.57′ E 22°10.27′) and Zletovska (N 40°58.54′ E 21°39.45′), and the map of the sampling area was previously published.[1] Fish were caught by electro fishing, using an electrofisher Samus 725G, according to the guidelines described in standard CEN EN 14011:2003. The captured fish were transported to the laboratory, and during transportation they were kept alive in a tank containing aerated river water, which was collected at each sampling site. In the laboratory, the fish were anesthetized with Clove Oil (Sigma Aldrich, USA) and euthanized, and then the liver, gills and gonads were dissected. Fish total lengths (cm) and total masses (g) were measured, and Fulton condition indices (FCI) were calculated using the formula provided by Rätz and Lloret,[33] whereas fish sex was determined histologically.[34] The liver and gills were put in liquid

Table 1 Basic biometric characteristics and total hepatic and gill cytosolic concentrations of Cd, Cu, Fe, Mo and Zn of five Vardar chub (*Squalius vardarensis*) specimens, which were caught in two Macedonian rivers (Bregalnica and Zletovska) in June 2015

Fish no.	River	Length/cm	Mass/g	Sex	FCI/g cm^{-3}		Cd/μg L^{-1}	Cu/μg L^{-1}	Fe/mg L^{-1}	Mo/μg L^{-1}	Zn/mg L^{-1}	TP/mg L^{-1}
1	Bregalnica	19.1	78.57	F	1.13	Liver	6.99	4.07×10^3	6.53	22.2	5.87	17.69
						Gills	4.04	46.2	5.26	2.79	14.8	13.25
2	Bregalnica	19.4	83.75	F	1.15	Liver	10.8	2.88×10^3	3.65	16.2	5.68	18.15
						Gills	2.32	44.6	4.53	2.41	13.9	13.15
3	Zletovska	16.2	45.94	M	1.08	Liver	175.0	7.21×10^3	11.1	27.6	10.0	19.74
						Gills	66.7	42.7	4.26	1.38	18.1	15.50
4	Zletovska	18.5	59.36	F	0.94	Liver	142.1	5.47×10^3	8.51	20.1	6.80	18.64
						Gills	52.3	45.4	4.48	1.62	22.2	13.67
5	Bregalnica	17.7	56.20	M	1.01	Liver	26.5	3.80×10^3	7.90	29.7	5.32	19.60
						Gills	2.28	44.5	3.63	2.16	10.0	14.08

nitrogen immediately upon dissection, and afterwards they were stored in a freezer at −80 °C. In total, five fish were analyzed in the course of this study, and their biometric parameters are given in Table 1.

Isolation of cytosolic fractions from Vardar chub liver and gills and their heat-treatment

Frozen Vardar chub livers and gills were put on ice and cut into small pieces, and then cooled homogenization buffer [100 mM Tris–HCl/base (Sigma, pH 8.1 at 4 °C) supplemented with reducing agent (1 mM dithiothreitol (DTT), Sigma)], w/v 1 : 5, was poured in. The obtained mixture was homogenized in a glass tube, which was put on ice, applying 10 strokes of a PTFE-coated pestle at 6000 rpm (Potter-Elvehjem homogenizer, Glas-Col, USA). Hepatic and gill cytosols were obtained by homogenate centrifugation at 50 000 × *g* for 2 h at 4 °C, using an Avanti J-E centrifuge (Beckman Coulter), *i.e.* the supernatants (S50) obtained after centrifugation corresponded to water-soluble cytosolic tissue fractions[35] containing lysosomes and microsomes.[36] The supernatants were aliquoted and stored in a freezer at −80 °C.

The heat-treatment of hepatic and gill cytosols was performed according to the slightly modified procedure described by Erk *et al.*[37] The cytosols were heated at 70 °C for 10 minutes using a DB-2D Dri-Block (Techne, UK). After the heat-treatment, the samples were kept on ice for 30 min at 4 °C, and then centrifuged at 10 000 × *g* for 15 min at 4 °C (Heraeus Biofuge Fresco, Kendro, USA). The supernatants (S10), containing heat-stable biomolecules, were separated from the pellets and stored at −80 °C.

Determination of total cytosolic protein concentrations

Total protein concentrations were measured in the hepatic and gill cytosols of Vardar chub according to Lowry, as previously described by Krasnići *et al.*[28] For protein determination we have used the Bio-Rad DC Protein Assay and the measurements were carried out on a spectrophotometer/fluorometer (Tecan, Infinite M200) at a wavelength of 750 nm. The concentrations were read from the calibration curve, which was constructed using five concentrations (0.25–2.0 mg mL^{-1}) of bovine serum albumin (Serva, Germany) dissolved in the homogenization buffer. Total protein concentrations in the gills and liver are presented separately for each fish as shown in Table 1.

SEC-HPLC fractionation of cytosols and heat-treated cytosols from Vardar chub liver and gills

The distribution of metals/nonmetals among biomolecules of different molecular masses in the cytosols and heat-treated cytosols from Vardar chub livers and gills was studied using SEC-HPLC (PerkinElmer HPLC system, series 200, USA), as previously described in detail by Krasnići *et al.*[27–29] Two types of SEC columns for globular proteins (GE Healthcare Bio-sciences, USA) were used: prepacked Tricorn™ Superdex 200 10/300 GL with a separation range of 10–600 kDa and a Tricorn™ Superdex 75 10/300 GL column with a separation range of 3–70 kDa. For each sample two chromatographic runs were carried out, and in each run a volume of 100 μL was applied on the column. The separation was achieved using 20 mM Tris–HCl/base (Sigma, pH 8.1 at 22 °C, flow 0.5 mL min^{-1}) as a mobile phase (isocratic mode) in both types of SEC columns. The fractions were collected in plastic tubes at one minute intervals using a fraction collector (FC 203B, Gilson, USA). For the column calibration, several protein standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, conalbumin, bovine albumin, ovalbumin, carbonic anhydrase, cytochrome *C*, and vitamin B12; Sigma, USA) were run through the column under the same conditions as the samples. The equation of the calibration straight line for Superdex 200 was: $y = -0.281x + 1.6473$; and for Superdex 75: $y = -0.3343x + 1.6664$ ($y = K_{av}$; $x = \log MM$). The void volume was determined by the use of blue dextran (defined MM: 2000 kDa), which was eluted in column Superdex 200 at 16.3 min and in Superdex 75 at 15.6 min.

AEC-HPLC separation of fractions of interest collected after SEC_{200}-HPLC and SEC_{75}-HPLC fractionation of cytosols and heat-treated cytosols from Vardar chub liver and gills

Fractions of interest collected from Vardar chub hepatic and gill cytosols using fractionation by SEC_{200}-HPLC or SEC_{75}-HPLC were pooled and preconcentrated to a volume of ∼200 μL by ultrafiltration using Amicon Ultra centrifugal filters (cut off at 3 kDa, Merck, Millipore, Ireland) and centrifuged at 14 000 × *g* at 4 °C in a Heraeus Biofuge Fresco centrifuge (Kendro, USA). Concentrated samples were then applied in volumes of 100 μL on anion-exchange column mono Q 5/50 GL (5 × 50 mm; GE Healthcare Bio-Sciences, Sweden) to perform AEC-HPLC separation. The separation and elution were achieved using

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4 mM Tris–HCl buffer (pH 7.4) as mobile phase A, and 250 mM ammonium acetate buffer prepared in 10 mM Tris–HCl (pH 7.4) as mobile phase B. The mobile phase flow was 1 mL min^{-1} and the gradient elution protocol was adjusted according to the procedure described by Rodríguez-Cea *et al.*[38] The protein absorbances were monitored at 254 and 280 nm using a Diode Array Detector (DAD) (PerkinElmer, series 200, USA). For each sample, two consecutive chromatographic runs were performed, and the fractions containing metals and biomolecules of interest were collected at 0.5 min intervals in plastic tubes using a fraction collector (FC 203B, Gilson, USA).

Measurements of metal/nonmetal concentrations in Vardar chub hepatic and gill cytosols, as well as in fractions obtained by SEC-HPLC and AEC-HPLC separations

The concentrations of Cd, Co, Cu, Fe, Mn, Mo, Se, and Zn were measured in the hepatic and gill cytosols of Vardar chub, which were ten times diluted with Milli-Q water and acidified with HNO_3 (*Suprapur*, Merck, Germany; final acid concentration in the samples: 0.65%) prior to measurements. Fractions of hepatic and gill cytosols collected after SEC-HPLC and AEC-HPLC separations were only acidified with HNO_3 (*Suprapur*, Merck, Germany, final acid concentration in the samples: 0.16%) prior to offline metal/nonmetal measurements. Indium (Fluka, Germany) was added as an internal standard (1 μg L^{-1}) in all samples.[39] The measurements were performed *via* HR ICP-MS (Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and a sample introduction kit consisting of a SeaSpray nebulizer and a cyclonic spray chamber Twister. Measurements of ^{82}Se, ^{98}Mo, and ^{111}Cd were operated in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium resolution mode. External calibrations were performed using multielement standard solution for trace elements (Analitika, Czech Republic). All standards were prepared in 1.3% HNO_3 (Suprapur; Merck, Germany) and supplemented with In (1 μg L^{-1}; Fluka, Germany). Limits of detection (LODs) were calculated as three standard deviations of ten consecutive measurements of trace elements in the blank solution (Tris–HCl/base, dithiothreitol, HNO_3). The LODs for trace elements measured within this study were as follows (in μg L^{-1}): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Mo, 0.004; Se, 0.138; and Zn, 2.40.[27–29] The accuracy of metal/nonmetal measurements *via* HR ICP-MS was checked by the analysis of the quality control sample (QC for trace metals, catalog no. 8072, lot no. 146142–146143, Burlington, Canada). A generally good agreement was observed between our data and the certified values, as can be seen from the obtained recoveries: Cd (96.2 ± 2.0%), Co (98.5 ± 2.7%), Cu (94.8 ± 4.0%), Fe (94.5 ± 4.3%), Mn (97.3 ± 2.9%), Mo (96.4 ± 13.4%), Se (98.2 ± 5.3%), and Zn (104.4 ± 15.5%).

Determination of exact molecular masses of selected cytosolic biomolecules from Vardar chub liver and gills and their identification *via* MALDI-TOF-MS and LC-MS/MS

The fractions, containing specific metal-binding biomolecules, were collected after one-dimensional (SEC-HPLC) and two-dimensional chromatographic separations (SEC-AEC-HPLC). They were further on pooled and preconcentrated by ultrafiltration using Amicon Ultra centrifugal filters (cut off at 3 kDa, Merck, Millipore, Ireland) and centrifuged at 14 000 × *g* at 4 °C in a Heraeus Biofuge Fresco centrifuge (Kendro, USA). Thus prepared samples were then analysed by MALDI TOF-MS (Bruker Daltonik Microflex series, Germany) and LC-MS/MS (the Bruker Daltonik amaZon ETD ion trap system, Germany).

Analyses on MALDI-TOF-MS were used to determine exact molecular masses of isolated Vardar chub hepatic and gill biomolecules of interest. For this purpose, 1 μL of preconcentrated samples was spotted in two replicates onto a 96-spot steel target plate (Bruker Daltonik, Germany) and allowed to visibly dry at room temperature. Subsequently, 1 μL of α-cyano-4-hydroxycinnamic acid (10 mg mL^{-1}; Bruker Daltonik, Germany) in 50% acetonitrile/2.5% trifluoroacetic acid was added on top of the samples as a matrix.

The analyses of LC-MS/MS were performed with an aim to identify, whenever possible, isolated cytosolic biomolecules obtained from Vardar chub liver and gills. For that purpose, preconcentrated samples were first dried and then redissolved in 20 mM ammonium bicarbonate. In this solution DTT was added to a final concentration of 10 mM in order to reduce possible disulfide bonds in the proteins. After 1 hour at room temperature, iodoacetamide (IAA) was added to the reaction mixture to a final conncentration of 54 mM and left in the dark at room temperature for another hour. Finally, trypsin (Trypsin Gold, Promega, USA) was added (1 μg per 100 μg of proteins) and the mixture was incubated overnight at room temperature. The reaction was stopped by the addition of formic acid.

Analyses of obtained samples of tryptic peptides were performed *via* low resolution ion trap LC-MS/MS. Peptides were loaded onto a Dionex Ultimate 3000 RSLC trap column (C18 resin, Acclaim "pepmap", 100 Å, 5 μm, 1 mm × 5 mm) in 1 μL of solution containing 0.1% formic acid and 2% acetonitrile, and desalted for 2 min with 0.1% formic acid at a flow rate of 20 μL min^{-1}. Peptides were separated on a cappilary column (C18 resin, Acclaim "pepmap", 100 Å, 2 μm, 0.3 mm × 150 mm) at a flow rate of 1 μL min^{-1}. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The 45 min multistep gradient consisted of mobile phase B: 1 min 5%, 30 min linear gradient to 45%, 1 min linear gradient to 90%, 4 min hold on 90%, 3 min linear gradient to 5%, 6 min hold on 5%. The Amazon ETD ion trap (Bruker Daltonik, Germany) mass spectrometer was operated at an ESI capillary voltage of −4500 V, while the high voltage end plate offset was −500 V. The nebulizer was set at 10 psi. The temperature of dry gas was set at 200 °C with a flow rate of 5 L min^{-1}. Helium was used as a collision gas. The fragmentation amplitude was set at 0.60 V and ramped between 30% and 300% of this value. Product ion spectra were sequentially recorded for each selecter precursor. The acquisition software was set up in autoMSMS mode using up to three precursor ions with active exclusion on (precursor exclusion after two MSMS spectra for 2 min). MS and MSMS spectra were acquired within a scan range from 300 to 1500 *m*/*z* using averages from five/two spectra and a scan rate of 8100 (*m*/*z*) s^{-1}. DataAnalysis software 4.0 (Bruker Daltonik GmbH, Germany) was used to extract MS and MS/MS

data and to create the Mascot (Matrix Science, UK) file for database search. Search parameters were set to: database NCBInr all entries, enzyme trypsin 1 miss cleavage, carbamidomethyl as global and methionine oxidation as variabile modification, and the mass tolerance for MS/MS spectra was 0.5 Da.

Data processing and statistical analyses

All basic calculations were done using Microsoft Excel 2007, whereas graphs were created using statistical program SigmaPlot 11.0 for Windows. Chromatographic results were processed using the TotalChrom Version 6.3.1 software (PerkinElmer, USA).

Results and discussion

SEC_{200}-HPLC analyses of trace element distributions in hepatic and gill cytosols of Vardar chub

SEC_{200}-HPLC analyses of trace element distributions in hepatic and gill cytosols of Vardar chub were previously done using specimens sampled in spring and autumn seasons of 2012 from three Macedonian rivers, Bregalnica, Zletovska and Kriva.[29] This study made it possible to define the molecular masses of cytosolic biomolecules that bind essential elements Co, Cu, Fe, Mn, Mo, Se and Zn, and nonessential and very toxic element Cd under the conditions of low and high metal exposure.[29] Furthermore, our goal was to characterize more specifically several of those biomolecules, which was the reason to repeat SEC_{200}-HPLC analyses on hepatic and gill cytosols of the same fish species following the new sampling campaign in June 2015 in Bregalnica and Zletovska rivers. We were, thus, able to confirm previously obtained results, and then to isolate the metal-containing fractions of interest which we needed for further analyses. The graphical presentation of SEC_{200}-HPLC analyses for Co, Cu, Fe, Mn, Mo, Se, Zn, and Cd in the hepatic cytosols and for Fe, Se, Zn, and Cd in the gill cytosols of Vardar chub is presented in the ESI† (Fig. S1 and S2), whereas the information on elution times and corresponding molecular masses of metal-binding biomolecules is presented in Table 2. Four elements (Co, Cu, Mn, and Mo) were not measured in the gill cytosols due to their very low cytosolic concentrations. The same as in our previous studies,[27–29] metal-binding biomolecules were defined as belonging to the high molecular mass region (HMM, >100 kDa), the medium molecular mass region (MMM, 30–100 kDa), the low molecular mass region (LMM, 10–30 kDa), and the very low molecular mass region (VLMM, <10 kDa).

Hepatic Co (Fig. S1a, ESI† and Table 2) was distributed between two peaks, with a higher Co quantity bound to HMM biomolecules (141–299 kDa) and a lower Co quantity bound to MMM biomolecules (40–109 kDa). Hepatic Mn (Fig. S1d, ESI† and Table 2) was distributed between two peaks, with a higher Mn quantity bound to HMM biomolecules (85–233 kDa) and a much lower Mn quantity bound to MMM biomolecules (15–66 kDa). Hepatic Mo (Fig. S1e, ESI† and Table 2) was distributed between four peaks covering a wide region of molecular masses, but a majority of Mo was bound to HMM biomolecules (66–385 kDa). Appreciable quantities of Mo were also bound to MMM biomolecules (11–51 kDa) and VLMM

Table 2 Elution times (t_e) and molecular masses (MM) of hepatic and gill cytosolic proteins of Vardar chub, which were comprised within metal-containing fractions obtained by cytosol separation using SEC_{200}-HPLC (Fig. S1 and S2, ESI). Presented data refer to maximums of trace element peaks and to peak spans (within the brackets)

			HMM peak 1[a]		HMM peak 2[a]		MMM peak[b]		LMM peak[c]		VLMM peak 1[d]		VLMM peak 2[d]	
Element		Organ	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
Essential elements	Co	Liver			20 (19–22)	233 (299–141)	25 (23–27)	66 (109–40)						
	Cu	Liver							31 (29–35)	15 (24–5)				
	Fe	Liver	18 (16–21)	385 (637–181)			27 (26–29)	40 (51–24)						
		Gills					27 (26–30)	40 (51–19)						
	Mn	Liver			22 (20–24)	141 (233–85)	28 (25–31)	31 (66–15)						
	Mo	Liver	15 (14–17)	820 (1054–495)	20 (18–25)	233 (385–66)	28–30 (26–32)	31–19 (51–11)			35 (33–37)	5 (9–3)		
	Se	Liver			22 (19–24)	141 (299–85)					37 (35–39)	3 (5–2)	42 (40–44)	0.9 (1.5–0.6)
		Gills									37 (35–39)	3 (5–2)	42 (40–46)	0.9 (1.5–0.3)
	Zn	Liver	15 (14–17)	820 (1054–495)			24 (19–28)	85 (299–31)	31 (29–34)	15 (24–7)				
		Gills	15 (14–17)	820 (1054–495)			24 (20–29)	85 (233–24)	31 (29–32)	15 (24–11)				
Non-essential element	Cd	Liver							31 (29–34)	15 (24–7)				
		Gills							31 (29–34)	15 (24–7)				

[a] HMM peak – peak maximum in the high molecular mass protein region (>100 kDa). [b] MMM peak – peak maximum in the medium molecular mass protein region (30–100 kDa). [c] LMM peak – peak maximum in the low molecular mass protein region (10–30 kDa). [d] VLMM peak – peak maximum in the very low molecular mass protein region (<10 kDa).

biomolecules (3–9 kDa). Predominant binding of Co, Mn, and Mo to HMM biomolecules in the liver of Vardar chub was consistent with previous observations on Vardar chub[29] and can be explained by the essential function of these metals in the activities of numerous enzymes.[40,41] Furthermore, both hepatic (Fig. S1f, ESI† and Table 2) and gill Se (Fig. S2b, ESI† and Table 2) in this study were mainly distributed between two VLMM peaks, with the major Se quantity bound to VLMM biomolecules of 0.3–1.5 kDa, which encompassed small peptides and free amino acids, such as selenomethionine.[42] High Se affinity for VLMM biomolecules in hepatic and gill cytosols of Vardar chub was already reported in our previous study, and could be associated to Se binding to small biomolecules involved in the defense against oxidative stress.[29,43,44]

Hepatic Cu (Fig. S1b, ESI† and Table 2) in this study was distributed within one peak, containing LMM biomolecules (5–24 kDa). The same was observed for hepatic (Fig. S1h, ESI† and Table 2) and gill Cd (Fig. S2d, ESI† and Table 2). This was consistent with the previous study on Vardar chub, where Cu- and Cd-binding LMM biomolecules were eluted within a single and sharp peak at the elution time of the MT standard, indicating high affinity of Cu and Cd for binding to MTs.[29] Such binding, suggesting a detoxification response of the organism, was previously reported for white sucker (*Catostomus commersonii*),[7] juvenile yellow perch (*Perca flavescens*),[45] and European eel (*Anguilla anguilla*).[46] Both hepatic (Fig. S1g, ESI† and Table 2) and gill Zn (Fig. S2c, ESI† and Table 2) were also found bound to LMM biomolecules (7–24 kDa), but this binding was more obvious and more intense in the liver, and indicated hepatic Zn association to MTs. This was consistent with the observations for both Cu and Cd, and was also in agreement with the previous reports on pearl cichlid (*Geophagus brasiliensis*) and sea catfish (*Netuma barba*),[38] as well as on European eel (*A. anguilla*).[46] However, notable quantities of hepatic and gill Zn were additionally found bound to HMM biomolecules (>495 kDa) and to MMM biomolecules (24–299 kDa). Large quantities of Zn eluted within HMM and MMM protein regions were also observed in our previous study in both Vardar chub organs,[29] indicating the essential role of Zn in the function of many proteins and enzymes,[47,48] such as transport protein albumin (66 kDa) and enzymes Cu–Zn superoxide dismutase (SOD, 32 kDa) and carboanhydrase (29 kDa).[49] Furthermore, potential Zn binding to MTs, which was more pronounced and clear in the liver than in the gills, was also observed in our former study on Vardar chub.[29]

In this study, hepatic Fe (Fig. S1c, ESI† and Table 2) was distributed between two comparable peaks, the first one referring to HMM biomolecules (181–637 kDa) and the second one to MMM biomolecules (24–51 kDa). In the gills (Fig. S2a, ESI† and Table 2), however, the majority of Fe was distributed within one peak containing MMM biomolecules (19–51 kDa), which coincided with the second Fe peak of the hepatic cytosol. This was in agreement with the previous finding on Fe distribution in the liver and gills of Vardar chub, with much more pronounced Fe presence in the HMM protein region in the liver,[29] most likely referring to Fe binding to ferritin (~450 kDa),[49] a primary iron storage protein.[50] The suggested explanation was that the absence of HMM Fe-binding biomolecules in the gills was the reflection of known function of the liver and not the gills in the process of Fe storage.[29] Similarly, Neves *et al.*[50] found ferritin expression in both the liver and brain of sea bass (*Dicentrarchus labrax*), but higher concentrations were measured in the liver, as the major organ of iron storage. On the other hand, we have found comparable Fe quantities eluted within the MMM protein region in both organs of Vardar chub, which possibly reflected Fe binding to transport or enzymatic proteins, such as hemoglobin (~65 kDa),[49] transferrin (~80 kDa),[49] myoglobin (~17 kDa),[49] catalase (subunits of ~60 kDa),[49] or some other proteins.

Since it is usually expected to find transport protein transferrin in the hepatic samples, as described by Neves *et al.*[50] in the study on the liver of sea bass (*D. labrax*), we have run the standard protein transferrin (76–81 kDa) through the SEC_{200}-column and recorded its elution time at the 22nd to 27th minute, with a maximum at approximately the 24th minute, corresponding to MM of 85 kDa (Fig. S3, ESI†). However, the corresponding peak was not observed in either hepatic or gill SEC_{200} Fe profiles of Vardar chub (Fig. S1c and S2a, ESI†). We have, furthermore, collected the MMM Fe-fractions from both liver and gills after the SEC_{200}-HPLC separation (t_e 25–30 min), and then recorded MALDI-TOF-MS spectra for the collected samples (Fig. S4a and b, ESI†), as well as for standard proteins transferrin (Sigma-Aldrich Co., USA) and Zn,Cu-SOD (Sigma-Aldrich Co., USA) (Fig. S4c and d, ESI†). We have included SOD because MMM Fe-fractions have partly overlapped with the tails of LMM Cu-fractions and MMM Zn-fractions (Table 2). A comparison of MALDI-TOF-MS spectra obtained for the samples and standards clearly indicated the absence of transferrin in our samples, but confirmed the probable presence of SOD, based on the concurrent appearance of similar peaks in the spectra of both hepatic and gill samples and of the SOD standard (peaks at 31 kDa, 15.5 kDa (2+), and 7.75 kDa (4+)). Since hemoglobin subunits have molecular mass of approximately 15 kDa,[51,52] peaks at 15.5 kDa could have additionally indicated the presence of hemoglobin in our samples, especially taking into consideration the reddish colour of the collected fractions. Several peaks observed below 10 kDa in the MALDI-TOF spectra of hepatic and gill samples have possibly indicated Fe-binding to biomolecules belonging to a low-molecular-mass iron pool, sometimes called the transit iron pool,[53] which contains small and soluble complexes that help in intracellular Fe transport from one Fe-binding protein to the other.[54]

Analysis of Vardar chub hepatic and gill MMM Fe-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC and MS techniques

Since the likely presence of non-Fe-binding proteins was established in MMM Fe-fractions obtained by SEC_{200}-HPLC separation, in the next step of our research we have collected these fractions from liver and gills (t_e 26–29 min, Fig. S1c and S2a, ESI†), purified them *via* AEC-HPLC (Fig. 1), and then analyzed Fe-binding proteins present in hepatic and gill samples *via*

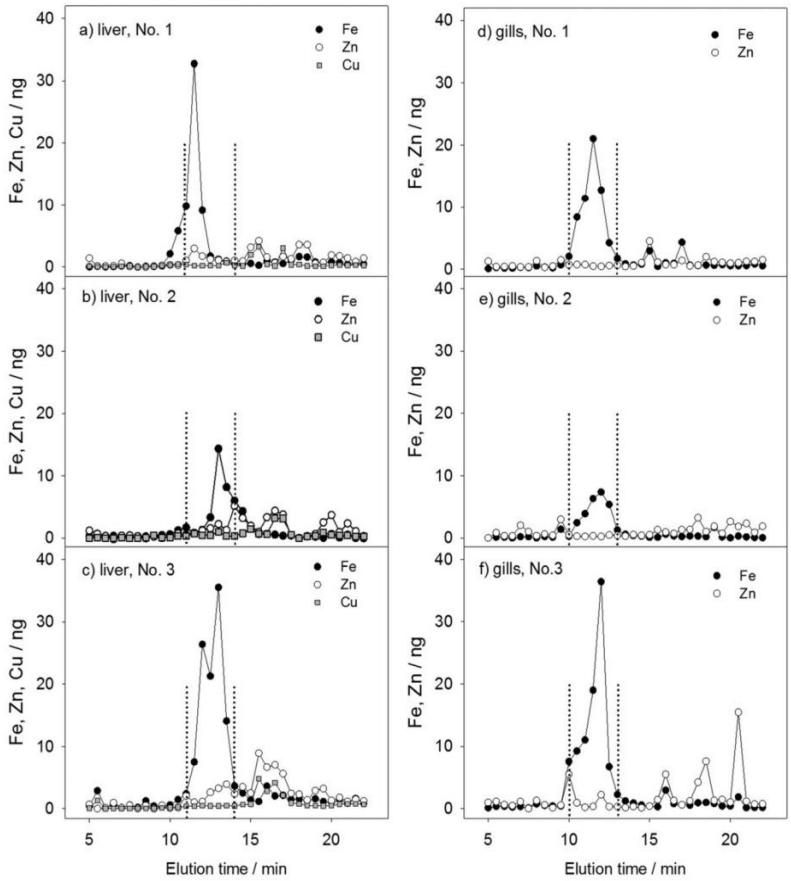

Fig. 1 AEC-HPLC analyses of hepatic and gill MMM Fe-peaks, which were obtained by SEC_{200}-HPLC separations (t_e 26–29 min). The obtained AEC distribution profiles of Fe, Zn, and Cu in three samples of liver (a–c) and Fe and Zn in three samples of gills (d–f) are presented in this figure as nanograms of metals eluted every half minute. The major Fe-containing peaks (liver: t_e 11–14 min; gills: t_e 10–13 min), which are marked by dotted lines, were further used for MS analyses.

MALDI-TOF-MS and LC-MS/MS (Fig. 2, Tables 3 and 4). AEC-separation was often applied after SEC-fractionation in the course of isolation and characterization of Fe-binding biomolecules, *e.g.* in human serum[55–57] and in fish (rainbow trout, *Oncorhynchus mykiss*).[52] During AEC-separation, Fe-binding biomolecules were eluted within single clear and sharp peaks, with elution time from 10.0 to 14.5 min for the liver (Fig. 1a–c) and from 9.5 to 13.5 min for gills (Fig. 1d–f), whereas the concentration of ammonium acetate (mobile phase B) needed for their elution generally ranged from 32.5 to 43.8 mM (Table 3). Small Zn and Cu peaks separated from Fe-peaks were observed (t_e 15.0–17.5 min), especially in the profiles of hepatic samples (Fig. 1a–c), showing the advantage of AEC-HPLC as a useful protein purification procedure.

To further characterize purified Fe-binding biomolecules, we have collected Fe-fractions eluted during AEC separation (liver: t_e 11–14 min; gills: t_e 10–13 min) and recorded their mass spectra *via* MALDI-TOF-MS. The MALDI spectra obtained for the hepatic Fe-fractions always showed one major peak at 15.4 kDa, and sometimes two additional smaller peaks at 31.5 kDa and 46.9 kDa (Fig. 2a). Recorded peaks most likely corresponded to hemoglobin (Hb) spectra, with characteristic Hb monomers (~15 kDa), dimers (~30 kDa) and trimers (~45 kDa). Hemoglobin, although a large protein of molecular mass of approximately 64.5 kDa, has four subunits each containing a polypeptide chain (α or β) and a heme group.[58] In the rainbow trout (*O. mykiss*) blood, five major α chains and four major β chains have been identified, with the mass range from 15.1 to 16.0 kDa,[52] corresponding well to our results. In the study on human blood, Yu *et al.*[51] have also observed the simultaneous occurrence of monomers and dimers after the separation of Hb on SDS-PAGE mini-gels. Accordingly, the peak at ~15 kDa most likely corresponded to either an α-chain, or a β-chain, and the peak at ~30 kDa probably corresponded to the covalent product of α–α, β–β, or α–β crosslinking, whereas the peak at 45 kDa probably corresponded to a covalently linked trimer.[51] The MALDI-TOF spectra obtained for the gill Fe-fractions also showed clear peaks at ~15 kDa, which could be attributed to Hb, but additional peaks were observed at lower molecular masses, indicating multiple charges on proteins

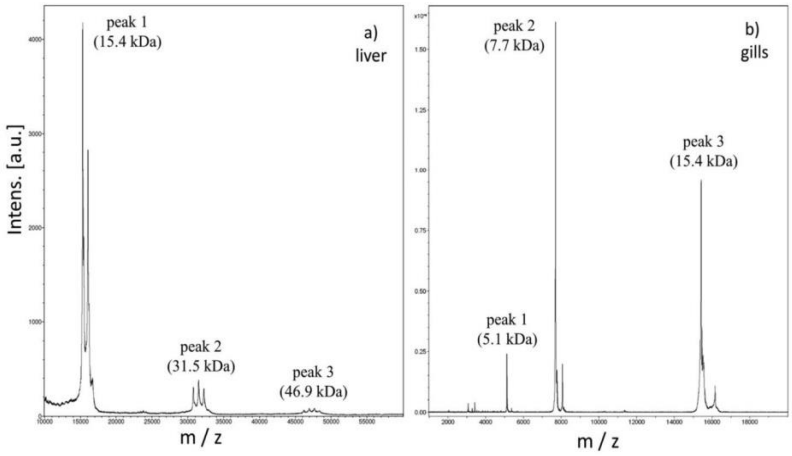

Fig. 2 Mass spectra obtained *via* MALDI-TOF-MS for MMM Fe-binding biomolecules, which were separated by SEC_{200}-HPLC followed by AEC-HPLC from hepatic cytosols (a) and gill cytosols (b) of Vardar chub (the spectra are presented for fish no. 2).

Table 3 Elution times (t_e) and concentrations of ammonium acetate (mobile phase B) needed for elution of hepatic and gill cytosolic biomolecules of Vardar chub during the AEC-HPLC separation of Fe-MMM (Fig. 1) and Cd-LMM (Fig. 3) fractions, which were obtained by prior SEC_{200}-HPLC fractionation (t_e 26–29 min and 30–34 min, respectively). Data for the AEC-HPLC separation of Cd-LMM fractions from fish no. 2 are not presented, due to low Cd concentrations and high dilution effect

	Sample	Fe-MMM fraction (Fig. 3)	t_e/min	c (NH_4OAc)/mM	Cd-LMM fraction (Fig. 5)	t_e/min	c (NH_4OAc)/mM
Liver	No. 1	Peak 1	10.0–12.5	32.5–43.8	Peak 1	9.5–12.0	32.5–43.8
					Peak 2	13.0–16.5	43.8–62.5
	No. 2	Peak 1	11.0–14.0	32.5–43.8	—	—	—
	No. 3	Peak 1	10.5–14.5	32.5–62.5	Peak 1	9.0–12.0	25.0–43.8
Gills	No. 1	Peak 1	10.0–13.0	32.5–43.8	Peak 1	9.5–12.0	32.5–43.8
					Peak 2	13.0–16.5	43.8–62.5
	No. 2	Peak 1	10.0–13.0	32.5–43.8	—	—	—
	No. 3	Peak 1	9.5–13.5	32.5–43.8	Peak 1	10.0–12.0	32.5–43.8
					Peak 2	12.5–16.0	43.8–62.5

(Fig. 2b), whereas the signs of multimers were not recorded. For example, in fish no. 1 (Fig. 2b), the other two peaks were probably double charged (peak 2: 7.7 kDa; 2+) or triple charged (peak 1: 5.1 kDa; 3+).

To identify Fe-binding biomolecules with certainty, we additionally analyzed AEC-collected hepatic and gill Fe-fractions *via* LC-MS/MS with a subsequent Mascot database search, which confirmed the Hb presence in soluble fractions of both the liver and gills of Vardar chub (Table 4). In both the liver and gills, subunit β of Hb (corresponding to subunits originating from various fish species) was identified, whereas subunit α was only found in one gill sample (fish no. 2, Table 4). The observation of subunit α can explain the MALDI spectra obtained for the gills of fish no. 2, which next to the peak of 15.3 kDa also contained two additional peaks of lower molecular masses (at 11.3 kDa and 13.5 kDa) which possibly corresponded to Hb subunit α. Lower molecular mass of the α-chain (15.1 kDa) compared to the β-chain (15.9 kDa) was also recorded in human Hb,[51] as well as in rat Hb (α: 15.3 kDa; β: 16.0 kDa).[59] Our results, therefore, undoubtedly confirmed the presence of blood in the samples of Vardar chub liver and gills, which was not surprising considering that organ perfusion was not performed prior to liver and gill dissection and the isolation of soluble tissue fractions. In addition, major binding of Fe to hemoglobin instead of transferrin is consistent with the findings by Fernández-Menéndez *et al.*,[59] who showed that in rat red blood cells less than 13% of total Fe was bound to transferrin, *i.e.* 87–93% of Fe was found bound to hemoglobin. Combination of SEC- and AEC-HPLC with MS analyses was proven to be a sensitive and optimal tool for the detection of Hb variants, as also pointed out by Kleinert *et al.*[60]

Analysis of Vardar chub hepatic and gill LMM Cd-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC

Furthermore, our aim was to better characterize the potential MT fraction from Vardar chub liver and gills, *i.e.* Cd, Cu, and Zn-binding LMM biomolecules observed after SEC_{200}-HPLC separation. Hyphenated techniques, such as combination of SEC-HPLC and AEC-HPLC with ICP-MS, allow us to distinguish different MT isoforms, which could bring much more insightful information on the regulation of essential elements such as Cu and Zn, and on the detoxification of metals such as Cd in

Table 4 Characterization and identification of MMM Fe-binding biomolecules from hepatic and gill cytosols of Vardar chub separated by SEC_{200}-HPLC (t_e 26–29 min) and AEC-HPLC (t_e 11–14 min), using MALDI-TOF (Fig. 2), and LC-MS/MS after protein trypsinization

	Fish	MM (kDa) (according to SEC_{200})	*m/z* (kDa) (according to MALDI-TOF)		Proteins identified by LC-MS/MS: Mascot search results
Liver	No. 1 (Fig. 4a)	24–51	Peak 1	15.4	Hemoglobin subunit beta (*Danio rerio*, score: 244; *Carassius auratus*, score: 181; *Arctogadus glacialis*, score: 147)
			Peak 2	31.5	
			Peak 3	46.9	
	No. 2	24–51	Peak 1	15.4	Hemoglobin subunit beta (*Danio rerio*, score: 225; *Carassius auratus*, score: 156; *Arctogadus glacialis*, score: 122)
			Peak 2	—	
			Peak 3	—	
	No. 3	24–51	Peak 1	15.4	Hemoglobin subunit beta (*Danio rerio*, score: 160; *Arctogadus glacialis*, score: 106)
			Peak 2	31.6	
			Peak 3	47.0	
Gills	No. 1 (Fig. 4b)	19–51	Peak 1	5.1	Hemoglobin subunit beta-1 (*Danio rerio*, score: 313)
			Peak 2	7.7	
			Peak 3	15.4	
	No. 2	19–51	Peak 1	11.3	Hemoglobin subunit beta (*Danio rerio*, score: 111; *Carassius auratus*, score: 96; *Arctogadus glacialis*, score: 85)
			Peak 2	13.5	Hemoglobin subunit alpha (*Cyprinus carpio*, score: 69)
			Peak 3	15.3	

various organisms.[61] Thus, we have collected hepatic and gill Cd-containing LMM fractions produced by SEC_{200}-HPLC separation (t_e 30–34 min) and analysed them by AEC-HPLC (Fig. 3a–d).

In general, two well resolved Cd-peaks were obtained by AEC-separation of Cd-containing LMM fractions from both the Vardar chub liver (Fig. 3a and b) and gills (Fig. 3c and d), with elution times from 9.0–12.0 minutes and 12.5–16.5 minutes, whereas the concentrations of ammonium acetate (mobile phase B) needed for their elution generally ranged from 32.5 to 43.8 mM and 43.8 to 62.5 mM, respectively (Table 3). The elution of these biomolecules did not require hard ionic strength conditions in the anion-exchange column, which is consistent with the previous studies on MTs obtained from several other fish species (*Cyprinus carpio*, *A. anguilla*, and *G. brasiliensis*), which reported MT elution at a concentration of 30–45 mM of ammonium acetate.[38,62,63] In addition to AEC-separation of hepatic and gill samples, we have also analyzed MT standards from rabbit liver (isoforms MT-I and MT-II) using the AEC-HPLC method (Fig. 3e and f). Examination of their absorbance profiles at 254 nm revealed that the MT-I standard eluted at 9.5–11.5 minutes, whereas the MT-II standard eluted at 13.0–16.0 minutes. Evidently, elution time of the MT-I standard coincided with the first Cd-containing peak, whereas the elution time of the MT-II standard coincided with the second Cd-containing peak of Vardar chub organs, further confirming that analyzed LMM Cd-containing fractions indeed contained MTs, specifically MT isoforms MT-I and MT-II. The Cd-binding biomolecules were previously investigated in the liver of two species of flatfish (*Limanda limanda* and *Microstomus kitt*) by Duquesne and Richard,[64] who also confirmed the existence of two isoforms of hepatic MT, with MT-II as a predominant isoform. Several other authors also reported Cd-binding to two MT isoforms, MT-I and MT-II, in organs of various fish species: in the liver of eel (*A. anguilla*)[46] and dab (*L. limanda*)[65] and in the liver and gills of goldfish (*Carassius auratus*), yellow catfish (*Pelteobagrus fulvidraco*), stone moroko (*Pseudorasbora parva*) and barbel chub (*Squaliobarbus curriculus*).[66] The obtained results in this study further demonstrated the importance of the multidimensional chromatographic approach in MT analyses, since the sole application of SEC-HPLC separation revealed Cd-binding to MTs, but was not able to reveal the presence of various isoforms.

The obtained AEC-peaks contained all three analyzed metals (Cd, Cu, and Zn) in hepatic samples (Fig. 3a and b), whereas in the gills they referred solely to Cd (Fig. 3c and d), since Cu was not measured due to its low cytosolic concentrations in the gills, and Zn was not detected in the MT region after AEC-separation. This was an indication of the absence of Zn binding to MTs in Vardar chub gills, as this was already anticipated based on the results of the SEC_{200}-HPLC analysis of gill cytosols (Fig. S2, ESI†). Furthermore, this was in agreement with the absence of the clear Zn peak at the elution time of MTs in the gills of European chub (*S. cephalus*) obtained from the Sulta River.[28] Observed differences between Zn distributions in the liver and in gills suggested a tissue-dependent physiological role of MT isoforms. Although both organs evidently contained MT-I and MT-II isoforms, it is possible that each isoform differed in the amino acid sequence between organs, presenting gill and hepatic subisoforms of MT-I and MT-II. The hepatic Zn-containing MT isoforms are therefore considered, not only as a means of detoxification, but also as a means of storage of essential metals, which can be donated to the other proteins when needed.[35,67] The gill Cd-containing MT isoforms, on the other hand, can be hypothesized to serve predominantly as a detoxification tool.

It was previously reported by Goenaga Infante *et al.*[2] that Cd was preferably bound to a major MT-I isoform in the rabbit liver standard and that the increase of the Cd amount bound to MTs in the liver of gibel carp (*Carassius auratus gibelio*) mainly reflected the induction of an MT-I isoform. Such predominant binding to MT-I was observed in our study only in one liver sample (fish no. 3, Fig. 3b), whereas in the same fish, Cd binding to MT-II was more pronounced in the gills (fish no. 3, Fig. 3d). Similarly, Li *et al.*[66] reported Cd binding only to MT-I in the liver of bighead carp (*Aristichthys nobilis*) and white amur bream (*Parabramis pekinensis*), whereas in the gills of the same

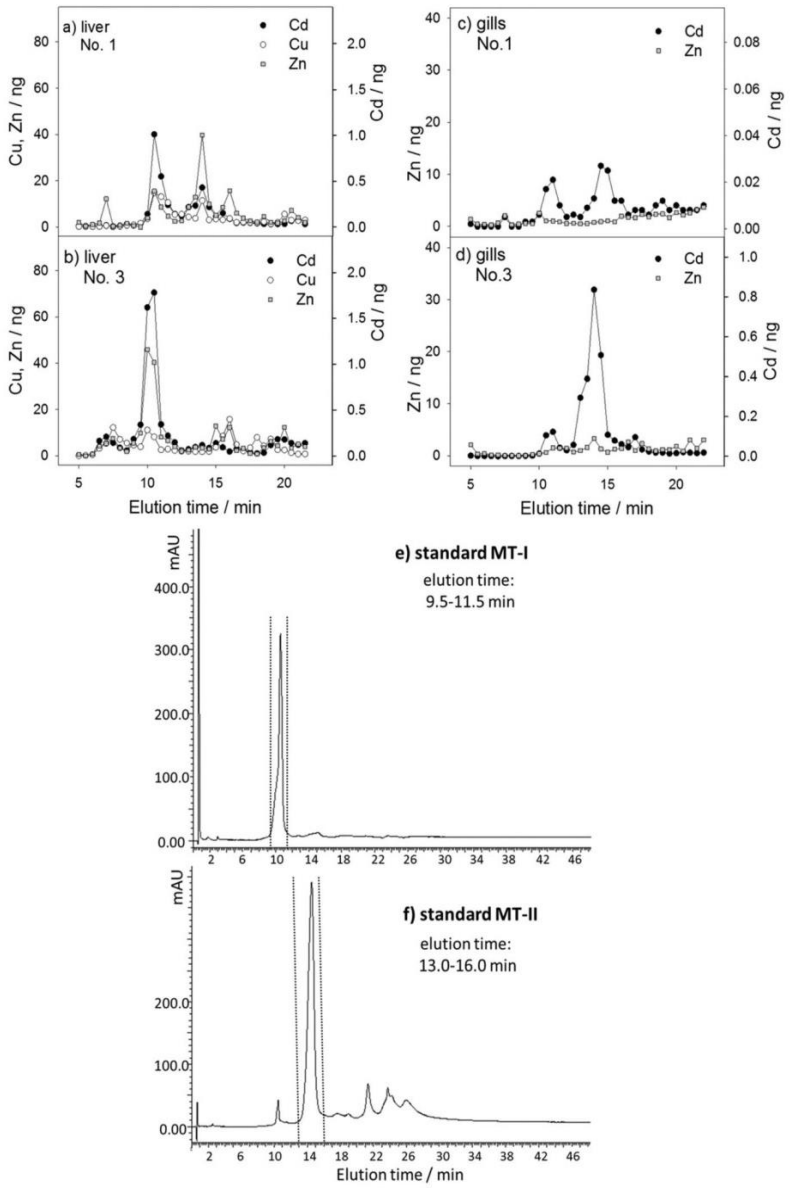

Fig. 3 AEC-HPLC analyses of hepatic and gill LMM Cd-peaks (presumably containing MTs), which were obtained by SEC_{200}-HPLC separations (t_e 30–34 min). The obtained AEC distribution profiles of Cd, Cu, and Zn in two samples of liver (a and b) and Cd and Zn in two samples of gills (c and d) are presented in this figure as nanograms of metals eluted every half minute. AEC-HPLC chromatographs recorded by UV detection at 254 nm are presented for standard proteins MT-I (e) and MT-II (f).

fish species Cd was bound to both MT-I and MT-II. Goenaga Infante *et al.*[2] further reported that, in contrast to Cd, the excess of intracellular Cu and Zn appears to be sequestered by four MT isoforms in the liver of gibel carp (*C. auratus gibelio*), including MT-I and MT-II isoforms, which were also detected *via* AEC-HPLC. This phenomenon of differential metal-binding by specific MT isoforms, which has been reported in various types of organisms (mammals, snails, and mussels),[68–70] might be related to different roles of each MT isoform in the metal detoxification and regulation.[38]

Further characterization of MT fractions by the application of MALDI-TOF-MS was done after heat-treatment and SEC_{75}-HPLC fractionation of hepatic and gill cytosols, which are presented in the next subsection.

SEC_{75}-HPLC analyses of trace element distributions in hepatic and gill cytosols before and after the heat-treatment

According to Rosabal *et al.*,[71] the heat-stable cytosolic fraction contains biomolecules, such as MTs, which are consistently involved in the detoxification of trace metals. MTs are

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heat-stable proteins, owing to a high content of cysteine residues (30% of their amino acids) which give heat-stability to peptides.[7] However, in addition to MTs, we have previously detected the presence of the other heat-stable biomolecules in Vardar chub liver and gills, namely VLMM biomolecules (<10 kDa) that bind Mo and Se.[29] Our aim, therefore, was to further purify cytosolic samples to obtain the fractions which contain solely heat-stable peptides and proteins characteristic of Vardar chub liver and gills, *i.e.* to remove by the heat-treatment at 70 °C high molecular mass proteins that could interfere with the characterization of heat-stable biomolecules.[16,37] We have performed SEC_{75}-HPLC analyses on both untreated and heat-treated hepatic and gill cytosols of Vardar chub to define the changes in the cytosolic distributions of several elements (Cd, Cu, Zn, Mo and Se) that occur after the cytosol heat-treatment. As already stated above in the introductory part of the section SEC_{200}-HPLC analyses of trace element distributions in hepatic and gill cytosols of Vardar chub, by performing this procedure we were able to confirm the results obtained during the 2012 campaign,[29] and to isolate the heat-stable metal-containing fractions that we wanted to further characterize. Distribution profiles of five mentioned elements in the hepatic and gill cytosols of Vardar chub before and after the heat-treatment are presented in the ESI† (Fig. S5 and S6), whereas their elution times and the molecular masses of the corresponding biomolecules are given in Table 5.

In our previous study, we have reported binding to heat-stable LMM biomolecules (10–30 kDa), presumably MTs, for Cd and Cu in the liver and Cd in the gills of Vardar chub.[29] In this study, elution within the LMM peak (9–31 kDa, Table 5) was again observed for both hepatic Cd (Fig. S5a and d, ESI†) and Cu (Fig. S5c and f, ESI†), as well as for gill Cd (Fig. S5g and i, ESI†). The observed LMM peaks remained mostly unchanged after the heat-treatment in the case of the liver (changes within 10%, Table 6) and slightly decreased in the case of the gills (changes up to 30%, Table 6), confirming the presence of Cd,Cu-binding heat-stable biomolecules, mostly MTs, in the Vardar chub liver and gills. As the heat-treatment causes a partial removal (~50% after 10 minutes at 70 °C) of the MT20 fraction (MT dimers) from the samples due to the presence of disulphide bonds within MT20 molecules,[37] the observed 30% decrease of the gill LMM peak after the heat-treatment most likely referred to the slight reduction of MT dimers.[37] MT monomers (MT10), on the other hand, are resistant to any physical or chemical treatment and do not change under the influence of increased temperature.[37] In the case of the Vardar chub gills, a small part of Cd was additionally eluted within the MMM peak (38–137 kDa, Fig. S5g, i, ESI† and Table 5), which visibly decreased (up to 65%, Table 6) after the heat-treatment, indicating the heat-sensitive nature of Cd-binding MMM biomolecules in the gills. This was a confirmation of the previous finding of Cd binding to MMM biomolecules only in the Vardar chub gills (60–110 kDa), which was hypothesized to refer to the undetoxified portion of gill Cd.[29]

Elution within the LMM peak (11–31 kDa, Table 5) was also observed for hepatic Zn (Fig. S5b and e, ESI†), but, as expected, not for gill Zn (Fig. S5h and j, ESI†), again confirming the absence of Zn-MT binding in the gills of Vardar chub. The hepatic Zn-LMM peak was proven to be relatively heat-stable, taking into consideration the rather low decrease of the LMM peak after the heat-treatment (up to 22%, Table 6). This small decrease of the Zn-LMM peak was probably related to the reduction of the MT20 fraction,[37] as already discussed above for the gill Cd-LMM fraction. The findings of this study are consistent with the previously reported Zn binding to heat-stable LMM biomolecules (10–30 kDa) in the liver and its absence in the gills of Vardar chub.[29] In this study, in both organs of Vardar chub, Zn was also eluted within the MMM peak (~20–137 kDa), which represented the minor part of cytosolic Zn in the liver (Fig. S5b and e, ESI†), and the major part in the gills (Fig. S5h and j, ESI†). After the heat-treatment, both hepatic and gill Zn-MMM peaks were almost completely removed (decrease up to 80%, Table 6), pointing to the heat-sensitivity of Zn-binding MMM proteins. Zinc binding to MMM biomolecules (30–140 kDa) and their almost complete removal by the heat-treatment was also previously reported for the Vardar chub liver and gills.[29]

In the untreated hepatic cytosols of Vardar chub, Mo was eluted within two peaks (Fig. S6a and c, ESI†), with the majority

Table 5 Elution times (t_e) and molecular masses (MM) of hepatic and gill cytosolic proteins of Vardar chub, which were comprised within metal-containing fractions obtained by separation of cytosols and heat-treated cytosols using SEC_{75}-HPLC (Fig. S5 and S6, ESI). Presented data refer to maximums of trace element peaks and to the peak spans (within the brackets)

			MMM peak 1[a]		MMM peak 2[a]		LMM peak[b]		VLMM peak 1[c]		VLMM peak 2[c]	
Element		Organ	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
Essential elements	Cu	Liver					23 (21–27)	20 (31–9)				
	Mo	Liver	16 (14–21)	89 (137–31)					28 (26–30)	7 (11–5)		
		Gills							28 (26–30)	7 (11–4.5)		
	Se	Liver	16 (14–18)	89 (137–58)	21 (19–25)	31 (47–13)			30 (28–32)	4.5 (6–3)	37 (35–39)	1 (1.6–0.5)
		Gills	16 (14–24)	89 (137–16)					30 (28–32)	4.5 (6–3)	38 (35–40)	0.8 (1.6–0.5)
	Zn	Liver	18 (14–20)	58 (137–38)			23 (21–26)	20 (31–11)				
		Gills	16 (14–23)	89 (137–20)								
Non-essential element	Cd	Liver					23 (21–26)	20 (31–11)				
		Gills	16 (14–20)	89 (137–38)			23 (21–26)	20 (31–11)				

[a] MMM peak – peak maximum in the medium molecular mass protein region (30–100 kDa). [b] LMM peak – peak maximum in the low molecular mass protein region (10–30 kDa). [c] VLMM peak – peak maximum in the very low molecular mass protein region (<10 kDa).

Table 6 Heat-treatment induced changes in distribution profiles of Cd, Zn, and Cu (Fig. S5, ESI) and Mo and Se (Fig. S6, ESI) in cytosols of Vardar chub liver and gills, as observed after SEC_{75}-HPLC fractionation. The results are presented as a percentage decrease (−) or increase (+) of metal quantity eluted within individual peaks (in the table expressed as molecular masses (MM) of eluted proteins) of heat-treated cytosols, when compared to untreated cytosols

		Liver		Gills	
	Protein MM	Fish no. 2	Fish no. 5	Fish no. 2	Fish no. 5
Cd	137–38 kDa	ND	ND	−65%	−45%
	31–11 kDa	−2%	+0.5%	−31%	−20%
Zn	137–20 kDa	−80%	−81%	−76%	−81%
	31–11 kDa	−12%	−22%	ND	ND
Cu	31–9 kDa	−2%	−6%	NM	NM
Mo	137–31 kDa	−83%	−80%	ND	ND
	11–5 kDa	+146	+272%	+7%	+27%
Se	137–58 kDa	−82%	−84%	−82%	−62%
	47–13 kDa	−73%	−80%	−82%	−64%
	6–3 kDa	−13%	−27%	+1%	0.5%
	1.6–0.5 kDa	+2%	−11%	+6%	+6%

ND – peak not detected; NM – metal not measured.

of Mo eluted within the first MMM peak (31–137 kDa, Table 5), and only a small amount eluted within the second VLMM peak (5–11 kDa, Table 5). However, after the cytosol heat-treatment, the hepatic Mo-MMM peak was almost completely removed (decrease of ∼80%, Table 6), whereas the Mo-VLMM peak markedly increased (146–272%, Table 6), suggesting that a part of Mo has shifted from the MMM region to the VLMM biomolecule region. The same finding was previously reported for the Vardar chub liver, and hypothesized as possible decomposition of heat-sensitive Mo-MMM-proteins to smaller, heat-stable, Mo-binding biomolecules.[29] In contrast, both before and after the heat-treatment, gill Mo (Fig. S6e and g, ESI†) was eluted almost completely within the VLMM peak (5–11 kDa, Table 5) which coincided with the second peak of the hepatic cytosol, again confirming the results of our previous study[29] and proving the heat-stability of gill Mo-VLMM biomolecules. The gill Mo-VLMM peak also slightly increased after the heat-treatment, but only up to 30% (Table 6). Since a small part of Mo was eluted in the MMM protein region, and was removed after the heat-treatment (Fig. S6e and g, ESI†), it was possible that a portion of Mo also shifted from the MMM to VLMM region, as observed for the liver, thus explaining a slight increase of the VLMM peak.

In the untreated hepatic (Fig. S6b and d, ESI†) and gill cytosols (Fig. S6f and h, ESI†) of Vardar chub, Se eluted within one or two peaks in the MMM protein region (∼10–140 kDa, Table 5), as well as within two VLMM peaks (3–6 kDa and 0.5–1.6 kDa, Table 5). After the cytosol heat treatment, Se-MMM peaks of both organs were almost completely removed (decrease up to 80%, Table 6), confirming their heat-sensitivity. In contrast, Se-VLMM peaks exhibited evident heat-stability, with hepatic Se-VLMM peaks having slightly decreased (up to 27%, Table 6), and gill Se-VLMM peaks having remained almost unchanged (increase up to 6%, Table 6) after the heat-treatment. These results were consistent with our previous report on Se in Vardar chub organs, indicating partial Se binding to small, heat-stable compounds within the hepatic and gill cytosols of Vardar chub.[29]

Our further aim in this study was to more closely characterize the heat-stable biomolecules that bind Cd (probable MT fractions), as well as those that bind Mo in the liver and gills of Vardar chub. To achieve this goal, we have collected the fractions of interest obtained by the above described SEC_{75}-HPLC fractionation of heat-treated Vardar chub hepatic and gill cytosols, and then analyzed them *via* AEC-HPLC and MALDI-TOF-MS.

Analysis of hepatic MT fractions of Vardar chub (isolated by heat-treatment and SEC_{75}-HPLC) using AEC-HPLC and MALDI-TOF-MS

We have collected LMM fractions containing Cd, Cu and Zn, which were produced by the SEC_{75}-HPLC separation of heat-treated hepatic cytosols (t_e 21–25 min), and analysed them by AEC-HPLC. The distribution profiles of Cu, Zn and Cd obtained by the combination of AEC-HPLC separation and subsequent measurements of HR ICP-MS are presented in Fig. 4 for the samples of hepatic cytosols purified by the heat-treatment, and they confirmed the results already presented for the untreated hepatic cytosols (Fig. 3a and b) in the section Analysis of Vardar chub hepatic and gill LMM Cd-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC. As can be seen, in both analyzed fish Cd, Cu, and Zn were eluted within two peaks, the first one from 9.5 to 12.5 min (concentration of ammonium acetate as mobile phase B from 32.5 to 43.8 mM) and the second one from 12.5 to 15 min (concentration of ammonium acetate as mobile phase B from 43.8 to 62.5 mM) (Fig. 4a and b). This was a confirmation of the above discussed association of Cd, Cu and Zn to two different MT isoforms in the liver of Vardar chub, where the elution time of standard MT-I (9.5–11.5 min; Fig. 3e) coincided with the first Cd, Cu, and Zn peak and the elution time of standard MT-II (13–16 min; Fig. 3f) coincided with the second Cd, Cu, and Zn peak.

Furthermore, we have collected these two hepatic fractions obtained by AEC-HPLC separation, marked as the L-Cd1 fraction (t_e 9.5–12 min) and the L-Cd2 fraction (t_e 12.5–15 min) and analyzed them *via* MALDI-TOF-MS, to determine their exact molecular masses and define if there are observable differences in the masses of MT-I and MT-II isoforms of the Vardar chub liver. First, we have recorded mass spectra for each of the MT standards (Fig. 5a and b) and established that molecular masses of MT-I (6.2 kDa) and MT-II (6.1 kDa) differed by only 100 Da. Molecular masses of major peaks in L-Cd1 and L-Cd2 fractions were the same (6.0 kDa; Table 7; Fig. 5c and d) and only 100–200 Da lower than molecular masses of MT standards. Considering the accuracy of the MALDI-TOF-MS instrument, as well as the possibility of some differences in MT saturation or in the content of specific metals bound to MTs, both of which can result in mass differences of a few hundred Da,[72] our findings suggested that the mass of the two MT isoforms isolated from the Vardar chub liver corresponded to the masses of analyzed MT-I and MT-II standards. MT isoforms I and II commonly differ from one another very little considering their molecular masses.[73] Accordingly, rabbit liver standards MT-I and MT-II without Zn (Enzo, Switzerland), which we have used

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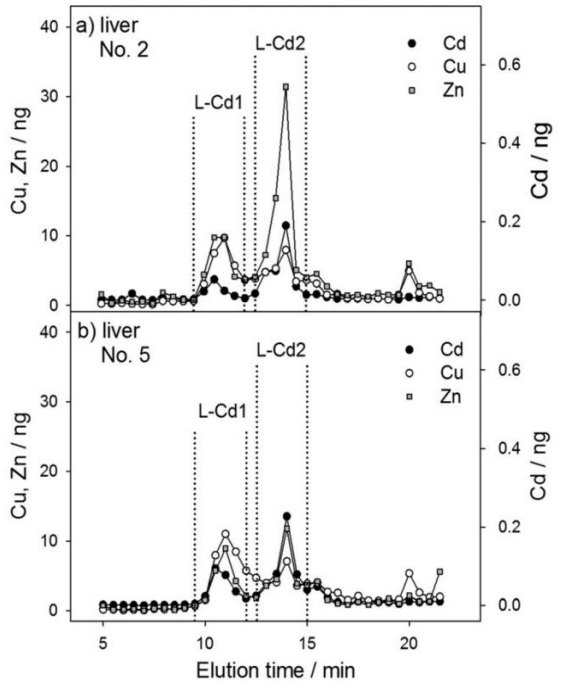

Fig. 4 AEC-HPLC analyses of hepatic LMM Cd-peaks, which were obtained by SEC_{75}-HPLC separations of heat-treated hepatic cytosols (t_e 21–25 min). The obtained AEC distribution profiles of Cd, Zn, and Cu in two samples of heat-treated hepatic cytosols (a and b) are presented in this figure as nanograms of metals eluted every half minute. The L-Cd1 peaks (t_e 9.5–12 min) and L-Cd2 peaks (t_e 12.5–15 min), which are marked by dotted lines, were further used for MS analyses.

in this study, were declared as both having the same molecular masses, amounting to 6.145 kDa, whereas their fully saturated forms (including seven Zn ions) have a mass of 6.603 kDa. Carpenè and Vašák[73] identified two MT isoforms (MT-1 and MT-2) in the liver of goldfish (*C. auratus*) by ion exchange chromatography and amino acid sequencing. The two isoforms differed in only one amino acid residue, with MT-1 containing 61 and MT-2 containing 62 residues.[73] So, it was evident that it is not possible to distinguish these two isoforms based solely on their masses, since the molecular masses of some MT isoforms are too close for successful separation *via* SEC-HPLC and mass spectrometry.[72] The application of AEC-HPLC as an intermediate step can be helpful in such cases. MT isoforms differ in amino acid composition other than cystein residues, which causes them to have different isoelectric points and different hydrophobicities,[74] and consequently their distinct separation by AEC-HPLC following SEC-HPLC can be expected. The two dimensional approach of MT separation *via* SEC-AEC-HPLC is often applied in various aquatic organisms, such as flatfish (*L. limanda*),[65] gibel carp (*C. auratus gibelio*),[2] eel (*A. anguilla*),[62] sea catfish (*N. barba*) and pearl cichlid (*G. brasiliensis*),[38] and molluscs.[75] Further similarity between the two MT isoforms from Vardar chub liver and two MT standards referred to the fact that in all four samples we have also detected smaller peaks probably corresponding to MT dimers, with the molecular masses equal to 12.0 kDa for the liver and 12.3–12.4 kDa for the standards. Concurrent presence of MT-monomers and MT-dimers was previously reported by Ivanković *et al.*[76] in both control and Cd-exposed mussels (*Mytilus galloprovincialis*).

In addition to the described MT peaks, in MALDI-TOF-MS spectra of L-Cd1 and L-Cd2 several other minor peaks were observed, as shown in Fig. 5c and d, which could present new molecular signatures that bind to Cd in fish liver. Minor peaks observed within the L-Cd1 fraction had molecular masses equal to 4.3 kDa, 4.4 kDa, 8.6 kDa and 9.3 kDa, whereas within the L-Cd2 fraction the following minor peaks were observed: 4.3 kDa, 4.4 kDa, 7.9 kDa, 8.2 kDa and 8.6 kDa. Since low resolution ion trap LC-MS/MS applied in this study could not reliably determine listed Cd-binding biomolecules, their investigation will be included in our future studies.

Analysis of gill MT fractions of Vardar chub (isolated by heat-treatment and SEC_{75}-HPLC) using MALDI-TOF-MS

To further characterize gill MTs, we have collected Cd-containing LMM fractions, which were produced by SEC_{75}-HPLC separation of heat-treated gill cytosols (t_e 21–25 min), and tried to analyse them by AEC-HPLC, the same as we have done for the liver. However, due to the low concentrations of analyzed metal and proteins in the samples, we were not able to obtain detectable results. Therefore, we have analyzed the LMM fractions collected after the SEC_{75}-HPLC separation (t_e 21–25 min) directly on MALDI-TOF-MS. The obtained spectrum presented in Fig. 5e showed three peaks, with a major peak at a mass of 4.9 kDa (Table 7). This peak did not seem as it referred to MTs. However, Maltez *et al.*[75] also found a major Cd-containing peak at a mass of 5.1 kDa in the snail *Marisa cornuarietis*. Although they initially inferred that this mass was too low if compared with already classified metallothionein-like proteins (MLPs), they eventually came to the conclusion, based on sequencing analysis, that the MLP isoform at a mass of 5.1 kDa could correspond to MT.[75] In our study, two smaller peaks were also obtained, one at 6.2 kDa which corresponded to masses of MT standards (6.1–6.2 kDa; Fig. 5a and b), and another at 3.1 kDa which probably corresponded to double charged MT molecules. Using such approach of MS analysis directly after the SEC-HPLC separation obviously has its disadvantages, as it did not provide the possibility to reliably discern two isoforms, whose presence was observed after the AEC-HPLC separation of SEC_{200}-HPLC fractions from untreated gill cytosols (Fig. 3c and d; Table 3) and discussed in the section Analysis of Vardar chub hepatic and gill LMM Cd-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC. However, our analyses did indicate that there were differences between MTs from the two organs. Our finding that gill MTs, unlike hepatic MTs, do not bind Zn, is consistent with the report by Noël-Lambot *et al.*[77] who claimed that gill MT of eels (*A. anguilla*), contrary to the liver, binds very small amounts of Zn and Cu. They even asserted that MT does not seem to be a normal constituent of the gills, but rather its synthesis in the gills is induced as a consequence of Cd exposure.[77] Van Campenhout *et al.*[78] found a similar type

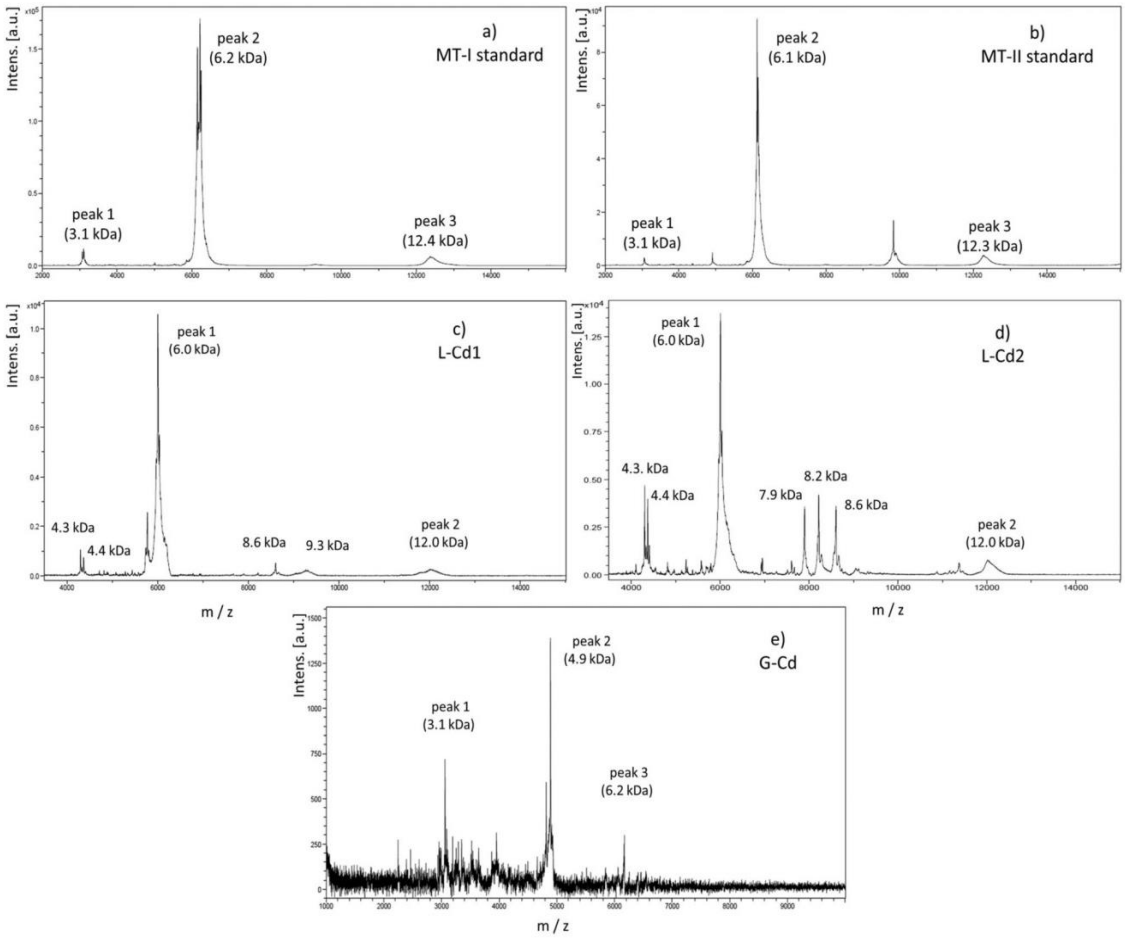

Fig. 5 Mass spectra obtained *via* MALDI-TOF-MS for the following samples: metallothionein standards MT-I (a) and MT-II (b); two heat stable LMM hepatic peaks, L-Cd1 (c) and L-Cd2 (d), which were separated by SEC_{75}-HPLC and AEC-HPLC from heat-treated hepatic cytosols of Vardar chub (the spectra are presented for fish no. 5); heat stable LMM Cd-containing peak, G-Cd (e), which was separated by SEC_{75}-HPLC from heat-treated gill cytosol of Vardar chub (the spectra are presented for fish no. 2).

of difference between the liver and kidneys of carp (*C. carpio*), with only 2% of cytosolic Zn bound to MTs in the kidneys, and over 30% in the liver. This difference between gill and hepatic MT was further confirmed by the fact that gills, in addition to a small amount of commonly found 6 kDa MT, contained a predominant MT isoform of lower molecular mass (~5 kDa).

Analysis of heat-stable Mo-binding biomolecules from hepatic and gill cytosols of Vardar chub (isolated by heat-treatment and SEC_{75}-HPLC) *via* MALDI-TOF-MS

Since there is no information available about heat-stable biomolecules that bind Mo, or about Mo-binding biomolecules of fish in general, it was interesting to further characterize such molecules in the hepatic and gill cytosols of Vardar chub. As stated in the literature, molecular characterization of Mo transporters, storage proteins and chaperones in fish, as well as genomic and proteomic studies concerning Mo-exposed fish have not yet been carried out.[79] Thus, we have collected Mo-containing VLMM fractions, which were produced by SEC_{75}-HPLC separation of heat-treated hepatic and gill cytosols (t_e 26–31 min), and tried to analyse and purify them by AEC-HPLC. The same as happened in the case of the gill MT, also happened during analyses of Mo fractions, *i.e.* we were not able to obtain detectable results due to the low concentrations of the analyzed metal and proteins in the samples of both liver and gills. Therefore, we have also analyzed the effect of Mo-containing VLMM fractions collected after SEC_{75}-HPLC separation (t_e 26–31 min) directly on MALDI-TOF-MS. This fraction, however, was partly overlapping with the minor Se peak (Fig. S6b, d, f and h, ESI;† Table 5), and our initial aim was to separate Mo-binding biomolecules from the traces of Se-binding biomolecules using AEC-HPLC. Since this was not successfully done, we could expect to find some minor peaks referring to small Se-binding biomolecules in the MALDI spectra. And, indeed, in the recorded spectra we have found a few unresolved small peaks (in the liver: 6.9 kDa and 9.6 kDa; Fig. 6a) or even

Table 7 MALDI-TOF determination of molecular masses of the following biomolecules: (1) heat-stable LMM Cd, Zn, Cu-binding biomolecules (L-Cd1 and L-Cd2, Fig. 5c and d) obtained from heat-treated hepatic cytosols of Vardar chub separated by SEC_{75}-HPLC (t_e 21–25 min) and AEC-HPLC (t_e 9.5–12 min and 12.5–15 min, respectively); (2) heat-stable LMM Cd-binding biomolecules (G-Cd, Fig. 5e) obtained from heat-treated gill cytosols of Vardar chub separated by SEC_{75}-HPLC (t_e 21–25 min); (3) heat-stable VLMM Mo-binding biomolecules (Fig. 6a and b) obtained from heat-treated hepatic and gill cytosols of Vardar chub separated by SEC_{75}-HPLC (t_e 26–31 min)

	L-Cd1 (AEC)		L-Cd2 (AEC)		G-Cd (SEC_{75})		Mo (SEC_{75})	
Sample	MM (kDa) according to SEC_{75}	*m*/*z* (kDa) according to MALDI-TOF	MM (kDa) according to SEC_{75}	*m*/*z* (kDa) according to MALDI-TOF	MM (kDa) according to SEC_{75}	*m*/*z* (kDa) according to MALDI-TOF	MM (kDa) according to SEC_{75}	*m*/*z* (kDa) according to MALDI-TOF
Liver no. 2	11–31	**Peak 1 : 6.0** Peak 2 : 12.0	11–31	**Peak 1 : 6.0** Peak 2 : 12.0	—	—	5–11	Peak 1 : 4.2 **Peak 2 : 8.5**
Liver no. 5	11–31	**Peak 1 : 6.0** Peak 2 : 12.0	11–31	**Peak 1 : 6.0** Peak 2 : 12.0	—	—	5–11	Peak 1 : 4.4 **Peak 2 : 8.5**
Gills no. 2	—	—	—	—	11–31	Peak 1 : 3.1 **Peak 2 : 4.9** Peak 3 : 6.2	4.5–11	**Peak 1 : 3.3** Peak 2 : 8.4
Gills no. 5	—	—	—	—	11–31	Peak 1 : 3.0 **Peak 2 : 4.9** Peak 3 : 6.2	4.5–11	**Peak 1 : 3.3** Peak 2 : 8.5

background noise (in the gills; Fig. 6b) which could possibly be associated to either Mo or Se (Fig. 6). The obtained MALDI spectra presented in Fig. 6a and b showed the presence of two clear peaks in both the organs. However, the liver major peak was observed at mass of 8.5 kDa, whereas another, much lower peak was obtained at a mass of 4.2–4.4 kDa, probably referring to double charged species (Table 7). In the gills, on the other hand, the major peak was observed at a mass of 3.3 kDa, whereas a smaller peak was recorded at a mass of 8.5 kDa, which corresponded to the mass of the major hepatic peak (Table 7). This could be associated with the results of SEC_{75}-HPLC distribution (Fig. S6a, c, e and g, ESI;† Table 5), where evident differences were seen between two organs. Thus, it is possible that the biomolecule with a mass of ~8 kDa, which was predominant in the liver, and only minor in the gills, refers to Mo-binding species which appeared after the heat-treatment of the cytosols, by the degradation of MMM heat-sensitive biomolecules (~30–130 kDa). In contrast, a smaller biomolecule at ~3 kDa possibly refers to heat-stable Mo-binding species which was initially present in the cytosol, even before the heat-treatment, and which was predominat in the gills and negligible in the liver. Since LC-MS/MS analysis with a subsequent Mascot search did not result in definite recognition of two Mo-binding biomolecules, due to the use of a low resolution ion trap mass spectrometer, we are currently performing sequencing with the aim of their final characterization.

Conclusions

Application of two-dimensional fractionation of cytosolic biomolecules from Vardar chub liver and gills by a combination of SEC-HPLC and AEC-HPLC, followed by analyses of isolated fractions by two mass spectrometry techniques, MALDI-TOF-MS and LC-MS/MS, with an aim to better characterize and identify several selected metal-binding biomolecules, resulted in the following findings: (1) MMM Fe-binding biomolecules from Vardar chub liver and gills, defined by SEC-HPLC to have molecular masses of ~20–50 kDa, were identified as hemoglobin subunit β in the liver, with the molecular masses of ~15 kDa according to MS, and hemoglobin subunits α and β in the gills, with the molecular masses of ~11 kDa, ~13 kDa and ~15 kDa according to MS; (2) AEC-HPLC separation of LMM Cd-binding biomolecules from both hepatic and gill cytosols of Vardar chub indicated Cd binding to two MT isoforms, MT-I and MT-II, in both studied organs; (3) heat-stable hepatic LMM Cd-binding biomolecules, defined by SEC-HPLC to have molecular masses of ~10–30 kDa, were identified as MTs of

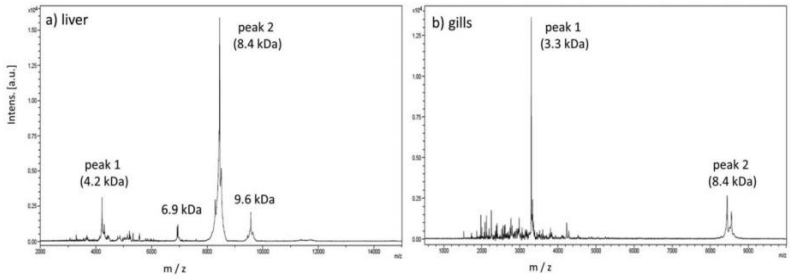

Fig. 6 Mass spectra obtained *via* MALDI-TOF-MS for heat-stable VLMM Mo-binding biomolecules, which were separated by SEC_{75}-HPLC from heat-treated hepatic (a) and gill (b) cytosols of Vardar chub (the spectra are presented for fish no. 2).

molecular masses equal to 6.0 kDa, according to MS, whereas gill LMM Cd-binding biomolecules were identified as MTs of molecular masses equal to 4.9 kDa, according to MS; (4) heat-stable VLMM Mo-binding biomolecules from the Vardar chub liver and gills, defined by SEC-HPLC to have molecular masses of 5–11 kDa, were identified as biomolecules of molecular masses equal to 3.3 kDa and 8.5 kDa, according to MS, with a smaller biomolecule being predominat in the gills and the larger one in the liver. Our study, thus, revealed the differences between the two studied organs regarding the characteristics of metal-binding biomolecules (different molecular masses of hemoglobin and MTs in the liver and gills) and regarding their prevalence (in the case of Mo). In addition, it was found that gill MT, unlike the MT hepatic isoform, does not bind Zn, indicating the possibility of different MT functions in these two organs. The need for simultaneous application of several analytical techniques in the research of cellular metal-binding biomolecules should be emphasized, to enable adequate separation and characterization of the studied biomolecules. SEC-HPLC, which can separate the biomolecules according to their masses, is not able to separate isoforms which differ in charge, whereas the masses recorded using this technique are in general higher than the masses obtained by MS analyses. Therefore, the use of multidimensional separations, as well as subsequent application of MS, as a method of higher sensitivity and accuracy in mass determination, is crucial for more reliable characterization of metal-binding proteins with the purpose of better understanding the metal behaviour within the cells.

Live subject statement

The research presented in this paper complied with the Croatian legislation (Ministry of Agriculture, Directive on protection of animals used for scientific purposes, Official Gazette 55/2013) and the EU legislation (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, 276/33) relevant for animal use for scientific purposes. The laboratory for Biological Effects of Metals, where the study was performed, is registered for killing the fish and work on the isolated organs, tissues and carcases of the animals that are killed for that purpose at the Administration for Veterinary Medicine and Food Safety of the Ministry of Agriculture (No. of the decision HR-POK-025).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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RASPRAVA

4. RASPRAVA

Metali predstavljaju važnu sastavnicu svakog vodenog okoliša te o njihovoj prisutnosti često ovisi i bioraznolikost vodenih ekosustava. Onečišćenje voda metalima je u porastu zbog tehnološkog napretka ljudske zajednice, pri čemu najznačajniju ulogu imaju industrija, rudarstvo, napredna poljoprivreda, otpad iz domaćinstava i promet. Zbog toksičnosti metala, njihovog trajnog zadržavanja u okolišu i mogućnosti akumulacije u tkivima vodenih organizama, onečišćenje vodenih ekosustava metalima može predstavljati ozbiljan problem (Harte i sur., 1991.). Za razliku od organskih zagađivala, metali se ne mogu kemijski niti biološki razgraditi. Stoga jednom uneseni u vodotoke mogu promijeniti kemijski oblik te postati manje ili više toksični, ali se ne mogu ukloniti iz biogeokemijskog kruženja (Sadiq, 1992.). Kao posljedica može doći do razvoja kroničnih oboljenja vodenih organizama te se poremećaji mogu pojaviti čak i na razini populacije (Holcombe i sur., 1976.). Neki metali u živim organizmima imaju važnu biološku ulogu (npr., Cu, Fe, Zn) te su nazvani esencijalnim metalima, a sastavni su dio enzima i drugih proteina, takozvanih metaloproteina (Smith i sur., 1997.). Metaloproteini su uključeni u transport elektrona i metala, skladištenje kisika, hidrolize kemijskih veza, redoks procese i sinteze bioloških spojeva (Gellein i sur., 2007.). Za niz drugih metala (npr., Cd, Pb) funkcije u živim organizmima nisu poznate te su stoga nazvani neesencijalnim metalima. Štetni utjecaj metala nastupa pri unosu neesencijalnih metala već u vrlo niskim količinama ili esencijalnih metala u prekomjernim količinama koje mogu narušiti homeostazu organizma. Na taj način može nastati štetno djelovanje na različitim organizacijskim razinama, počevši od stanice, preko pojedinačnih organizama pa sve do populacije, što napokon može štetno utjecati i na zdravlje ljudi koji vodene organizme koriste u prehrani (Livingstone, 1993.).

Za ovo istraživanje kao bioindikatorski organizmi izabrane su dvije vrste riba (klen, *S. cephalus* Linnaeus, 1758., i vardarski klen, *S. vardarensis* Karaman, 1928.). Dosadašnja istraživanja na ovim bioindikatorskim organizmima provedena u svrhu procjene izloženosti metalima bila su utemeljena na određivanju ukupnih ili citosolskih koncentracija metala bioakumuliranih u njihovim organima (npr., Casiot i sur., 2009.; Dragun i sur., 2007.; 2009.; 2012.; 2013.a; 2013.b; 2016.; 2019.; Duman i Kar, 2012.; Podrug i sur., 2009.; Rašković i sur., 2018.). Metali akumulirani u organima riba raspodijeljeni su u različite unutarstanične odjeljke (npr., citosol, granule, organele, stanične membrane). Pretpostavlja se da toksičnost metala za vodene organizme potječe od reakcija u citosolu, putem njihovog nespecifičnog

vezanja na fiziološki važne molekule te posljedične inaktivacije tih biomolekula (Mason i Jenkins, 1995.). Stoga, ukupne i citosolske koncentracije metala ne daju potpunu informaciju o metabolizmu, bioraspoloživosti te mogućim toksičnim učincima metala, kao ni o njihovoj detoksikaciji (de la Calle Guntińas i sur., 2002.). Svi navedeni procesi na staničnoj i molekularnoj razini ovise o vezanju metala na različite stanične biomolekule, a te biomolekule, kao ni biološka funkcija i mehanizmi toksičnosti mnogih metala u vodenim organizmima, još uvijek nisu dovoljno istraženi. Tek je nekoliko biomolekula na koje se metali vežu u stanicama vodenih organizama dosad prepoznato i iscrpno opisano npr., metalotioneini (MT) (Cd, Cu, Zn, Rodriguez-Cea i sur., 2003.; Goenaga Infante i sur., 2003.; Van Campenhout i sur., 2008.; Li i sur., 2018.), transferin (Fe, Neves i sur, 2009.; Mn, Schäfer, 2004.), hemoglobin (Fe, Fago i sur., 2002), peptidi koji vežu Se (Yamashita i Yamashita, 2010.). Stoga je cilj ovoga rada bio primijeniti moderne analitičke pristupe u opisivanju i prepoznavanju odabranih biomolekula koje vežu metale u jetrima i škrgama dviju vrsta klena, što će dalje u raspravi biti iscrpno izneseno.

4.1. Raspodjela odabranih esencijalnih (Co, Cu, Fe, Mn, Mo, Se, Zn) i neesencijalnih (Cd, Pb) elemenata među citosolskim biomolekulama različitih molekulskih masa u jetrima i škrgama klena (*S. cephalus*) iz rijeke Sutle

U ovome smo dijelu istraživanja opisali temeljne raspodjele devet odabranih metala/nemetala među citosolskim biomolekulama različitih molekulskih masa u jetrima i škrgama klenova (*S. cephalus*), odnosno utvrdili smo raspone molekulskih masa citosolskih biomolekula koje vežu esencijalne elemente Co, Cu, Fe, Mn, Mo, Se i Zn te neesencijalne elemente Cd i Pb u uvjetima niske izloženosti u okolišu. Nadalje, opisali smo i promjene raspodjela koje se javljaju pri povišenoj bioakumulaciji navedenih elemenata. Rezultati ovog dijela istraživanja objavljeni su u radovima pod rednim brojem 1 i 2 u popisu znanstvenih radova (str. 35-56).

Kao ciljne organe za ove analize odabrali smo jetra klena, jer odražavaju dugoročno pohranjivanje metala, te škrge, jer odražavaju koncentracije metala u vodi u kojoj ribe prebivaju (Romeo i sur., 1999.). Kako su ribe migratorni organizmi koji ne obitavaju samo na jednom području rijeke, prednost je škrga kao ciljnog organa što odražavaju trenutnu izloženost metalima u vodi u kojoj se ribe nalaze, dok se u jetrima kao glavnom detoksikacijskom organu može uočiti utjecaj dugotrajne izloženosti, koja može biti vezana i uz neki drugi dio riječnog toka, a ne isključivo uz onaj u kojemu je riba ulovljena (Giguère i

sur., 2004.; Romeo i sur., 1999.). Prema nekim se istraživanjima u škrgama uslijed izravnog dodira s vodom može očekivati brzi odgovor na promjene u razini izloženosti metalima, poput Cd (Barišić i sur., 2015.; Kraemer i sur., 2005.). U škrgama se očekuje niža razina bioakumulacije za većinu elemenata u odnosu na jetra, budući da se akumulirani metali iz škrga krvlju prenose u jetra i druge organe (Souza i sur., 2013.).

Klenovi korišteni u ovome istraživanju uzorkovani su u rijeci Sutli u Hrvatskoj u rujnu 2009. godine. Koncentracije otopljenih metala u vodi rijeke Sutle u jesen 2009. godine bile su većinom usporedive s nezagađenim vodotocima ili umjereno povišene (Co, 0,06-0,42 μg L^{-1}; Cu, 0,17-3,74 μg L^{-1}; Fe, 3,1- 80,5 μg L^{-1}; Mn, 0,4-261,1 μg L^{-1}; Mo, 0,5-20,1 μg L^{-1}; Zn, <5,0 μg L^{-1}; Cd, 0,01-0,31 μg L^{-1}; Pb, ≤1,18 μg L^{-1}; Dragun i sur., 2011.). Izuzetak su predstavljale uočljivo povišene koncentracije otopljenog Fe, Mn, Cd i Pb na pojedinim ograničenim područjima ove rijeke, koje su omogućile praćenje promjena citosolskih raspodjela tih metala u jetrima i škrgama klena pri povišenoj izloženosti u riječnoj vodi.

Prema našim saznanjima, rezultati dobiveni u ovome istraživanju prvi su podaci o citosolskim raspodjelama devet odabranih elemenata u jetrima i škrgama klena, kao bionidikatorskog organizma. Uobičajeni prvi korak u prepoznavanju biomolekula koje vežu metale predstavlja frakcioniranje citosola primjenom tekućinske kromatografije visoke djelotvornosti s isključenjem po veličini (SEC-HPLC) te određivanje koncentracija metala i nemetala u frakcijama koje sadrže biomolekule određenih molekulskih masa primjenom masenog spektrometra s induktivno spregnutom plazmom (ICP-MS) (Michalke i Schramel, 2004.; Montes-Bayón i sur., 2003.). Na taj se način može doći do informacija o profilima raspodjele pojedinih elemenata među citosolskim biomolekulama različitih molekulskih masa, što predstavlja početnu točku u identifikaciji tih biomolekula. Radi jednostavnijeg opisivanja profila raspodjele dobivenih primjenom SEC_{200}-HPLC-a (kolona Superdex 200, raspon razdvajanja 10-600 kDa) i ICP-MS-a visoke rezolucije (HR), definirali smo četiri glavne skupine biomolekula koje vežu metale, uzimajući u obzir njihovu molekulsku masu:

1. VMM ili biomolekule visoke molekulske mase (>100 kDa);
2. SMM ili biomolekule srednje molekulske mase (30-100 kDa);
3. NMM ili biomolekule niske molekulske mase (10-30 kDa);
4. JNMM ili biomolekule jako niske molekulske mase (<10 kDa).

Među analiziranim elementima, esencijalni metali Co, Mo, Mn i Zn imali su najuži raspon citosolskih koncentracija u jetrima i škrgama klena. U usporedbi s prethodnim istraživanjima i uzimajući u obzir nisku izloženost klenova ovim metalima u rijeci Sutli,

citosolske koncentracije Co (jetra: 2,1-5,1 ng mL^{-1}; škrge: 1,02-1,60 ng mL^{-1}), Mo (jetra: 8,7-38,5 ng mL^{-1}; škrge: 0,56-8,41 ng mL^{-1}), Mn (jetra: 73,5-252,8 ng mL^{-1}; škrge: 33,7-69,1 ng mL^{-1}) i Zn (jetra: 3,0-11,1 μg mL^{-1}; škrge: 9,6-21,0 μg mL^{-1}) u jetrima i škrgama klenova bilo je moguće definirati kao bazalne razine (Dragun i sur., 2013.a; 2013.b; 2016.; Podrug i sur., 2009.). Stoga je za navedena četiri metala bilo moguće utvrditi samo temeljnu raspodjelu među biomolekulama različitih molekulskih masa, karakterističnu za uvjete niske izloženosti metalima u riječnoj vodi.

Kobalt je esencijalni element za ribe i druge organizme, a ima važnu ulogu u izgradnji kobalamina (vitamina B12), u kojemu čini 4,5% molekulske mase (Blust, 2012.). Kobalt u suvišku može djelovati toksično na ribe, jer kationi kobalta (Co^{2+}) ometanjem transporta Ca mogu ometati normalnu funkciju škrga, uključujući ionsku regulaciju, održavanje acidobazne ravnoteže, prijenos plinova te izlučivanje suvišnog dušika (Hille, 1992.; Richards i Playle, 1998.). Kobalt nadalje može sudjelovati u procesima stvaranja slobodnih radikala, što rezultira proizvodnjom reaktivnih vrsta kisika (Battaglia i sur., 2009.; Olivieri i sur., 2001.; Wang i sur., 1993.). No, Co je ipak manje toksičan za ribe u usporedbi s toksičnim učincima drugih metalnih iona (Kubrak i sur., 2011.; Marr i sur., 1998.). Niska citosolska koncentracija Co u jetrima mogla bi se objasniti njegovim većim afinitetom za akumulaciju u crijevima i bubrezima nego u jetrima pri umjerenoj izloženosti, što je uočeno u istraživanjima na različitim vrstama riba (Baudin i Fritsch, 1989.), dok uloga jetre postaje važnija tek nakon povećane izloženosti kobaltu (Mukherjee i Kaviraj, 2009.). Profili raspodjele Co među citosolskim biomolekulama u jetrima i škrgama klena ukazali su na značajnu prisutnost ovoga metala u području VMM biomolekula (jetra: 61-407 kDa; škrge: 20-310 kDa), dok je dio Co bio raspodijeljen i između dva JNMM područja citosolskih biomolekula (maksimumi na ~4 kDa i 1-2 kDa), pri čemu drugo područje odgovara molekulskoj masi kobalamina (1.3 kDa; Kirschbaum, 1981.). Raspodjela Co između triju navedenih molekulskih područja u jetrima klena bila je jasnija, a pikovi su bili viši, uži i oštriji u odnosu na škrge, kao posljedica ~2-3 puta viših citosolskih koncentracija Co u jetrima nego u škrgama. Nadalje, prisutnost Co u jetrima bila je dominantno vezana uz VMM biomolekule, dok je u škrgama bila usporediva u svim trima skupinama biomolekula. Iako je najpoznatija uloga Co vezana uz izgradnju strukture kobalamina, profili raspodjele Co u jetrima klena ukazuju na značajnu ulogu te potrebu daljnjeg istraživanja VMM biomolekula koje vežu Co.

Molibden je važan esencijalni mikronutrijent koji čini katalitički centar više od pedeset enzima (Ricketts, 2009.) te služi kao kofaktor za najmanje sedam enzima (Beers i

Berkow, 1998.). Reid (2002.) je uočio da se Mo, za razliku od Co, akumulira u jetrima riba neovisno o dozi te da ne postoji poznati homeostatski kontrolni sustav za Mo u ribama unatoč činjenici da je riječ o bitnom mikronutrijentu. Niske citosolske koncentracije Mo u jetrima i škrgama klena stoga mogu biti objašenjene otkrićem Anana i sur. (2002.), koji su u istraživanjima na kornjačama također izmjerili niske koncentracije Mo u citosolu uslijed specifične akumulacije Mo u jezgri i mitohondrijima. Zbog naročito niskih citosolskih koncentracija Mo u škrgama (0,56-8,41 ng mL^{-1}; Dragun i sur., 2016), profile raspodjele bilo je moguće odrediti samo za jetra klena. Najveći dio Mo u jetrima bio je eluiran u području VMM biomolekula (62-407 kDa), koje obuhvaćaju poznate enzime kojima je Mo kofaktor, poput aldehid oksidaze, ~130 kDa (Uchida i sur., 2003.), sulfit oksidaze, ~120 kDa (Johnson i Rajagopalan, 1976.) te Fe-Mo flavoprotein ksantin oksidaze, 275 kDa (Truglio i sur., 2002.). Manji dio Mo bio je eluiran u području JNMM (maksimum na 7,1 kDa). Usporedbom sa standardom MT (MM eluiranja nakon SEC-HPLC razdvajanja: 12,5 kDa) utvrdili smo da se Mo u jetrima klena ne veže na MT pri izloženosti molibdenu u riječnoj vodi do 20 μg L^{-1}. Naši su rezultati bili u skladu s tvrdnjama Rickettsa (2009.) i Reida (2011.) da MT nije uključen u detoksikaciju molibdena kod potočne pastrve (*Salmo trutta*) i kalifornijske pastrve (*Oncorhynchus mykiss*), čak ni pri izloženosti do 1000 mg L^{-1}.

Mangan u živim organizmima ima esencijalnu ulogu u aktivnosti raznih enzima, poput Mn-superoksid dismutaze, piruvat karboksilaze, jetrene arginaze, glutamin sintetaze i oksalacetat dekarboksilaze te ga možemo naći i u proteinima poput β-globulina i albumina (Fridovich i Freeman, 1986.; Garcia i sur., 2006.; Libor i sur., 1979.; Mogobe i sur., 2015.; Singh i Singh 1990.). Niske koncentracije Mn u citosolima jetara i škrga klena unatoč povremeno visokoj izloženosti u riječnoj vodi moguće je objasniti postojanjem homeostatske kontrole, ali i, slično Mo, većom zastupljenošću ovoga metala u mitohondrijima nego u citosolu. Dva najvažnija manganova metaloenzima kod sisavaca, Mn-superoksid dismutaza, jedan od najvažnijih unutarstaničnih antioksidacijskih enzima, te piruvat karboksilaza, lokalizirana su u mitohondrijima. Profili raspodjele Mn u jetrima ukazuju na njegovu prisutnost u tri biomolekulska područja, koja pokrivaju široko područje molekulskih masa (7-400 kDa): VMM biomolekule (62-407 kDa), SMM biomolekule (36-62 kDa) te NMM biomolekule (7-36 kDa). Budući da su koncentracije Mn u citosolu škrga bile čak do 8 puta niže u usporedbi s koncentracijama Mn u citosolu jetara, pikovi Mn u škrgama bili su slabije razlučeni te je Mn bio raspodijeljen unutar istog području molekulskih masa kao i u jetrima, ali u samo dva biomolekulska područja. Prvo područje pokriva VMM i SMM biomolekule

(35-310 kDa), a drugo odgovara NMM piku jetara (2-35 kDa). Molekulske mase biomolekula s kojima je eluiran najveći dio mangana (VMM i SMM) obuhvaćaju i molekulske mase enzima arginaze (100 kDa; Wolf i sur., 2007.) i Mn-superoksid dismutaze (~88-95 kDa; Fridovich i Freeman, 1986.), kao i molekulske mase poznatih transportnih proteina, albumina (66 kDa) i transferina (80 kDa; Martin-Antonio i sur., 2009.). Albumin sudjeluje u transportu Mn iz probavnog sustava u jetra, dok transferin veže Mn u jetrima te u tom obliku predstavlja izvor Mn za prijenos u druga tkiva (Schäfer, 2004.).

Cink je esencijalni element za sve stanice svih poznatih organizama te je nakon Fe drugi najzastupljeniji element u tragovima u većini kralježnjaka (Vallee, 1986.). Neophodan je za različite osnovne biološke procese, uključujući metabolizam proteina, nukleinskih kiselina, ugljikohidrata i lipida, a također je uključen i u rad imunološkog sustava, neurotransmisiju i staničnu signalizaciju (Beyersmann, 2002.; Coleman, 1992.; Murakami i Hirano, 2008.). Sastavni je dio enzima koji kataliziraju više od 50 različitih biokemijskih reakcija te je sastavni dio i proteina uključenih u ekspresiju gena (Hogstrand i sur, 1991.; de la Calle Guntiñas i sur., 2002.). Visoke koncentracije cinka u stanicama mogu izazvati toksičnost za vodene organizme, zbog čega je Agencija za zaštitu okoliša SAD-a uključila Zn u popis prioritetnih zagađivala (USEPA, 2002.). Međutim, ribe su sposobne regulirati akumulaciju Zn i održavati ga u uskim koncentracijskim rasponima čak i pri vrlo viskoj izloženosti u okolišu (Watanabe i sur., 1997). Istraživanje na jetrima grgeča (*Perca fluviatilis*) (Hogstrand i sur. 1991.) pokazalo je da stostruko povećanje ukupne koncentracije Zn u vodi dovodi do samo 20% povišene akumulacije Zn u jetrima grgeča (Hogstrand i sur., 1991.). Kako su koncentracije otopljenog Zn u vodi rijeke Sutle bile vrlo niske (<5 μg L^{-1}; Dragun i sur., 2011.), bilo je očekivano da će koncentracije citosolskog Zn u jetrima i škrgama klena biti održane unutar bazalnog raspona. Raspodjela Zn u jetrima i škrgama klenova karakterizirana je slabom razlučivošću pikova koji pokrivaju širok raspon molekulskih masa (~10 do >600 kDa), što ukazuje na vezanje Zn na veliki broj staničnih biomolekula. Takav rezultat je u skladu sa strukturnim i katalitičkim ulogama Zn u brojnim proteinima i enzimima. Potvrđeno je kako kod ljudi više od 3.000 proteina veže Zn, što predstavlja 10% cjelokupnog ljudskog genoma, dok je kod riba utvrđeno da 10% svih gena u sekvenciranom genomu nosi oznaku vezanja Zn (Andreini i sur., 2006.; Passerini i sur., 2007.). Profili raspodjele Zn u jetrima klena obuhvaćali su, dakle, tri glavna pika: VMM (~400 do >600 kDa), SMM (~35-400 kDa) i NMM (~9-27). Profili raspodjele Zn u škrgama klena obuhvaćali su četiri glavna pika: VMM1 (~400 do >600 kDa), VMM2 (10-310 kDa), JNMM1

(1-5) i JNMM2 (<1). Molekulske mase VMM i SMM biomolekula koje vežu Zn u jetrima i škrgama klena obuhvaćaju, na primjer, molekulske mase enzima alkohol dehidrogenaze (150 kDa), transportnog proteina albumina (66 kDa), Cu-Zn superoksid dismutaze (32,5 kDa) i ugljične anhidraze (29 kDa) (Sanz-Medel i sur., 2003.; Szpunar i Lobinski, 1999.). U jetrima je zabilježeno i eluiranje Zn koje se podudara s vremenom eluiranja MT, što je u skladu sa značajnom ulogom MT, proteina niskih molekulskih masa bogatih cisteinom, u detoksikaciji toksičnih metala, poput Cd, i održavanju homeostaze esencijalnih metala, poput Zn i Cu (Coyle i sur., 2002.; Huang i sur., 2004.). U škrgama jasno razlučeni pik cinka u području eluiranja MT nije bio zabilježen, ali je uočeno eluiranje Zn u JNMM području biomolekula (<5 kDa), i to samo u uzorcima škrga s višim citosolskom koncentracijama cinka.

Za razliku od esencijalnih metala Co, Mo, Mn i Zn, preostala tri analizirana esencijalna elementa, metali Fe i Cu te nemetal Se, imali su širi raspon citosolskih koncentracija u jetrima klena, dok su u škrgama bili prisutni u relativno uskom koncentracijskom području: Fe (jetra: 2,3-16,8 μg mL^{-1}; škrge: 3,27-5,50 μg mL^{-1}), Cu (jetra: 0,45-3,87 μg mL^{-1}; škrge: 40,7-76,0 ng mL^{-1}) i Se (jetra: 25,6-229,2 ng mL^{-1}; škrge: 59,6-147,2 ng mL^{-1}). Ovakvi rezultati ukazuju na pojačanu akumulaciju navedenih elemenata u jetrima klena, dijelom zbog povećane izloženosti (na primjer, Fe), a dijelom zbog manje izražene regulacije unosa i pohranjivanja. Zbog širokog koncentracijskog raspona navedena tri elementa u jetrima klena, bilo je moguće osim opisivanja temeljnih profila raspodjele metala/nemetala među biomolekulama različitih molekulskih masa zabilježiti i promjene u njihovim raspodjelama nastale uslijed povišene akumulacije u jetrima pri umjereno povišenoj izloženosti u riječnoj vodi. Usporedno će biti opisane i temeljne raspodjele Fe, Cu i Se u škrgama klena.

Željezo je metal esencijalan za život, jer ima mnogobrojne uloge u fiziološkim funkcijama živih organizama. Sastavni je dio metaloproteina hemoglobina koji sudjeluje u prijenosu kisika krvlju te citokrom oksidaze koja sudjeluje u mitohondrijskom prijenosu elektrona (Bury i sur., 2012.). Također ima važnu ulogu u sintezi DNA (Bury i sur., 2012.) te u obrani od bakterijskih infekcija (Vidal i sur., 1993.). Temeljni profili raspodjele Fe među citosolskim biomolekulama, kako u jetrima, tako i u škrgama klena, ukazali su na prisutnost željeza u dva područja citosolskih biomolekula: VMM (~150-700 kDa) i SMM (~10-80 kDa). Opravdano je pretpostaviti kako VMM biomolekule koje vežu Fe (maksimum na ~400 kDa) odgovaraju proteinu feritinu (450 kDa), koji je predominantno prisutan u jetrenom tkivu, a služi za pohranu te održavanje Fe u citoplazmi u topljivom, bioraspoloživom i netoksičnom

obliku (Martin-Antonio i sur., 2009.; Szpunar i Lobinski, 1999.). Prisutnost Fe u području SMM biomolekula vjerojatno je rezultat vezanja Fe na poznate proteine raznih funkcija, poput krvnog proteina hemoglobina (65 kDa), enzima katalaze (60 kDa) (Martin-Antonio i sur., 2009.) ili transportnog proteina mioglobina (17 kDa) (Wolf i sur., 2007.). Razlika između jetara i škrga prvenstveno se očitovala u povišenoj prisutnosti Fe u feritinskom piku u jetrima, dok je u škrgama Fe dominantno prisutno u području SMM biomolekula. Nadalje, u jetrima je povišenje citosolskih koncentracija Fe bilo praćeno porastom feritinskog pika, što ukazuje na značajnu ulogu jetre, ali ne i škrga, u skladištenju Fe. Neves i sur. (2009.) su također potvrdili jaču ekspresiju feritina u jetrima, kao glavnom organu za pohranu Fe, u usporedbi s mozgom brancina (*Dicentrarchus labrax*).

Bakar je neophodan za normalno funkcioniranje svih stanica, a važan je sastavni dio proteina i enzima (Mumtaz, 2002.). Esencijalni je element za sve aerobne organizme budući da se njegov redoks potencijal koristi pomoću mitohondrijske citokrom c oksidaze u staničnom disanju (Solomon i Lowery, 1993.). Neophodan je i za niz drugih metaboličkih procesa (npr., stvaranje vezivnog tkiva, stvaranje i održavanje mijelina, uklanjanje slobodnih radikala superoksida) (Gaetke i sur., 2014.), jer ima važnu ulogu kao kofaktor brojnih enzima i metaloproteina (Hauser-Davis i sur., 2012.). Glavni organ za pohranjivanje i održavanje homeostaze Cu su jetra, gdje se sintetizira protein koji sadrži Cu, ceruloplazmin, koji se izlučuje u krv te predstavlja izvor Cu za sve ostale organe (Harris, 2000.). Profili raspodjele Cu među citosolskim biomolekulama u jetrima klena ukazali su na njegovu najizraženiju prisutnost u području NMM biomolekula (maksimum na 16 kDa), što odgovara standardu MT, pa se može pretpostaviti da se u citosolu jetara Cu dominantno veže na taj protein. Naši su rezultati pokazali da u citosolu jetara klena MT ima dominantnu ulogu u vezanju, a tako i detoksikaciji Cu. To je potvrđeno i porastom prisutnosti Cu u najvećoj mjeri u području MT pri povišenju citosolskih koncentracija Cu u jetrima. Manja količina Cu u jetrima bila je raspodijeljena i unutar SMM biomolekulskog područja (27-62 kDa), što može ukazivati na vezanje Cu na poznate biomolekule raznih funkcija, poput superoksid dismutaze (32 kDa, Richardson i sur., 1975.) ili ugljične anhidraze (29 kDa). Sanchez i sur. (2005.) smatrali su kako vezanje Cu na biomolekule superoksid dismutaze ukazuje na bitnu ulogu Cu u zaštiti od oksidacijskog stresa, dok su Vutukuru i sur. (2006.) ukazali na vezanje Cu na superoksid dismutazu kao na potencijalni rizik od inhibicijskog učinka na taj antioksidacijski enzim, što znači da je potrebno razmotriti značaj prisutnosti Cu u području molekulskih masa viših od molekulske mase MT. Raspodjela Cu u škrgama klena otkrila je da se značajan dio ovoga

metala pojavljuje u području VMM biomolekula (~60 do >600 kDa), što nije uočeno kod jetara klena. No, slično jetrima, dominantna količina Cu eluirana je u području SMM biomolekula (5-60 kDa), koje obuhvaćaju i molekulsku masu MT. Vezanje na MT ipak je bilo izraženije i jasnije u jetrima, vjerojatno zbog značajnije uloge jetara u pohranjivanju Cu. Zanimljivo je da je, za razliku od jetara, u uzorcima s povišenom koncentracijom citosolskog Cu u škrgama bilo uočeno pojačano vezanje Cu na VMM biomolekule, koje mogu obuhvaćati albumin (66 kDa), ceruloplazmin (151 kDa; Boivin i sur., 2001.), β-amilazu (200 kDa) ili transkuprein (270 kDa; Liu i sur., 2007.), a koje može ukazivati na povećani rizik od toksičnog učinka bakra.

Selen je nemetal, esencijalni element za žive organizme, ali s vrlo uskim rasponom esencijalnosti i toksičnosti (Jukola i sur., 1996.). Biološka uloga Se primarno se odnosi na njegovu ugradnju u proteine te ga, na primjer, nalazimo kao sastavni dio glutation peroksidaze i vitamina E (Watanabe i sur., 1997.). Međutim, za većinu selenoproteina riba funkcije još nisu poznate te se vrlo malo zna o njegovom metaboliziranju u ribama (Hauser-Davis i sur., 2012.). Profili raspodjele Se u jetrima klena pokazali su da je mali dio Se u jetrima eluiran u VMM području (~60-400 kDa), a najveći dio u NMM području (~7-60 kDa), što je ukazivalo na vjerojatno vezanje ovoga nemetala na poznate selenoproteine koji su katalitički aktivni u redoks procesima (Hauser-Davis i sur., 2012.), poput glutation peroksidaze (85 kDa, Shulgin i sur., 2008.), jodotironin dejodinaze (~10-30 kDa) ili tioredoksin reduktaze (66 kDa, Larsson, 1973.). Paliwal i sur. (1986.) su u svojim istraživanjima utvrdili kako i određena izoforma MT pokazuje afinitet za jedinstveno vezanje Se, dok su Ferrarello i sur. (2002.) istaknuli da vezanje Se na MT ima sinergističku zaštitnu uloga protiv toksičnosti drugih teških metala. S druge strane, Iwai i sur. (1988.) utvrdili su da se radioaktivno obilježeni Se uglavnom eluira u području molekulskih masa većih od MT, što je u skladu s našim rezultatima. I u našem je istraživanju na jetrima klena uočeno da je samo veoma mala količina Se prisutna u rubnom području NMM biomolekula, što može ukazivati na vezanje na MT. Nadalje, manja je količina Se u jetrima klena bila raspodijeljena i u području JNMM biomolekula (<2 kDa). Profili raspodjele Se među citosolskim biomolekulama u škrgama bili su usporedivi s profilima u jetrima te su obuhvaćali tri pika (VMM, NMM i JNMM), uz tu razliku što se u škrgama većina Se veže uz JNMM biomolekule. Moguće je pretpostaviti kako se radi o selenospojevima jako niskih molekulskih masa koji su učinkoviti u obrani organizama od oksidacijskog stresa, poput nedavno identificiranog organskog selenovog spoja u tuni (*Thunnus orientalis),* selenoneina (~0.5 kDa;

Yamashita i Yamashita, 2010.; Yamashita i sur., 2012.), ili selenometionina (~0.2 kDa; Klotz i sur., 2003.). Povećanje citosolskih koncentracija Se u jetrima rezultiralo je izraženijom prisutnošću u NMM području, dok se povećanje citosolskih koncentracija Se u škrgama uglavnom odražavalo u povećanoj prisutnosti u JNMM području, što je bilo u skladu s temeljnim profilima raspodjele obaju organa.

Dobro je poznato da su neesencijalni elementi Cd i Pb toksični i pri vrlo niskoj izloženosti, uslijed činjenice da u organizmima riba nije razvijena učinkovita regulacija bioakumulacije neesencijalnih elemenata (Heath, 1995.). U jetrima klena uočeno je da su od svih ispitivanih metala najširi koncentracijski raspon imali upravo Cd (3,4-59,4 ng mL^{-1}) i Pb (<granice detekcije (0.10 ng mL^{-1}) do 44,2 ng mL^{-1}). Bioakumulacija u škrgama za oba metala bila je nešto slabije izražena pa su analize raspodjele provedene samo za Cd (0,48-1,03 ng mL^{-1}), dok profile za Pb nije bilo moguće jasno definirati. Prepoznavanje staničnih komponenata, odnosno biomolekula na koje se vežu Cd i Pb u jetrima te Cd u škrgama pri nižim i višim razinama izloženosti značajan je korak u razumijevanju njihovih potencijalnih toksičnih učinaka na klena.

Kadmij je neesencijalni metal čiji je toksični učinak otkriven početkom 20. stoljeća kada je porasla njegova uporaba u industriji (Schäfer i sur., 1999.). Veže se na metaloproteine kompeticijom sa Fe, Mn, Zn, Cu i drugim esencijalnim elementima, pri čemu dolazi do pogrešnog smatanja proteina, proizvodnje reaktivnih kisikovih radikala ili reaktivnih spojeva dušika, što sve može dovesti do oksidacijskih oštećenja te smrti stanice (Sarkar i sur., 2013.; Sedak i sur., 2015.). Temeljna raspodjela Cd u jetrima i škrgama klena ukazuje na dominantnu prisutnost Cd u područje NMM biomolekula (~5-30 kDa), što po vremenu eluiranja i molekulskoj masi odgovara standardu proteina MT. Pri porastu citosolskih koncentracija Cd u jetri 10-15 puta, većina dodatne bioakumulirane količine Cd veže se i dalje na MT, što potvrđuje dobro poznati visoki afinitet Cd za vezanje na MT, kao mehanizam zaštite od toksičnosti (Roesijadi, 1992.; Park i sur., 2001.). No, pri povišenju citosolskih koncentracija Cd u jetri, uočeno je i dodatno vezanje Cd na SMM biomolekule (~35-100 kDa), što je pokazatelj da pri povećanoj bioakumulaciji Cd u jetrima možda ne dolazi do njegove potpune detoksikacije putem vezanja na MT, već se dio Cd veže i na druge biomolekule viših molekulskih masa, što ostavlja mogućnost razvoja toksičnih učinaka. U škrgama već pri relativno niskim citosolskim koncentracijama postoji naznaka raspodjele Cd unutar područja VMM i SMM biomolekula. Poznata je činjenica da se detoksikacija Cd odvija primarno putem glutationa (GSH) i MT pa nepotpuna detoksikacija Cd može dovesti

do kompeticije toga metala s esencijalnim metalima za vezna mjesta na drugim važnim biomolekulama te potencijalno dovesti do staničnih oštećenja (McGeer i sur., 2012.). Stoga je potrebno provesti daljnja istraživanja, po mogućnosti pri izloženosti višim koncentracijama Cd, kako bi bilo točnije utvrđeno na koje se biomolekule osim MT Cd veže kada je u stanicama prisutan u povišenim koncentracijama te odražava li njegovo vezanje na SMM biomolekule dodatni oblik detoksikacije ili mogućnost za razvoj toksičnih učinaka. Matz i Krone (2007.) te Kwong i sur. (2011.) u svojim su istraživanjima predložili da osim GSH i MT u fiziološkim promjenama vezanima uz zaštitu stanica kod riba izloženih Cd značajnu ulogu igra i indukcija proteina toplinskog stresa, HSP70 i HSP90, čije su molekulske mase također obuhvaćene kadmijevim SMM-pikom, kao i molekulska masa transferina (80 kDa), koji je nedavno prepoznat kao glavni protein koji veže Cd u krvnoj plazmi šarana (*Cyprinus carpio*) (De Smet i sur., 2001.).

Olovo se ubraja u metale koji su izrazito štetni za zdravlje (Permyakov, 2009.) te je kao i Cd europskom Okvirnom direktivom o vodama uključeno u popis prioritetnih toksičnih zagađivala (EU ODV; EPCEU, 2008.). Toksični učinci olova povezani su s njegovim interakcijama s enzimima, čija je aktivnost ovisna o prisutnosti slobodnih sulfhidrilnih (SH) skupina s kojima olovo stvara merkaptide (Duraković i Labar, 2000.; Goering, 1993.). Organizam tada te skupine više ne može koristiti za stvaranje hemoglobina i citokroma. Već vrlo niske koncentracije olova inhibiraju i Na^+,K^+-ATP-azu, što rezultira povećanom krhkošću eritrocita i skraćivanjem njihova vijeka trajanja (Duraković i Labar, 2000.). Olovo se u organizmu najviše apsorbira u kostima, a od mekih tkiva u jetrima i bubrezima (Sedak i sur., 2015.). Profili raspodjele Pb u jetrima klena ukazali su na dominantnu prisutnost Pb u području SMM biomolekula (~35-240 kDa), što može ukazivati na njegov potencijal za toksično djelovanje. Preostala količina Pb bila je raspodijeljena između nekoliko manjih pikova među kojima se ističe NMM pik (~9-20 kDa), koji odgovara području eluiranja MT. O eluiranju Pb u području MT izvijestili su i Pavičić i sur. (1993.) kod dagnji (*Mytilus sp.*) izloženih smjesi metala (Cd, Cu i Pb). No, nekoliko je drugih autora izvijestilo o izostanku indukcije MT olovom u tkivima riba (Reichert i sur., 1979; Roesijadi i Robinson, 1994). Budući da je Pb slabiji inducer MT u usporedbi s drugim metalima kao što su Cd i Zn (Waalkes i Klaassen, 1985.), moguće je da je uočeno vezanje Pb na MT povezano sa stvaranjem inkluzijskog tijela prilikom detoksikacije Pb uklapanjem u granule (Mager, 2012.).

4.2. Raspodjela esencijalnih (Co, Fe, Mn, Mo, Se, Cu i Zn) i neesencijalnog (Cd) elementa među citosolskim biomolekulama različitih molekulskih masa u jetrima i škrgama vardarskog klena (*S. vardarensis*) iz triju makedonskih rijeka

Istraživanje provedeno na klenu (*S. cephalus*) nadalje je prošireno na srodnu vrstu, pripadnika istoga roda *Squalius*, vardarskog klena (*S. vardarensis*) iz triju različito onečišćenih makedonskih rijeka. Cilj je bio utvrditi temeljne profile raspodjele među citosolskim biomolekulama različitih molekulskih masa, kao i njihove promjene za iste elemente kao i kod klena (*S. cephalus*), odnosno za esencijalne elemente Co, Fe, Mn, Mo, Se, Cu i Zn te neesencijalne elemente Cd i Pb, u jetrima i škrgama kao ciljnim organima, pod različitim uvjetima izloženosti metalima. Rezultati ovog dijela istraživanja objavljeni su u radovima pod rednim brojem 3 u popisu znanstvenih radova (str. 57-70). Instrumentalna mogućnost detektiranja pojedinih elemenata, međutim, ograničila je naše istraživanje na određivanje raspodjela Co, Fe, Mn, Mo, Se, Cu, Zn i Cd u jetrima te Fe, Mo, Se, Zn i Cd u škrgama vardarskog klena. Ovakav istraživački pristup omogućio nam je usporedbu raspodjela, odnosno sudbine navedenih elemenata u jetrima i škrgama dvaju srodnih ribljih vrsta te procjenu mogu li se navedene vrste koristiti usporedno u monitoringu udaljenih slatkovodnih sustava.

Vardarski klenovi korišteni u ovome istraživanju uzorkovani su u rijekama Bregalnici, Krivoj Reci i Zletovskoj Reci u sjeveroistočnoj Makedoniji u svibnju i lipnju 2012. godine. Rijeka Bregalnica karakterizirana je blažim onečišćenjem metalima u usporedbi s drugim dvjema rijekama, a izvor onečišćenja predstavljalo je ispiranje poljoprivrednog tla, u najvećoj mjeri rižinih polja (Ramani i sur., 2014.; Stipaničev i sur., 2017.). Rijeke Kriva i Zletovska izložene su utjecaju otpadnih voda iz aktivnih rudnika Pb i Zn, Toranica i Zletovo (Ramani i sur., 2014.). Od analiziranih osam elemenata, u rijeci Bregalnici nađene su najviše koncentracije otopljenog Fe i Mo (Fe, 61,3±3,4 μg L^{-1}; Mo, 0,950±0,010 μg L^{-1}), u Zletovskoj Reci otopljenog Cd, Co, Cu, Mn i Zn (Cd, 0,272±0,002 μg L^{-1}; Co, 1,51±0,01 μg L^{-1}; Cu, 3,38±0,07 μg L^{-1}; Mn, 351,9±6,5 μg L^{-1}; Zn, 197,0±2,9 μg L^{-1}) te u Krivoj Reci otopljenog Cd (Cd, 0,270±0,009 μg L^{-1}) (Ramani i sur., 2014.). U makedonskim rijekama povišene koncentracije u riječnoj vodi u usporedbi s rijekom Sutlom, uslijed utjecaja aktivnih rudnika, nađene su za metale Mn i Zn, što nam je omogućilo da promjene koje se događaju u citosolskim raspodjelama pri izrazito visokoj izloženosti u riječnoj vodi utvrdimo za veći broj elemenata. Zanimljivo je spomenuti da su u vardarskim klenovima iz navedenih rijeka provedene i histološke analize na više organa (Barišić i sur., 2015.; Jordanova i sur., 2016.,

2017.) te su zapažena izraženija oštećenja na škrgama, jetrima i slezeni riba iz rijeka onečišćenih rudničkim otpadom, a napose iz Zletovske Reke, u kojoj je zabilježena i najizraženija bioakumulacija brojnih metala u jetrima i škrgama vardarskih klenova (Dragun i sur., 2019.). Kao i u istraživanju na klenu iz rijeke Sutle, za analize smo primijenili kombinaciju frakcioniranja citosola jetara i škrga pomoću SEC_{200}-HPLC-a te mjerenja metala/nemetala pomoću HR ICP-MS-a, kao i svrstavanje biomolekula na temelju njihovih molekulskih masa u četiri skupine (VMM, SMM, NMM i JNMM). Budući da su neke od biomolekula koje vežu metale i služe u njihovoj detoksikaciji, poput MT, toplinski stabilne na vrlo visokim temperaturama (Dragun i sur., 2009.; Lavradas i sur., 2016.), istraživanje na vardarskom klenu je osim na ukupnim citosolima provedeno i na citosolima toplinski obrađenima na 70°C (Erk i sur., 2002.). Metali vezani na toplinski stabilne proteine i peptide smatraju se detoksiciranim frakcijama metala u stanici, dok su biomolekule osjetljive na visoke temperature definirane kao stanična frakcija osjetljiva na metale i podložna njihovom toksičnom djelovanju (Giguère i sur., 2006.; Goto i Wallace, 2010., Rosabal i sur., 2015.). Uz iznimku iscrpnih studija toplinski stabilnih proteina MT (npr., Goenaga Infante i sur., 2006.; Hauser-Davis i sur., 2012., Mason i sur., 2004.; Rodríguez-Cea i sur., 2003.), prema našim saznanjima u znanstvenoj literaturi nema informacija o drugim toplinski stabilnim biomolekulama koje vežu metale/nemetale u jetrima i škrgama riba. Za analize raspodjela metala/nemetala u toplinski obrađenim citosolima primijenjena je SEC_{75}-HPLC metoda (kolona Superdex 75, raspon razdvajanja 3-70 kDa) te je u citosolima jetara i škrga vardarskog klena utvrđena prisutnost toplinski stabilnih molekula koje vežu Mo, Se, Cu, Zn i Cd.

Citosolske koncentracije Co u analiziranim jetrima vardarskog klena iz triju makedonskih rijeka nalazile su se u rasponu od 2,56-5,54 ng mL^{-1}. Kobalt je bio raspodijeljen među četiri pika, jedan u VMM području, jedan u SMM i dva u JNMM području. Najveći dio citosolskog Co u jetrima bio je vezan uz VMM biomolekule (~110-380 kDa), što je u skladu s prethodnim istraživanjima koja su pokazala da ioni Co imaju visoki afinitet za vezanje na enzimske proteine visokih molekulskih masa (Paustenbach i sur., 2013.; Wojcieszek i Ruzik, 2016.). Dominantno vezanje Co za VMM biomolekule uočeno je i u jetrima pastrve (*Salmo trutta*; Dragun i sur., 2018.), kao i u jetrima grgeča (*P. flavescens*; Caron i sur., 2018.). Znatno manje količine Co bile su vezane uz SMM biomolekule (~30-85 kDa), kao i uz JNMM biomolekule (<5 kDa), unatoč već prije spomenutoj značajnoj ulozi Co u izgradnji kobalamina (vitamin B12; Blust, 2012.), kao i značajnoj ulozi jetara u njegovom metabolizmu

(Wang i sur., 2001.). Zbog relativno uskog raspona citosolskih koncentracija Co u analiziranim uzorcima, razlike u njegovoj citosolskoj raspodjeli pri različitim razinama bioakumulacije bile su jedva uočljive. Povišenjem citosolskih koncentracija Co u jetrima vardarskog klena dolazi tek do slabog porasta u VMM području, dok u drugim biomolekulskim područjima nije bilo razlike, zbog čega bi se dodatna pozornost trebala posvetiti upravo proučavanju VMM biomolekula na koje se veže Co. Zbog niskih citosolskih koncentracija Co u škrgama, njegovu raspodjelu nije bilo moguće odrediti za taj organ vardarskog klena.

Citosolske koncentracije Fe u analiziranim vardarskim klenovima iz triju makedonskih rijeka nalazile su se u rasponu od 6,4 do 14,7 μg mL^{-1} te u jetrima od 5,5 do 28,5 μg mL^{-1} u škrgama. Profili raspodjele Fe među citosolskim biomolekulama, kako u jetrima tako i u škrgama vardarskog klena, ukazali su na njegovu prisutnost u dva područja citosolskih biomolekula, u VMM području (~230-630 kDa) te u SMM području (24-51 kDa), što je uočeno i u jetrima potočne pastrve (*S. trutta*; Dragun i sur., 2018.). Razlika raspodjele Fe između dvaju organa bila je u tome što je u jetrima količina Fe eluirana unutar VMM područja bila usporediva ili viša nego u SMM području, dok je u škrgama većina Fe bila eluirana unutar SMM područja te je prisutnost Fe u VMM području bila zabilježena samo u uzorku s najvišom citosolskom koncentracijom Fe (28,5 μg mL^{-1}). Kao što je već navedeno u istraživanju na klenu (*S. cephalus*), VMM područje obuhvaća molekulsku masu skladišnog proteina feritina (450 kDa) (Szpunar i Łobiński, 1999.; Martin-Antonio i sur., 2009.), dok bi raspodjela Fe unutar SMM područja mogla biti rezultat vezanja Fe na razne poznate funkcionalne proteine željeza. Povišenje citosolskih koncentracija Fe u jetrima rezultiralo je povećanim eluiranjem Fe u VMM feritinskom području što je u skladu s pojačanom sintezom feritina pri povećanoj dostupnosti Fe (Torti i Torti, 2002.). Nasuprot tome, u škrgama je povećanje citosolskog Fe uglavnom rezultiralo povećanim eluiranjem unutar SMM biomolekulskog područja, izuzev u uzorku s najvišom citosolskom koncentracijom Fe. Dobiveni rezultati potvrdili su značajniju ulogu jetre nego škrga vardarskog klena u skladištenju Fe.

Citosolske koncentracije Mn u analiziranim jetrima vardarskog klena iz triju makedonskih rijeka nalazile su se u rasponu 214,5 do 408,6 ng mL^{-1}. Profili raspodjele za esencijalni element Mn određeni su samo za jetra zbog niske citosolske koncentracije u škrgama vardarskog klena. Mangan je u jetrima bio raspodijeljen unutar dvaju slabo razlučenih pikova u VMM (~85-380 kDa) i SMM (24-85 kDa) području biomolekula, pri

čemu SMM područje obuhvaća molekulske mase proteina uključenih u transport Mn, albumina (66 kDa) i transferina (80 kDa; Martin-Antonio i sur., 2009.), kao što je već uočeno i kod klena (*S. cephalus*). Sličan profil raspodjele Mn zabilježen je i u jetrima potočne pastrve (*S. trutta*; Dragun i sur., 2018.). Profili raspodjele Mn bili su usporedivi u svim uzorcima jetara vardarskog klena iz svih triju rijeka, a pri porastu citosolskih koncentracija ponekad je došlo do porasta eluiranja Mn u VMM području, a ponekad u SMM području biomolekula.

Citosolske koncentracije Mo u analiziranim vardarskim klenovima iz triju makedonskih rijeka nalazile su se u rasponu od 12,6 do 26,6 ng mL^{-1} u jetrima te od 2,55 do 3,05 ng mL^{-1} u škrgama. Profili citosolskih raspodjela za esencijalni element Mo određeni su samo za jetra zbog znatno nižih citosolskih koncentracija u škrgama vardarskog klena. Molibden je u jetrima bio raspodijeljen unutar širokog područja molekulskih masa u VMM području biomolekula (~100 do >600 kDa), što ukazuje na njegovo vezanje za veliki broj citosolskih biomolekula, uključujući i različite enzime kojima Mo služi kao kofaktor (Beers i Berkow, 1998.), poput aldehid oksidaze (~130 kDa; Uchida i sur., 2003.), sulfid oksidaze (~ 120 kDa; Johnson i Rajagopalan, 1976.) ili Fe-Mo flavoprotein ksantin oksidaze (275 kDa, Truglio i sur., 2002.). Slični profili raspodjele, s eluiranjem Mo u području biomolekula molekulskih masa od 100 do 500 kDa, opisani su za jetra potočne pastrve (*S. trutta*; Dragun i sur., 2018.) te jetra grgeča (*P. flavescens*; Caron i sur., 2018.). Nadalje, manji dio Mo bio je eluiran i u području JNMM biomolekula (3-7 kDa). Povišenje citosolskih koncentracija u jetrima vardarskog klena odrazilo se na njegovom pojačanom eluiranju dominantno unutar VMM područja, potvrđujući prevladajuće vezanje Mo na VMM biomolekule. Nakon toplinske obrade citosola jetara, povećava se prisutnost Mo u području JNMM biomolekula (5-9 kDa), što ukazuje na preraspodjelu Mo među citosolskim biomolekulama ili na razgradnju većih proteina/peptida osjetljivih na visoke temperature na manje toplinski stabilne molekule koje vežu Mo. Primjenom SEC_{75}-HPLC-a uspjeli smo provesti analizu citosolske raspodjele Mo i za škrge. U škrgama također nalazimo Mo vezan na toplinski stabilne biomolekule unutar JNMM područja (5-11 kDa), ali je njegova prisutnost usporediva prije i nakon toplinske obrade.

Citosolske koncentracije Se u analiziranim vardarskim klenovima iz triju makedonskih rijeka nalazile su se u rasponu od 85,8 do 631,1 ng mL^{-1} u jetrima te od 57,9 do 469,0 ng mL^{-1} u škrgama. Profili raspodjele Se u jetrima vardarskog klena pokazuju njegovu prisutnost u širokom biomolekulskom području, i to vezanog na VMM (~110-300 kDa), NMM (~20-50 kDa) i JNMM biomolekule (2-5 kDa i <2 kDa). Raspodjela Se u škrgama

razlikovala se samo po izostanku vezanja Se na VMM biomolekule, a bila je karakterizirana eluiranjem Se u oštrom, jasno odvojenom piku u JNMM području (<2 kDa), što može ukazivati na prisutnost nisko molekulskih selenospojeva koji djeluju kao jaki čistači slobodnih radikala te su učinkoviti u obrani od oksidacijskog stresa (npr., selenometionin, ~0,2 kDa, Klotz i sur., 2003.; selenonein, ~0,5 kDa, Yamashita i Yamashita, 2010.; Yamashita i sur., 2012.). Pri povišenim koncentracijama Se u škrgama javlja se povećanje Se-pika isključivo u JNMM području, dok se u jetrima javlja i povišenje NMM pika, što može ukazivati na vezanje Se na selenoproteine katalitički aktivne u redoks procesima, poput glutation peroksidaze, jodotironin dejodinaze i tioredoksin reduktaze (Hauser-Davis i sur., 2012.). Nadalje, u citosolima jetara vardarskog klena utvrđena je prisutnost toplinski stabilnih biomolekula koje vežu Se unutar dvaju JNMM područja (3-6 kDa; 0,5-1,6 kDa), a u škrgama samo unutar drugog JNMM područja (0,5-1,3 kDa), što je moguće povezati s nekim peptidima ili aminokiselinama koji sadrže Se, poput selenocisteina (Se-Cys) i selenometioneina (Se-Met) (Rahmanto i Davies, 2012.).

Citosolske koncentracije Cu u analiziranim jetrima vardarskog klena iz triju makedonskih rijeka nalazile su se u rasponu od 1,67 do 6,54 μg mL^{-1}. Profili citosolskih raspodjela za esencijalni element Cu određeni su samo za jetra zbog znatno nižih citosolskih koncentracija u škrgama vardarskog klena. Profili raspodjele Cu među citosolskim biomolekulama u jetrima vardarskog klena ukazali su na dominantnu prisutnost ovoga metala samo u području NMM biomolekula (7-40 kDa), što odgovara molekulskoj masi i vremenu eluiranja standarda MT te ukazuje na vezanje Cu na MT. Dominantno vezanje Cu za MT-frakciju opisano je i za jetra grgeča (*P. flavescens*; Caron i sur., 2018.), jegulje (*Anguilla anguilla*; Van Campenhout i sur., 2008.; Rodriguez-Cea i sur., 2003.) i babuške (*Carassius gibelio*; Van Campenhout i sur., 2010.). Povišenjem citosolskih koncentracija Cu dolazi do vidljivog porasta u MT-području, što je u skladu s najvećim kapacitetom za indukciju MT u tkivima koja su aktivna u uzimanju, skladištenju i izlučivanju elemenata u tragovima, poput škrga, crijeva, jetara i bubrega (Roesijadi i Robinson, 1994.). Nadalje, pri povišenju citosolske koncentracije Cu opaženo je i širenje NMM pika prema višim molekulskim masama, što bi moglo ukazivati na vezanje Cu i na druge citosolske biomolekule pri njegovoj povišenoj bioakumulaciji, na primjer superoksid dismutazu (32 kDa), kao znak bitne uloge Cu u zaštiti stanica od oksidacijskog stresa (Sanchez i sur., 2005.).

Citosolske koncentracije Zn u analiziranim vardarskim klenovima iz triju makedonskih rijeka nalazile su se u rasponu od 3,51-8,37 μg mL^{-1} u jetrima te od 4,28-14,4

μg mL^{-1} u škrgama. U jetrima vardarskog klena Zn je bio raspodijeljen u tri biomolekulska područja: VMM (>500 kDa), SMM (~30-400 kDa) i NMM (7-24 kDa), pri čemu je najizraženija prisutnost Zn bila u uskom, oštrom piku u NMM području što odgovara vremenu eluiranja i molekulskoj masi MT, koji sudjeluje u transportu i homeostazi Zn (Sakulsak, 2012.). Usporedivi profili Zn dobiveni su za jetra potočne pastrve (*S. trutta*) iz rijeke Krke (Dragun i sur., 2018.). U škrgama, manji dio Zn eluiran je u VMM području (>400 kDa), a najveći dio unutar jednog širokog područja molekulskih masa s maksimumom u SMM području (~10-300 kDa, s maksimumom na 85 kDa), koje obuhvaća i neke dobro poznate proteine koji sadrže Zn, poput alkohol dehidrogenaze (150 kDa, Szpunar i Łobiński, 1999.). Za razliku od jetara, u škrgama jasno vezanje Zn na MT područje nije bilo uočeno. Raspodjela Zn među biomolekulama u jetrima i škrgama bila je usporediva u svim uzorcima iz svih triju rijeka i nije bilo moguće jasno utvrditi promjene u raspodjeli Zn kao rezultat njegove povećane bioakumulacije.

Citosolske koncentracije Cd u analiziranim vardarskim klenovima iz triju makedonskih rijeka nalazile su se u rasponu od 2,38 do 68,2 ng mL^{-1} u jetrima te od 0,34 do 30,0 ng mL^{-1} u škrgama. Slično bakru, i Cd je bio raspodijeljen unutar jednog jasnog i uskog NMM područja biomolekula (7-24 kDa) u oba organa vardarskog klena, što po vremenu eluiranja i molekulskoj masi odgovara standardu MT. Kadmij je snažan inducer MT, koji služi kao mehanizam zaštite od njegove toksičnosti (Park i sur., 2001.; Roesijadi, 1992.). Povišenje citosolskih koncentracija Cd u uzorcima jetara i škrga vardarskog klena iz Zletovske Reke i Krive Reke, koje su izložene utjecaju onečišćenja iz rudnika, rezultiralo je proporcionalnim povećanjem eluiranja Cd unutar MT područja, dok vezanje Cd na druge biomolekule nije bilo uočeno. Dominantno eluiranje Cd u MT frakciji, kao i porast prisutnosti Cd vezanog za MT pri povišenju njegove bioakumulacije u jetrima zabilježen je i kod potočne pastrve (*S. trutta*; Dragun i sur. 2018.). Ovakav rezultat upućuje na gotovo potpunu detoksikaciju Cd u oba organa vardarskog klena u ispitivanim uvjetima, što su već prije Lavradas i sur. (2016.) pokazali za smeđe dagnje (*Perna perna*). Analizom toplinski obrađenih citosola jetara i škrga vardarskog klena potvrđena je prisutnost toplinskih stabilnih proteina koji vežu Cd, najvjerojatnije MT, u području NMM biomolekula (~10-30 kDa). Potvrđeno je i istovremeno vezanje Cu i Zn na te iste toplinske stabilne biomolekule u jetrima vardarskog klena. No, zanimljiv je nalaz izostanak vezanja Zn na toplinski stabilnu MT frakciju u škrgama vardarskog klena, što je u skladu s rezultatima prethodno dobivenim za klena (*S. cephalus*).

Rezultati ostvareni za dvije vrste klena (klen, *S. cephalus*, i vardarski klen, *S. vardarensis*) pokazali su da su raspodjele metala/nemetala među citosolskim biomolekulama različitih molekulskih masa u jetrima i škrgama kao ciljnim organima većinom jednake u objema vrstama, što omogućuje usporedbu rezultata dobivenih primjenom ovih dviju srodnih vrsta u monitoringu udaljenih slatkovodnih sustava. Između dviju vrsta klena nije bilo razlika u citosolskim raspodjelama Fe, Se i Cd u škrgama, kao ni u citosolskim raspodjelama Co, Cu, Fe, Mn, Mo i Se u jetrima. Manje razlike zabilježene su za citosolske raspodjele Zn u jetrima i škrgama te Cd u jetrima. Iako je u jetrima obiju vrsta klena uočeno vezanje Zn na NMM biomolekule (MT), to je vezanje bilo puno jasnije vidljivo u jetrima vardarskog klena. Nadalje, vezanje Zn na JNMM biomolekule (<5 kDa) u škrgama uočeno je samo kod klena (*S. cephalus*). U jetrima klena pri višim je koncentracijama uz vezanje Cd na NMM biomolekule (MT) uočeno i vezanje Cd na SMM biomolekule (~35-100 kDa), što nije uočeno kod vardarskog klena gdje je sav Cd bio vezan na MT. Postoji mogućnost da izraženije vezanje Zn i Cd na MT u jetrima vardarskog klena ukazuje na snažniju sposobnost detoksikacije te vrste ili na prilagodbu uvjetima povećane izloženosti metalima u rijekama onečišćenim rudničkim otpadom.

4.3. Karakterizacija i identifikacija odabranih citosolskih biomolekula u jetrima i škrgama vardarskog klena (*S. vardarensis*) primjenom dvodimenzionalne tekućinske kromatografije visoke djelotvornosti (SEC-AEC-HPLC) i dviju tehnika spektrometrije masa (MALDI-TOF-MS i LC-MS/MS)

Nakon opisanih temeljnih raspodjela devet odabranih metala/nemetala u jetrima i škrgama klena (*S. cephalus*) i vardarskog klena (*S. vardarensis*), sljedeći je korak u istraživanju bio pobliža karakterizacija i identifikacija nekoliko odabranih biomolekula koje vežu Fe, Cd, Cu, Zn i Mo u jetrima i škrgama vardarskog klena. Radi nastavka istraživanja, provedeno je dodatno uzorkovanje u makedonskim rijekama Bregalnici i Zletovskoj Reci, u lipnju 2015. godine. Na jetrima i škrgama vardarskih klenova uzorkovanih 2015. godine ponovljena su razdvajanja pomoću SEC_{200}-HPLC-a i SEC_{75}-HPLC-a te određivanje profila raspodjele pomoću HR ICP-MS-a, radi preciznog definiranja vremena eluiranja odabranih biomolekula te prikupljanja frakcija odgovarajućih molekulskih masa za njihove daljnje analize. Dobiveni profili raspodjele bili su podudarni s profilima raspodjele definiranima za organe vardarskih klenova uzorkovanih u proljeće 2012. godine. Rezultati ovog dijela istraživanja objavljeni su u radovima pod rednim brojem 4 u popisu znanstvenih radova (str. 71-89).

Tekućinska kromatografija visoke djelotvornosti s isključivanjem po veličini (SEC-HPLC) često se koristi kao osnovna metoda za razdvajanje metaloproteina i drugih biomolekula koje vežu metale/nemetale, a zasniva se na načelu molekularnih sita koja omogućuju razdvajanje molekula prema njihovoj veličini te u manjoj mjeri prema njihovom obliku (Szpunar, 2005.). Međutim, isključivom primjenom SEC-HPLC-a kao metode razdvajanja ne bismo bili u mogućnosti precizno odrediti molekulske mase biomolekula na koje se vežu metali/nemetali u organima riba, jer ona omogućava samo grubo razdvajanje biomolekula i približno određivanje njihovih molekulskih masa na temelju usporedbe sa proteinskim standardima poznatih molekulskih masa. Za zahtijevan korak preciznije karakterizacije te konačnog prepoznavanja citosolskih biomolekula, potrebno je primijeniti dodatne analitičke postupke, poput raznih tehnika spektrometrije masa (npr., MALDI-TOF-MS i LC-MS/MS), za čiju je primjenu potrebno prethodno izolirati biomolekule od interesa. No, sama SEC-HPLC metoda nije uvijek dovoljna za razdvajanje i izoliranje tih biomolekula sa svrhom njihovog daljnjeg analiziranja pomoću spektrometra masa, jer više molekula približno jednakih molekulskih masa može biti eluirano u isto vrijeme, odnosno može doći do preklapanja pikova različitih citosolskih biomolekula. Budući da je očekivano da će većina frakcija izdvojenih pomoću SEC-HPLC-a sadržavati smjesu više citosolskih biomolekula, preporučljiva je primjena dodatnih tehnika razdvajanja, kako bismo što uspješnije pročistili i izdvojili biomolekule od interesa. Stoga smo za daljnju karakterizaciju biomolekula koje vežu metale u jetrima i škrgama vardarskog klena, nakon prvog stupnja razdvajanja pomoću SEC-HPLC-a, primijenili anionsko-izmjenjivačku kromatografiju (AEC-HPLC), koja uz osnovno razdvajanje biomolekula na temelju njihovih molekulskih masa doprinosi izdvajanju biomolekula od interesa njihovim razdvajanjem na temelju razlike u naboju. Analiza tako izdvojenih biomolekula provedena je metodama MALDI-TOF-MS i LC-MS/MS. Iako navedene tehnike pružaju veliki broj mogućnosti u istraživanjima (Montes-Bayon i sur., 2003.), što je prepoznato u mnogim znanstvenim područjima, primjena istraživanja specijacije metala u stanicama, poznatog i kao metalomika (Szpunar, 2004.), u okolišnim istraživanjima nije još dovoljno zastupljena. Takva se istraživanja uglavnom provode u strogo kontroliranim laboratorijskim uvjetima, najčešće nakon izlaganja samo jednome metalu (Ju i sur., 2011.), dok su multielementna istraživanja u stvarnim okolišnim uvjetima iznimno rijetka (npr., Caron i sur., 2018., Rosabal i sur., 2016.; Urien i sur., 2018.). Stoga je primjena opisane analitičke metodologije u istraživanju citosolskih biomolekula koje vežu metale u škrgama i jetrima vardarskog klena iz rijeka izrazito onečišćenih metalima omogućila bolji uvid u

sudbinu i ponašanje metala u stanicama ciljnih organa tog značajnog bioindikatorskog organizma.

U ovome dijelu istraživanja, pozornost je bila usmjerena na analize sljedećih biomolekula:

(1) biomolekula koje u jetrima i škrgama vardarskog klena vežu Fe, a razdvajanjem pomoću SEC_{200}-HPLC-a bile su eluirane u području molekulskih masa ~25-50 kDa;
(2) toplinski stabilnih biomolekula koje u jetrima i škrgama vardarskog klena vežu Cd, Cu i Zn, a razdvajanjem pomoću SEC_{200}-HPLC-a bile su eluirane u području molekulskih masa ~5-25 kDa te razdvajanjem pomoću SEC_{75}-HPLC-a u području molekulskih masa ~10-30 kDa (vjerojatna metalotioneinska frakcija);
(3) toplinski stabilnih biomolekula koje u jetrima i škrgama vardarskog klena vežu Mo, a razdvajanjem pomoću SEC_{75}-HPLC-a bile su eluirane u području molekulskih masa ~5-10 kDa.

4.3.1. Biomolekule koje vežu Fe

Primjenom SEC_{200}-HPLC-a u razdvajanju citosolskih frakcija organa vardarskih klenova uzorkovanih 2015. godine, Fe je u jetrima bilo podjednako raspodijeljeno između dvaju biomolekulskih područja, VMM (~180-640 kDa) i SMM (~25-50 kDa), dok je u većini uzoraka škrga Fe bilo raspodijeljeno unutar samo jednog biomolekulskog područja, SMM (~20-50 kDa). Ovi rezultati u skladu su s prethodnim rezultatima o raspodjeli Fe u jetrima i škrgama vardarskih klenova uzorkovanih 2012. godine, kao i klenova uzorkovanih u rijeci Sutli. Za analize spektrometrijom masa prikupljene su SMM frakcije (~20-50 kDa) iz jetara i škrga vardarskih klenova, koje su se djelomično, u rubnim područjima, preklapale sa SEC_{200}-HPLC pikovima Cu i Zn. MALDI-TOF-MS analizom potvrđena je moguća prisutnost Cu,Zn-superoksid dismutaze u izdvojenim SMM frakcijama, budući da su u njihovim spektrima masa zabilježeni *m/z* pikovi koji su odgovarali masenom profilu standarda superoksid dismutaze (*m*/*z*: 31 kDa, 15,5 kDa (2+) i 7,75 kDa (4+)).

Sa svrhom pročišćavanja SMM frakcija željeza i uklanjanja primjesa Cu i Zn, proveden je dodatni korak razdvajanja primjenom AEC-HPLC-a. AEC-HPLC često se primjenjuje tijekom izolacije i karakterizacije biomolekula koje vežu Fe nakon početnog razdvajanja pomoću SEC-HPLC-a, na primjer u ljudskom serumu (Mestek i sur., 2007.; Montes-Bayón i sur., 1999.; Muniz i sur., 2001.) ili ribama (npr., kalifornijska pastrva, *O. mykiss*; Fago i sur., 2002.). U ovome istraživanju, primjenom AEC-HPLC-a ostvareno je

uspješno razdvajanje biomolekula koje vežu Fe od biomolekula koje vežu Cu i Zn, što upućuje na prednost primjene ove tehnike u postupcima finog razdvajanja biomolekula.

Za konačnu karakterizaciju izdvojenih SMM biomolekula koje vežu Fe, razdvojenih i pročišćenih dvodimenzionalnom kromatografijom, primijenjene su dvije tehnike spektrometrije masa, MALDI-TOF-MS i LC-MS/MS. MALDI-TOF-MS spektri u jetrima su uvijek pokazivali glavni *m/z* pik na 15,4 kDa, dok su u nekim uzorcima jetara bila prisutna i dva dodatna niža *m/z* pika na 31,5 kDa i 46,9 kDa, što je najvjerojatnije ukazivalo na spektre hemoglobina, s karakterističnim hemoglobinskim monomerima (~15 kDa), dimerima (~30 kDa) i trimerima (~45 kDa). Iako je hemoglobin protein molekulske mase približno 64,5 kDa, on sadrži četiri polipeptidne podjedinice, pri čemu se svaka podjedinica sastoji od α ili β polipeptidnog lanca i prostetičke skupine hema (Perutz i sur., 1960.). Naši rezultati za jetra podudaraju se s istraživanjima provedenima na kalifornijskoj pastrvi (*O. mykiss*), kod koje je bilo identificirano pet glavnih α lanaca i četiri glavna β lanca, s rasponom molekulskih masa od 15,1 do 16,0 kDa (Fago i sur., 2002.). Yu i sur. (1997.) u analizama ljudske krvi također su uočili istovremenu pojavu monomera i dimera nakon razdvajanja hemoglobina pomoću SDS-poliakrilamidne gel elektroforeze. Sukladno tome, *m/z* pik na ~15 kDa najvjerojatnije odgovara α-lancu ili β-lancu, *m/z* pik na ~30 kDa vjerojatno odgovara kovalentnom produktu α-α, β-β ili α-β povezivanja, dok *m/z* pik na ~45 kDa vjerojatno odgovara kovalentno vezanom trimeru (Yu i sur., 1997.). MALDI-TOF-MS spektri dobiveni za SMM frakcije Fe u škrgama također su pokazali jasne *m/z* pikove na ~15 kDa, koje je moguće pripisati hemoglobinskim monomerima. No, opaženi su i dodatni *m/z* pikovi na nižim molekulskim masama, što ukazuje na vjerojatnu prisutnost višestruko nabijenih biomolekula (na primjer, dvostruko nabijenih (*m/z*: 7,7 kDa; 2+) ili trostruko nabijenih (*m/z*: 5,1 kDa; 3+)), dok istovremeno u škrgama pojava multimera nije zabilježena.

Uporabom LC-MS/MS-a i pretraživanjem baza Mascot sustavom, u prikupljenim je i pročišćenim SMM frakcijama jetara i škrga vardarskog klena potvrđena prisutnost hemoglobina. I u jetrima i u škrgama identificirana je β podjedinica hemoglobina, dok je α-podjedinica identificirana samo u jednom uzorku škrga. U MALDI-TOF masenom spektru škrga u kojemu je pronađena α podjedinica, pored *m/z* pika na 15,3 kDa, uočena su i dva dodatna *m/z* pika nižih molekulskih masa, 11,3 kDa i 13,5 kDa, za koje se može pretpostaviti da odgovaraju upravo hemoglobinskoj α podjedinici. Niža molekulska masa α-podjedinice (15,1 kDa) u usporedbi s β-podjedinicom (15,9 kDa) već je i prije zabilježena u ljudskom

hemoglobinu (Yu i sur., 1997.), kao i hemoglobinu štakora (α: 15,3 kDa, β: 16.0 kDa) (Fernández-Menéndez i sur., 2018.).

Naši su rezultati nedvojbeno potvrdili prisutnost krvi u jetrima i škrgama vardarskog klena, što nije bilo iznenađujuće budući da izdvajanju organa i citosolskih frakcija nije prethodila perfuzija organa. U uzorcima jetara obično je očekivano da dio Fe bude vezan na transportni protein transferin (80 kDa; Neves i sur., 2009.). No, kao što je vidljivo iz gore prikazanih rezultata, u našem je istraživanju Fe i u jetrima i u škrgama bilo raspodijeljeno u području biomolekula čije su molekulske mase bile ili znatno više ili nešto niže od molekulske mase transferina. Predominantno vezanje Fe na hemoglobin, a ne transferin, u skladu je s nalazima Fernández-Menéndez i sur. (2018.) koji su pokazali da je u crvenim krvnim stanicama štakora tek 13% ukupnog Fe vezano na transferin. Povezivanje dvodimenzionalne tekućinske kromatografije visoke djelotvornosti (SEC-AEC-HPLC) i raznih tehnika spektrometrije masa (HR ICP-MS; MALDI-TOF-MS; LC-MS/MS) pokazalo se osjetljivim i optimalnim načinom detekcije, karakterizacije i identifikacije biomolekula koje vežu Fe, kao što je već i ranije zabilježeno tijekom istraživanja hemoglobina (Kleinert i sur., 2008.).

4.3.2. Toplinski stabilne biomolekule koje vežu Cd, Cu i Zn

Razdvajanjem citosolskih frakcija jetara i škrga vardarskih klenova uzorkovanih 2015. godine primjenom SEC_{200}-HPLC-a zabilježeno je vezanje Cd, Cu i Zn u području NMM biomolekula (~5-25 kDa), vjerojatno MT, kao i dodatno vezanje Zn u području VMM (~500 do >600 kDa) i SMM biomolekula (~25-300 kDa). Rezultati za Cu odnose se samo na jetrima vardarskog klena jer zbog niskih citosolskih koncentracija nije bilo moguće postojećom analitičkom opremom provesti analize Cu u škrgama. Ostvareni rezultati podudaraju se s rezultatima o raspodjeli Cd, Cu i Zn u jetrima i škrgama vardarskih klenova uzorkovanih 2012. godine. Uzimajući u obzir značajnu funkciju MT u održavanju homeostaze i detoksikaciji Zn, Cu i Cd, u daljnjem je tijeku istraživanja radi prepoznavanja MT izoformi u organima vardarskog klena primijenjen dodatni korak razdvajanja NMM citosolskih biomolekula (~5-20 kDa, dobivenih pomoću SEC_{200}-HPLC-a) na temelju njihovog naboja, primjenom AEC-HPLC-a.

Znanstvenici su u dosadašnjim istraživanjima utvrdili postojanje različitog broja izoformi MT u raznim vodenim organizmima. Tako su kod kalifornijske pasrtve (*O. mykiss*) (Kay i sur., 1986.), morskog ježinca (*Echinoid*ea) (Wilkinson i Nemer, 1987.) i oštrige

(*Ostrea edulis*) (Roesijadi i sur., 1989.) opisane dvije izoforme, dok su kod hlapa (*Homarus gammarus*) utvrđene tri izoforme MT. Istraživanje MT izoformi može značajno doprinijeti razumijevanju regulacije esencijalnih elemenata poput Cu i Zn te detoksikacije neesencijalnih metala poput Cd u različitim organizmima (Szpunar i Łobiński, 1999). Izoforme MT razlikuju se u aminokiselinskom sastavu, ne uključujući cistein, zbog čega imaju različite izoelektrične točke i različitu hidrofobnost, što omogućuje njihovo razdvajanje pomoću AEC-HPLC-a. AEC-HPLC razdvajanjem NMM biomolekula (~5-20 kDa) iz jetara i škrga vardarskog klena dobivena su dva dobro razdvojena AEC-pika koja su u oba organa imala identična vremena eluiranja. Jetreni AEC-pikovi sadržavali su sva tri analizirana metala (Cd, Cu i Zn), dok je AEC-pik u škrgama sadržavao samo Cd, a vezanje Zn u MT-frakciji škrga nije bilo potvrđeno. Prvi AEC-pik podudarao se s vremenom eluiranja standardne otopine izoforme MT-I, dok se drugi pik podudarao s vremenom eluiranja standardne otopine izoforme MT-II, potvrđujući vezanje Cd, Cu i Zn u jetrima te Cd u škrgama na dvije MT izoforme. Znanstvenici Duquesne i Richard (1994.) također su u svojim istraživanjima u jetrima dviju vrsta riba (obični iverak, *Limanda limanda*, i maloglavi iverak, *Microstomus kitt*) potvrdili postojanje dviju izoformi MT, s dominatnom prisutnošću izoforme MT-II. Nekoliko drugih autora također je izvijestilo o vezivanju Cd za dvije MT izoforme, MT-I i MT-II, u organima raznih vrsta riba: u jetrima jegulje (*A. anguilla*) i iverka (*L. limanda*) te u jetrima i škrgama zlatne ribice (*Carassius auratus*), žutog soma (*Pelteobagrus fulvidraco*), bezribice (*Pseudorasbora parva*) i grdobine (*Squaliobarbus curriculus*) (Giugere i sur., 2006.; Lacorn i sur., 2001.; Li i sur., 2018.). Izostanak vezanja Zn na MT u škrgama vardarskog klena bio je u skladu s našim prethodnim rezultatima. Uočene razlike između jetara i škrga u vezanju Zn na MT ukazale su na moguće razlike u fiziološkim ulogama izoformi MT u različitim tkivima i organima. Iako su u oba organa nađene obje izoforme, MT-I i MT-II, moguće je da se aminokiselinski slijedovi pojedinih izoformi razlikuju između organa te tako predstavljaju subizoforme MT-I i MT-II svojstvene škrgama i jetrima. Stoga je moguće da jetrene subizoforme metalotioneina koje sadrže i Zn i Cu imaju istovremeno ulogu i u detoksikaciji nesencijalnih i u pohrani esencijalnih metala te po potrebi mogu poslužiti kao izvor tih metala za druge proteine. S druge strane, moguće je da subizoforme MT svojstvene škrgama, koje sadrže Cd, a ne sadrže Zn, imaju prvenstveno ulogu u detoksikaciji. Nadalje, moguće je da te MT izoforme uz Cd sadrže i Cu, ali to nije bilo moguće utvrditi zbog jako niskih citosolskih koncentracija Cu u škrgama.

Za razliku od biomolekula koje vežu Fe, a kod kojih je nakon dvodimenzionalnog kromatografskog razdvajanja provedena analiza spektrometrijom masa, NMM biomolekule koje vežu Cd, Cu i Zn dodatno smo toplinski obradili prije daljnjih analiza, kao dodatni stupanj pročišćavanja toplinskih stabilnih proteina metalotioneina. Toplinski obrađeni citosoli jetara vardarskog klena razdvojeni su SEC_{75}-HPLC-om te je izdvojena NMM frakcija (~10-30 kDa), koja je nadalje frakcionirana pomoću AEC-HPLC-a, čime je potvrđeno vezanje Cd, Cu i Zn na dvije MT izoforme, MT-I i MT-II. Dvije dobivene AEC-frakcije analizirali smo pomoću MALDI-TOF-MS-a, kako bismo odredili njihovu točnu molekulsku masu i utvrdili postoje li uočljive razlike u masama MT-I i MT-II izoformi iz jetara vardarskog klena. Na temelju dobivenih masenih spektara utvrđeno je kako obje MT izoforme u jetrima vardarskog klena imaju jednaku molekulsku masu od 6,0 kDa, što je samo 100-200 Da niže od molekulskih masa standarda MT-I (6,2 kDa) i MT-II (6,1 kDa), koji se međusobno razlikuju za samo 100 Da. Uzimajući u obzir točnost MALDI-TOF-MS instrumenta, kao i mogućnost razlika u zasićenju MT ili u sadržaju specifičnih metala vezanih na MT, koji mogu rezultirati razlikama od nekoliko stotina Da u masi (Chassaigne i Łobiński, 1998), naša su istraživanja pokazala da je masa dviju MT izoformi izoliranih iz jetara vardarskog klena podudarna s masama analiziranih MT-I i MT-II standarda. Budući da se MT izoforme međusobno vrlo malo razlikuju u masi (Carpené i Vašák, 1989), očigledno ih nije moguće razdvojiti samo primjenom SEC-HPLC-a te je vidljiva važnost dodatne primjene AEC-HPLC-a, odnosno dvodimenzionalnog razdvajanja. Carpené i Vašák (1989.) identificirali su dvije izoforme MT (MT-I i MT-II) u jetrima zlatne ribice (*C. auratus*) koristeći također anionsko izmjenjivačku kromatografiju i sekvencioniranje aminokiselina, dok se uporaba dvodimenzionalnog pristupa razdvajanju MT (SEC-AEC-HPLC) često primjenjuje u istraživanjima različitih vodenih organizmima, poput riba iverka (*L. limanda*; Lacorn i sur., 2001.), babuške (*C. auratus gibelio*; Goenaga Infante i sur., 2006.), jegulje (*A. anguilla*; Rodríguez-Cea i sur., 2003.), morskog soma (*Netuma barba*) i bisernog ciklida (*Geophagus brasiliensis*) (Rodríguez-Cea i sur., 2003.) te mekušaca (Maltez i sur., 2009.).

Daljnja sličnost između dviju izoformi MT iz jetara vardarskog klena i dvaju standarda (MT-I i MT-II) odnosi se na činjenicu da su i kod jednih i kod drugih u masenim spektrima detektirani i niži pikovi na 12,0 kDa za jetra te 12,3-12,4 kDa za MT standarde, a koji vjerojatno odgovaraju MT dimerima. Da osim MT izoformi postoje i MT varijante različitih molekulskih masa, odnosno monomeri (10 kDa, MT-10) i dimeri (20 kDa, MT-20), potvrđeno je u prijašnjim istraživanjima na raznim organizmima, i to na ribama

(mozambičkoj tilapiji (*Oreochromis mossambicus*), Chan, 1994.; Wu i sur., 1999.; vrsti iz reda Somovki (*Heteropneustes fossilis*), Chatterjee i Maiti, 1987.; morskoj mački bljedici (*Scyliorhinus canicula*), Hidalgo i sur., 1988.), na štakorima (Hidalgo i sur., 1988.), na školjkašima (oštrigama (*O. edulis*), Fowler i sur., 1986.; dagnjama (*Mytilus galloprovincialis*), Frazier i sur., 1985.; Ivanković i sur., 2002.) i na puževima (*Gastropoda*) (Langston i Zhou, 1978.).

Toplinski obrađeni citosoli škrga vardarskog klena također su bili razdvojeni SEC_{75}-HPLC-om te je izdvojena NMM frakcija (~10-30 kDa). No, ona nije dalje frakcionirana pomoću AEC-HPLC-a zbog nemogućnosti detekcije uslijed prevelikog razrijeđenja, već je provedena izravna analiza pomoću MALDI-TOF-MS-a. U dobivenim masenim spektrima NMM frakcije škrga zabilježena su tri *m/z* pika, od kojih je najuočljiviji bio *m/z* pik na 4,9 kDa, koji zbog svoje relativno niske molekulske mase nije djelovao kao da predstavlja pik metalotioneina. No, i Maltez i sur. (2009.) su pronašli izraziti pik kadmija na 5,1 kDa provodeći istraživanje na pužu (*Marisa cornuarietis*). Iako su u početku zaključili kako je ova molekulska masa preniska u usporedbi s već klasificiranim metalotioneinima ili metalotioneinu sličnim proteinima (MLP), analizirajući aminokiselinski slijed ipak su zaključili kako je moguće da MLP izoforma na 5,1 kDa odgovara metalotioneinu. U masenim spektrima škrga vardarskog klena zabilježena su i dva manja *m/z* pika, jedan na 6,2 kDa koji odgovara molekulskoj masi standarda MT (6,1-6,2 kDa) te drugi na 3,1 kDa koji se vjerojatno odnosi na dvostruko nabijeni molekulski ion MT. Upotreba ovakvog pristupa analizi biomolekula primjenom masene spektrometrije nakon samo jednog stupnja razdvajanja citosola (SEC-HPLC) očito ima svoje nedostatke, budući da ne osigurava mogućnost pouzdanog razlučivanja dviju MT izoformi. No, s druge strane, rezultati dobiveni našim istraživanjima ukazali su na postojanje razlika MT prisutnih u jetrima i u škrgama. Kao prvo, naše otkriće da vezanje Zn na MT u škrgama, za razliku od MT u jetrima, nije bilo moguće detektirati, u skladu je s otkrićem Noël-Lambota i sur. (1978.) da MT u škrgama jegulja (*A. anguilla*), za razliku od MT u jetrima, vežu vrlo male količine Zn i Cu. Ovi su znanstvenici također tvrdili kako MT nije prirodno prisutan u škrgama, već njegovu sintezu u škrgama inducira izloženost kadmiju (Noël-Lambota i sur., 1978.). Van Campenhout i sur. (2004.) primjetili su sličnu razliku između MT u jetrima i bubrezima šarana (*C. carpio*), gdje je samo 2% citosolskog Zn bilo vezano za MT u bubrezima, a više od 30% u jetrima. Razlika između MT u škrgama i MT u jetrima vardarskog klena dodatno je potvrđena činjenicom da je u škrgama dominantno prisutna MT izoforma niže molekulske mase (~5 kDa), uz prisutnost vrlo male količine uobičajene izoforme MT, molekulske mase 6 kDa.

4.3.3. Toplinski stabilne biomolekule koje vežu Mo

U znanstvenoj je literaturi navedeno kako zasad nije provedena molekularna karakterizacija transportera molibdena, niti proteina za pohranjivanje Mo u ribama, kao ni genomička i proteomička istraživanja riba izloženih molibdenu (Reid, 2011.). Tijekom istraživanja na vardarskim klenovima uzorkovanima 2012. godine, analizom toplinski obrađenih citosola jetara i škrga utvrdili smo postojanje toplinski stabilnih citosolskih biomolekula koje vežu Mo, molekulskih masa u rasponu ~5-10 kDa. Kako su te biomolekule zasad neistražene, cilj nam je bio provesti njihovu dodatnu karakterizaciju u jetrima i škrgama vardarskih klenova uzorkovanih 2015. godine te je u tu svrhu ponovljeno razdvajanje citosola pomoću SEC_{75}-HPLC-a, prije i poslije toplinske obrade. U jetrenim citosolima prije toplinske obrade najveći je dio Mo bio raspodijeljen u području SMM biomolekula (~30-140 kDa), dok je samo manji dio bio prisutan u području JNMM biomolekula (~5-10 kDa). No, nakon toplinske obrade citosola, Mo-SMM pik bio je gotovo potpuno uklonjen, dok je uočeno izrazito povećanje Mo-JNMM pika, ukazujući na mogućnost da je dio Mo prešao iz SMM područja u JNMM područje. Pretpostavka je da se to događa uslijed raspadanja toplinski osjetljivih Mo-SMM proteina u manje, toplinski stabilne molekule jako niskih molekulskih masa. Nasuprot tome, Mo je u škrgama i prije i poslije toplinske obrade bio vezan na JNMM biomolekule (~5-10 kDa). Profili raspodjele Mo dobiveni za vardarske klenove iz 2015. godine podudarali su se s rezultatima dobivenim za vardarske klenove uzorkovane 2012. godine.

Kako dodatno razdvajanje pomoću AEC-HPLC-a nije bilo moguće provesti zbog niskih citosolskih koncentracija Mo, molibdenske JNMM frakcije iz obaju organa (~5-10 kDa) dobivene pomoću SEC_{75}-HPLC-a izravno su analizirane primjenom MALDI-TOF-MS-a. U masenim spektrima obaju organa bila su prisutna dva jasno razlučena *m/z* pika. U jetrima je *m/z* pik najvećeg intenziteta bio zabilježen na 8,5 kDa, dok je drugi niži *m/z* pik bio zabilježen na 4,2-4,4 kDa te se vjerojatno radilo o dvostruko nabijenom molekuskom ionu. Za razliku od jetre, u škrgama je *m/z* pik većeg intenziteta bio zabilježen na 3,3 kDa, dok je niži *m/z* pik bio zabilježen na 8,5 kDa te je odgovarao masi glavnog jetrenog *m/z* pika. Moguće je pretpostaviti kako se biomolekule molekulske mase 8,5 kDa, dominantno prisutne u jetrima, a puno manje u škrgama, odnose na JNMM biomolekule koje vežu Mo, a koje se u citosolu pojavljuju tek nakon toplinske obrade, vjerojatno raspadanjem SMM biomolekula. Nasuprot

tome, manje biomolekule na ~3 kDa, dominantno prisutne u škrgama, vjerojatno se odnose na toplinski stabilne biomolekule koje su prisutne u citosolu škrga čak i prije toplinske obrade. Budući da zbog upotrebe spektrometra masa niskog razlučivanja, LC-MS/MS analiza s naknadnim Mascot pretraživanjem nije rezultirala definitivnim prepoznavanjem tih dviju toplinskih stabilnih biomolekula koje vežu Mo, analize smo proširili na njihovo sekvenciranje koje je trenutno u tijeku. U masenim spektrima u jetrima zabilježeno je i nekoliko manjih pikova na 6,9 kDa i 9,6 kDa, koji zasad nisu identificirani, također zbog niskog razlučivanja primijenjenog spektrometra masa.

ZAKLJUČCI

5. ZAKLJUČCI

1. Analiziranje raspodjela odabranih elemenata među citosolskim biomolekulama u jetrima i škrgama dvaju srodnih bioindikatorskih organizama, klena (*Squalius cephalus*) i vardarskog klena (*Squalius vardarensis*), kombiniranom primjenom kromatografskih tehnika i nekoliko tehnika spektrometrije masa provedeno u sklopu izrade ove doktorske disertacije predstavlja početni korak u istraživanju unutarstanične sudbine i ponašanja metala u organima riba. Dobiveni rezultati predstavljaju osnovu za razvoj novih biomarkera izloženosti, odnosno toksičnih učinaka metala.

2. Primjenom SEC-HPLC-a i HR ICP-MS-a određene su raspodjele esencijalnih elemenata Co, Cu, Fe, Mn, Mo, Se i Zn te neesencijalnih elemenata Cd i Pb među citosolskim biomolekulama različitih molekulskih masa u jetrima i škrgama klena (*S. cephalus*) iz umjereno onečišćene rijeke Sutle i vardarskog klena (*S. vardarensis*) iz makedonskih rijeka pod utjecajem aktivnih rudnika, pri uvjetima niskih i povišenih izloženosti metalima u riječnoj vodi. Od dobivenih se rezultata posebno može istaknuti sljedeće:

    - raspodjela kobalta predominantno u području biomolekula visokih molekulskih masa (~20-410 kDa) te u dva područja biomolekula jako niskih molekulskih masa (~2-9 kDa i <2 kDa) u jetrima obiju vrsta klena te u škrgama klena *S. cephalus*; biomolekule molekulskih masa ispod 2 kDa odgovaraju molekulskoj masi kobalamina;
    - raspodjela bakra predominantno u području biomolekula niskih molekulskih masa (~5-40 kDa), čija molekulska masa i vrijeme eluiranja odgovaraju MT u jetrima obiju vrsta klena te u škrgama klena *S. cephalus*;
    - raspodjela željeza u području biomolekula visokih molekulskih masa (~140-700 kDa) koje obuhvaća molekulsku masu feritina, proteina koji služi za pohranu željeza, te u području biomolekula srednjih molekulskih masa (~10-80 kDa) u jetrima i škrgama obiju vrsta klena;
    - raspodjela mangana u širokom području molekulskih masa od 2 do 400 kDa, s najvećom prisutnošću u području biomolekula visokih molekulskih masa (~60-400 kDa) te znatno manjom u području biomolekula srednjih molekulskih masa (~25-85kDa) koje obuhvaćaju i molekulske mase transportnih proteina

albumina i transferina, u jetrima obiju vrsta klena i u škrgama klena *S. cephalus*;

- raspodjela molibdena predominantno u području biomolekula visokih molekulskih masa (~60 do >600 kDa), koje obuhvaćaju molekulske mase enzima poput aldehid oksidaze, sulfit oksidaze i Fe-Mo flavoprotein ksantin oksidaze, te raspodjela manjeg dijela Mo među biomolekulama niskih molekulskih masa (~3-12 kDa) u jetrima obiju vrsta klena;
- raspodjela selena u širokom području molekulskih masa (~1-400 kDa), i to predominantno vezanog na biomolekule niskih molekulskih masa (~20-60 kDa) i jako niskih molekulskih masa (<5 kDa) u jetrima obiju vrsta klena; u škrgama obiju vrsta klena većina Se je bila raspodijeljena među biomolekulama jako niskih molekulskih masa, ispod 5 kDa, koje obuhvaćaju molekulske mase poznatih selenospojeva, selenoneina i selenometionina, koji sudjeluju u obrani organizma od oksidativnog stresa;
- raspodjela cinka u području biomolekula visokih molekulskih masa (>400 kDa) i srednjih molekulskih masa (~10-380 kDa) u jetrima i škrgama obiju vrsta klena te izražena prisutnost u području biomolekula niskih molekulskih masa (~7-27 kDa), koje odgovaraju molekulskoj masi proteina MT, samo u jetrima obiju vrsta klena; samo u škrgama klena *S. cephalus* Zn se pojavljuje i u području biomolekula jako niskih molekulskih masa (<5 kDa);
- raspodjela kadmija predominantno u području biomolekula niskih molekulskih masa (~5-30 kDa), što odgovara molekulskoj masi i vremenu eluiranja proteina MT u jetrima i škrgama obiju vrsta klena;
- raspodjela olova predominantno u području biomolekula srednjih molekulskih masa (~35-240 kDa) u jetrima klena, što može ukazivati na njegov potencijal za toksično djelovanje; manji dio olova bio je raspodijeljen u području biomolekula niskih molekulskih masa (~9-20 kDa), što odgovara području eluiranja MT.

3. Analiza utjecaja različitih razina izloženosti metalima u riječnoj vodi na njihovu citosolsku raspodjelu u jetrima i škrgama klena i vardarskog klena pokazala je da povećanje izloženosti ne dovodi do kvalitativnih promjena u raspodjeli tih metala među citosolskim biomolekulama. Pri povišenoj izloženosti dolazi samo do kvantitativnih promjena, odnosno do povećanog vezanja pojedinih metala na iste biomolekule na koje se vežu i pri niskoj izloženosti, poput vezanja Cd na MT.

4. Raspodjela pojedinih metala među citosolskim biomolekulama različita je u jetrima i škrgama obiju vrsta klenova (*S. cephalus* i *S. vardarensis*) ovisno o različitim funkcijama organa. Na primjer, željezo je u jetrima većinom vezano na biomolekule visokih molekulskih masa, vjerojatno skladišni protein feritin, dok je u škrgama to vezanje zanemarivo, a većina je Fe vezana na biomolekule srednjih molekulskih masa. Nadalje, za cink je u jetrima uočeno izrazito vezanje na protein MT koji sudjeluje u održavanju homeostaze cinka, dok u škrgama takvo vezanje nije zabilježeno.

5. Raspodjele metala (Co, Cu, Fe, Mn, Mo, Se, Zn i Cd) među citosolskim biomolekulama pri niskoj i povišenoj izloženosti usporedive su u dvjema srodnim vrstama klena (*S. cephalus* i *S. vardarensis*), što omogućuje usporedbu rezultata dobivenih njihovom primjenom u monitoringu udaljenih slatkovodnih sustava. Manje razlike zabilježene su za citosolske raspodjele Zn i Cd u jetrima dviju vrsta. Iako je u jetrima obiju vrsta klena uočeno vezanje Zn i Cd na niskomolekulske biomolekule, vjerojatno MT, to je vezanje bilo puno jasnije izraženo u jetrima vardarskog klena, što možda ukazuje na snažniju sposobnost detoksikacije te vrste ili na prilagodbu uvjetima povećane izloženosti metalima u rijekama onečišćenim rudničkim otpadom.

6. Analizom toplinski obrađenih citosola dvaju organa vardarskog klena primjenom SEC-HPLC-a i HR ICP-MS-a potvrđena je prisutnost toplinskih stabilnih biomolekula (~10-30 kDa) koje vežu Cd, Cu i Zn u jetrima te Cd u škrgama. Utvrđeno je i postojanje toplinski stabilnih biomolekula vrlo niskih molekulskih masa (≤10 kDa) koje vežu Mo i Se.

7. Primjenom dviju tehnika spektrometrije masa (MALDI-TOF-MS i LC-MS/MS) nakon dvodimenzionalnog kromatografskog razdvajanja provedeno je precizno određivanje molekulskih masa i identifikacija nekoliko odabranih biomolekula koje vežu Fe, Cd, Cu, Zn i Mo u jetrima i škrgama vardarskog klena:

    - analizom biomolekula srednjih molekulskih masa (~20-50 kDa) koje vežu Fe, primjenom MALDI-TOF-MS-a utvrđena je prisutnost molekulskih iona na ~15.5 kDa, ~31kDa i ~47 kDa u jetrima, što odgovara monomerima, dimerima i trimerima hemoglobinskih podjedinica; u škrgama je utvrđena prisutnost molekulskih iona i na nižim masama (na ~11 kDa, ~13 kDa i ~15 kDa); uporabom LC-MS/MS-a u oba je organa identificirana β podjedinica hemoglobina, dok je α-podjedinica identificirana samo u škrgama;
    - AEC-HPLC razdvajanjem citosolskih biomolekula niskih molekulskih masa (~5-25 kDa) utvrđeno je vezanje Cd, Cu i Zn u jetrima te Cd u škrgama na dvije MT izoforme, MT-I i MT-II;
    - toplinski stabilnim biomolekulama niskih molekulskih masa koje vežu Cd (~10-30 kDa), primjenom MALDI-TOF-MS-a određena je molekulska masa od 6,0 kDa u jetrima te 4,9 kDa u škrgama, što odgovara literaturnim podacima za MT;
    - toplinski stabilnim biomolekulama jako niskih molekulskih masa (~5-10 kDa) koje vežu Mo, primjenom MALDI-TOF-MS-a određene su molekulske mase od 3,3 kDa i 8,5 kDa, pri čemu su biomolekule mase 8,5 kDa dominantno prisutne u jetrima, a biomolekule mase 3,3 kDa u škrgama.

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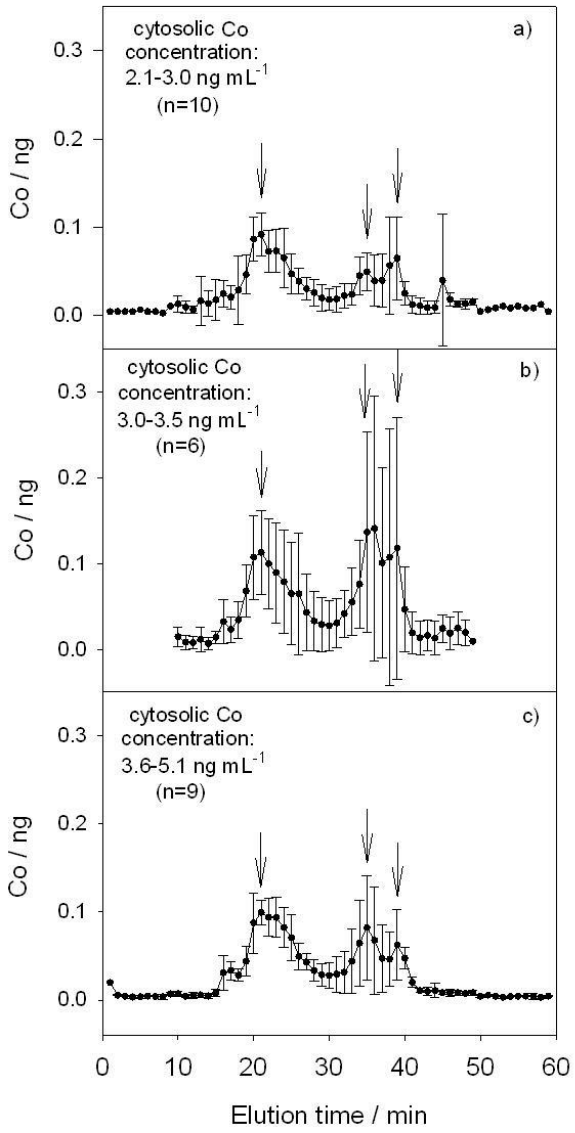
PRILOZI

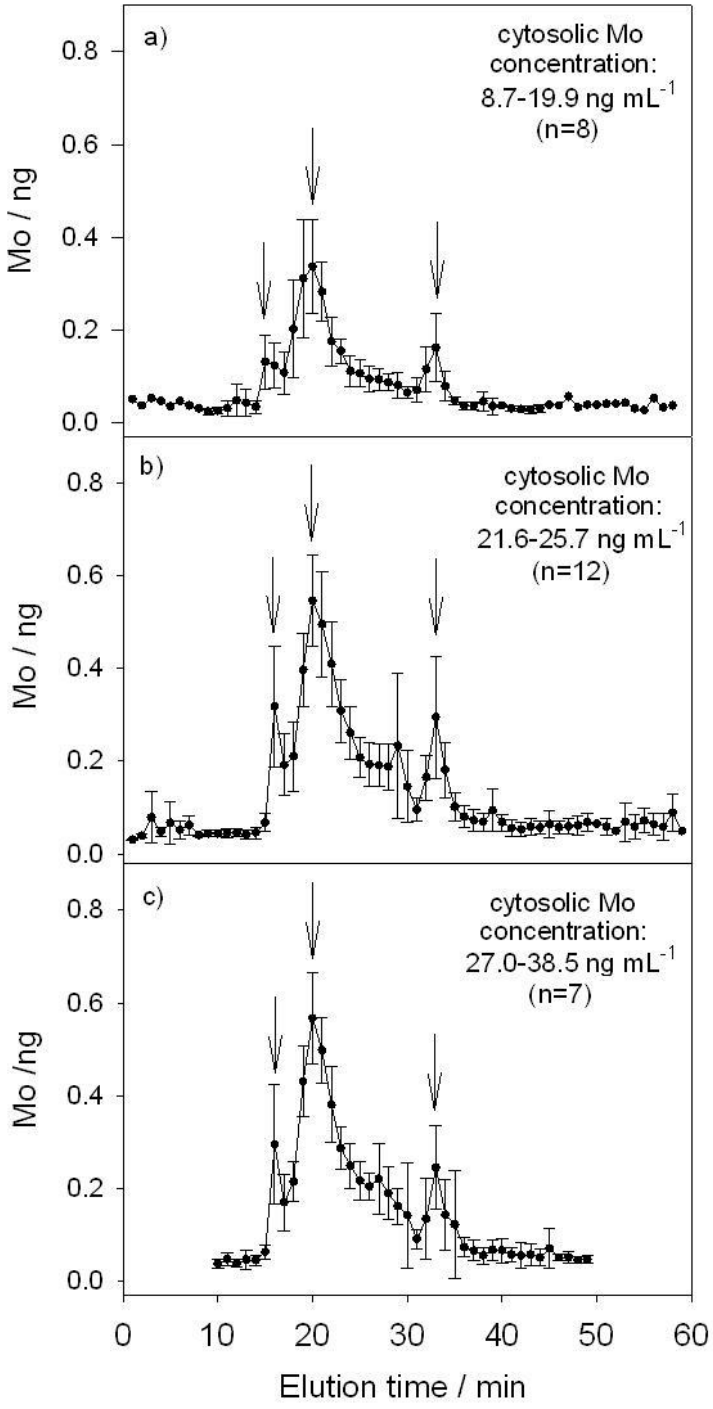
7. PRILOZI

7.1. Dodatne informacije uz rad:

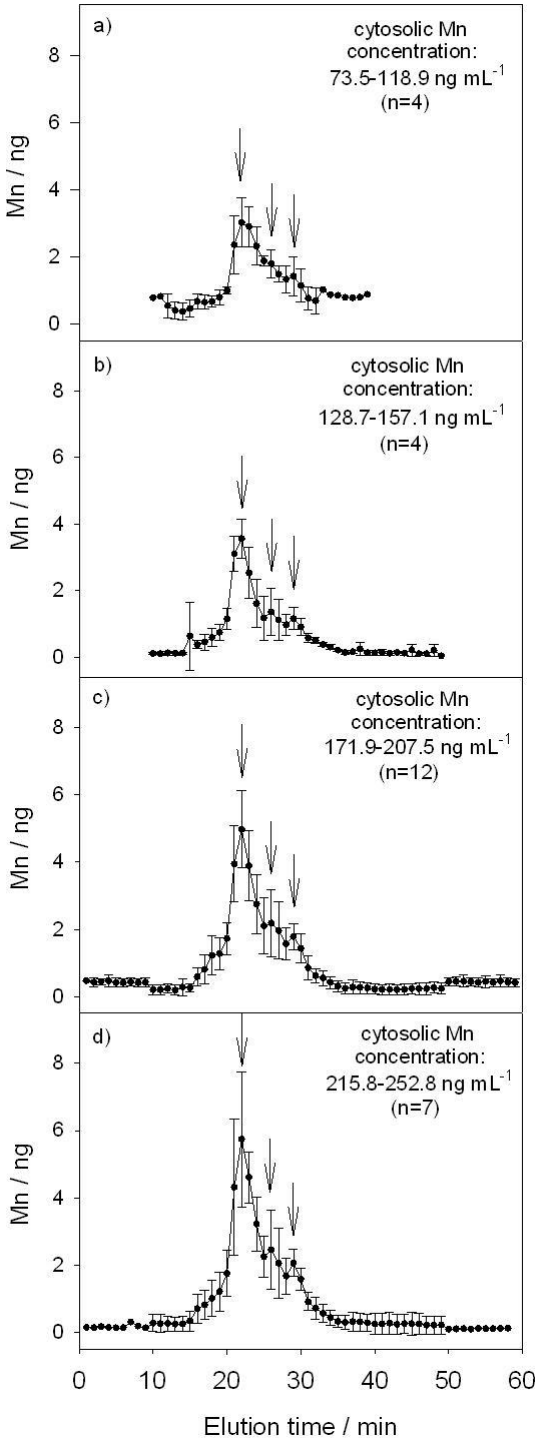
Krasnići N, Dragun Z, Erk M, Raspor B: Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se, Zn) and nonessential (Cd, Pb) trace elements among protein fractions from hepatic cytosol of European chub (*Squalius cephalus* L.). Environmental Science and Pollution Research 20 (2013), 4: 2340-2351. doi:10.1007/s11356-012-1105-8



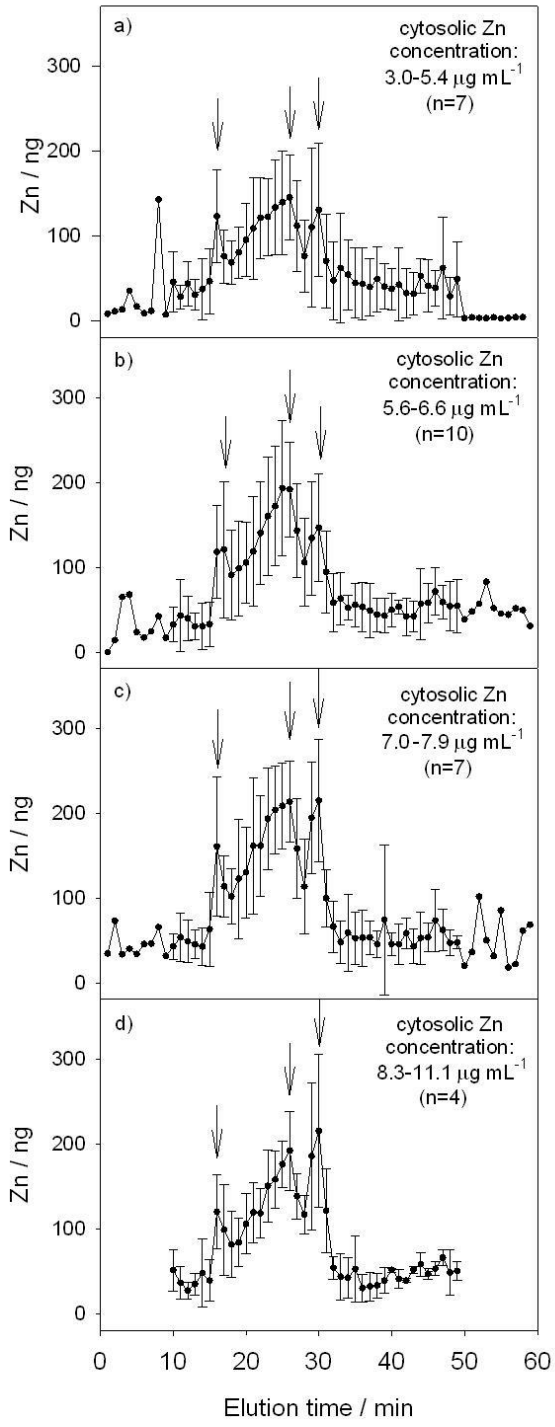
Slika 1. Raspodjele Co među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u tri skupine, prema citosolskim koncentracijama Co (ng mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI I**.



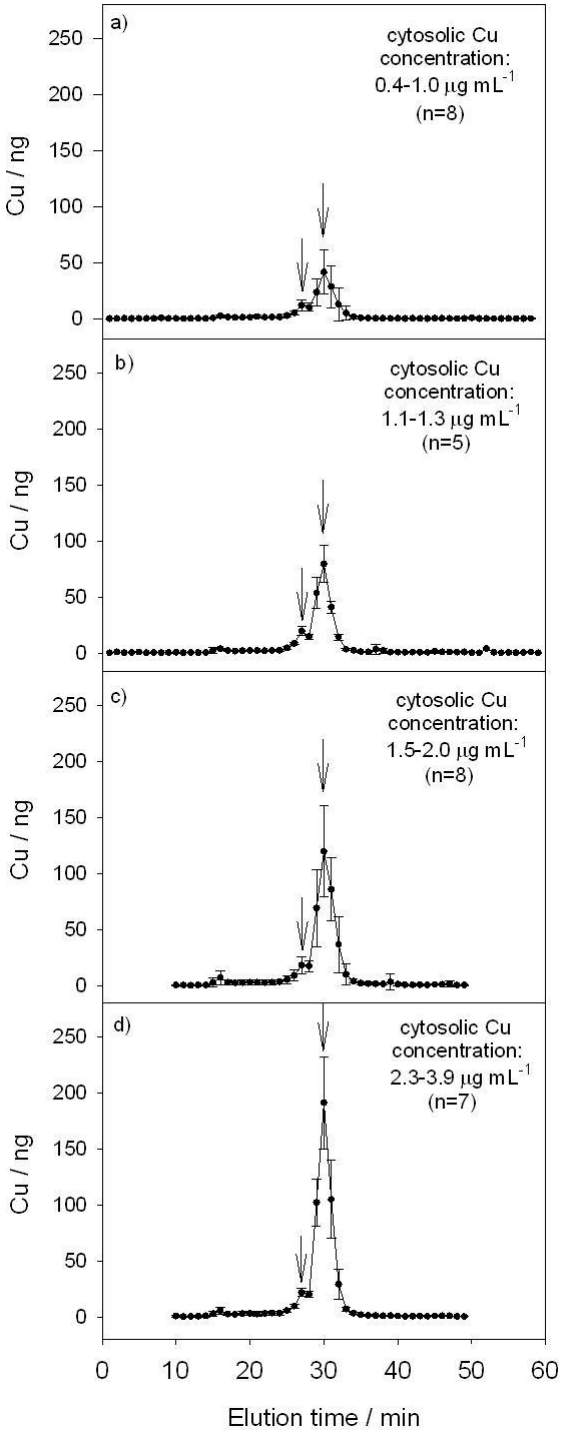
Slika 2. Raspodjele Mo među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u tri skupine, prema citosolskim koncentracijama Mo (ng mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI II**.



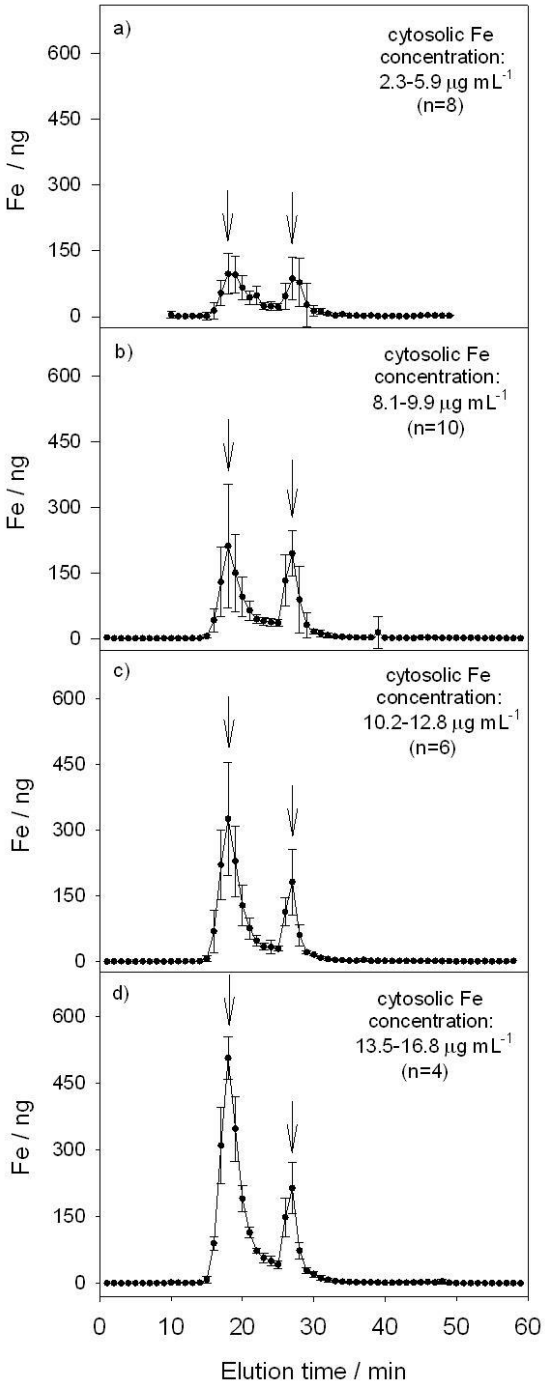
Slika 3. Raspodjele Mn među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u četiri skupine, prema citosolskim koncentracijama Mn (ng mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI III**.



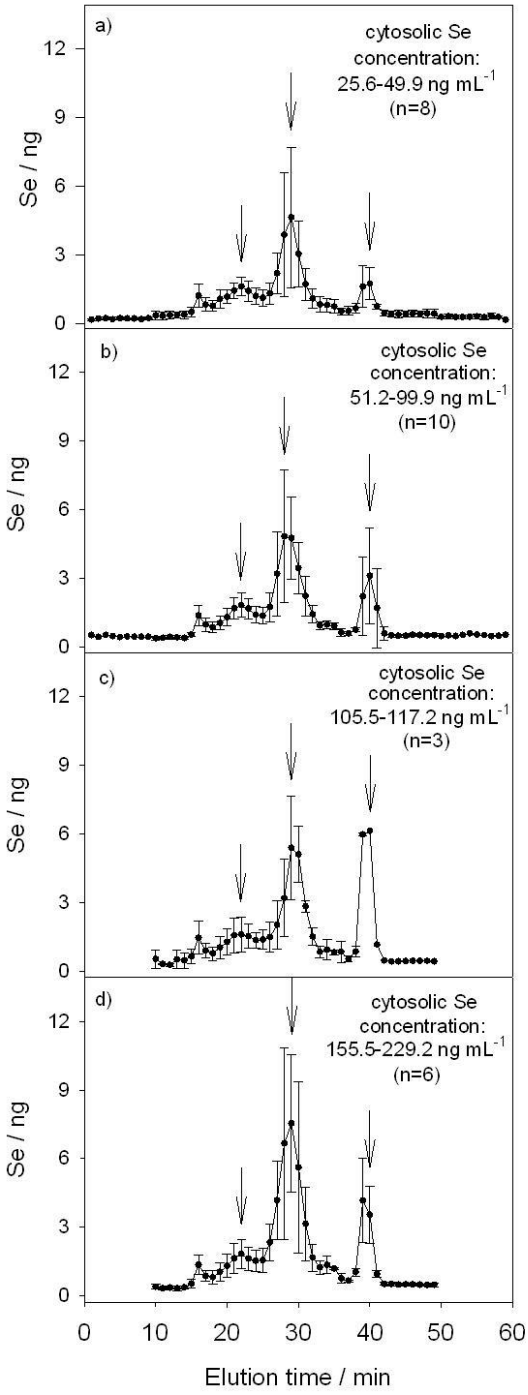
Slika 4. Raspodjele Zn među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u četiri skupine, prema citosolskim koncentracijama Zn (μg mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI IV**.



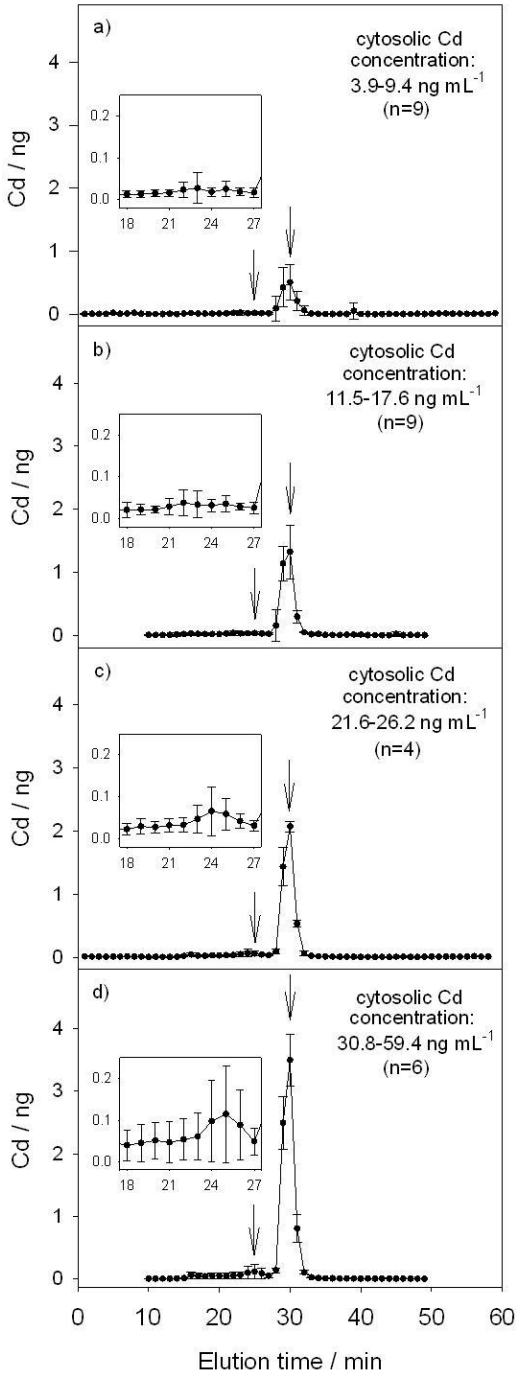
Slika 5. Raspodjele Cu među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u četiri skupine, prema citosolskim koncentracijama Cu (μg mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI V**.



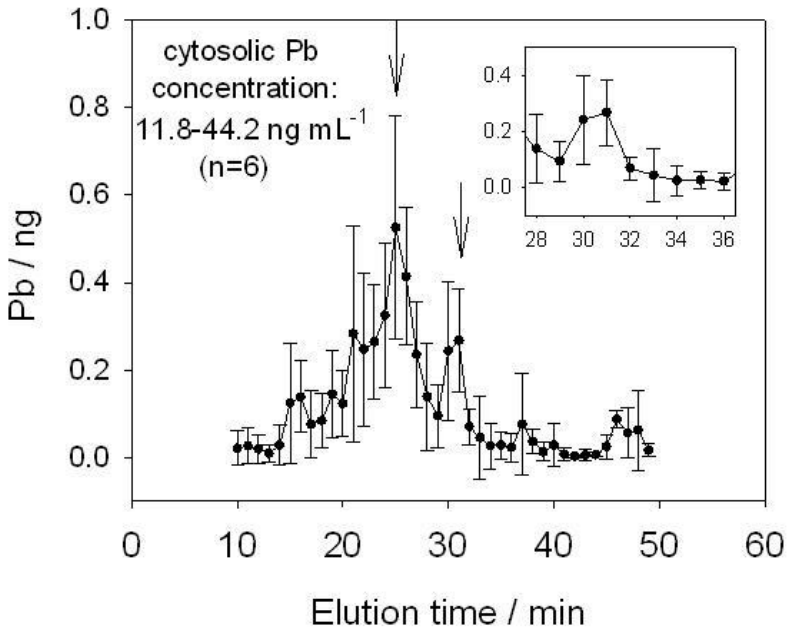
Slika 6. Raspodjele Fe među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u četiri skupine, prema citosolskim koncentracijama Fe (μg mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI VI**.



Slika 7. Raspodjele Se među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u četiri skupine, prema citosolskim koncentracijama Se (ng mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI VII**.



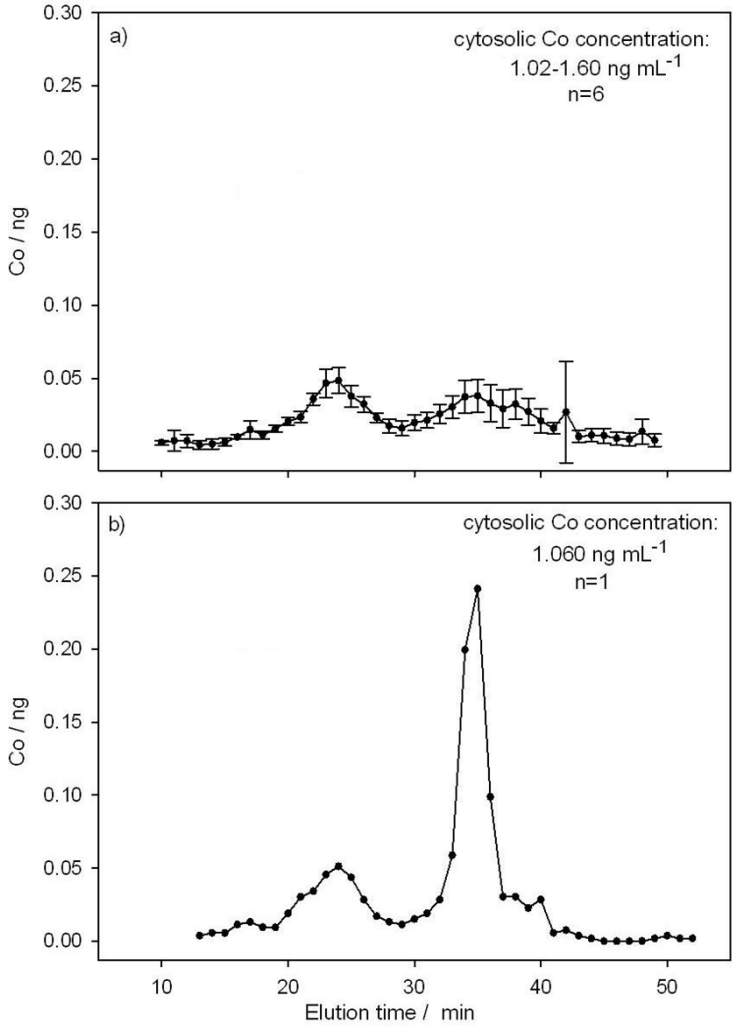
Slika 8. Raspodjele Cd među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u četiri skupine, prema citosolskim koncentracijama Cd (ng mL^{-1}) u jetrima klenova. Oznaka slike u radu **ESI VIII**.



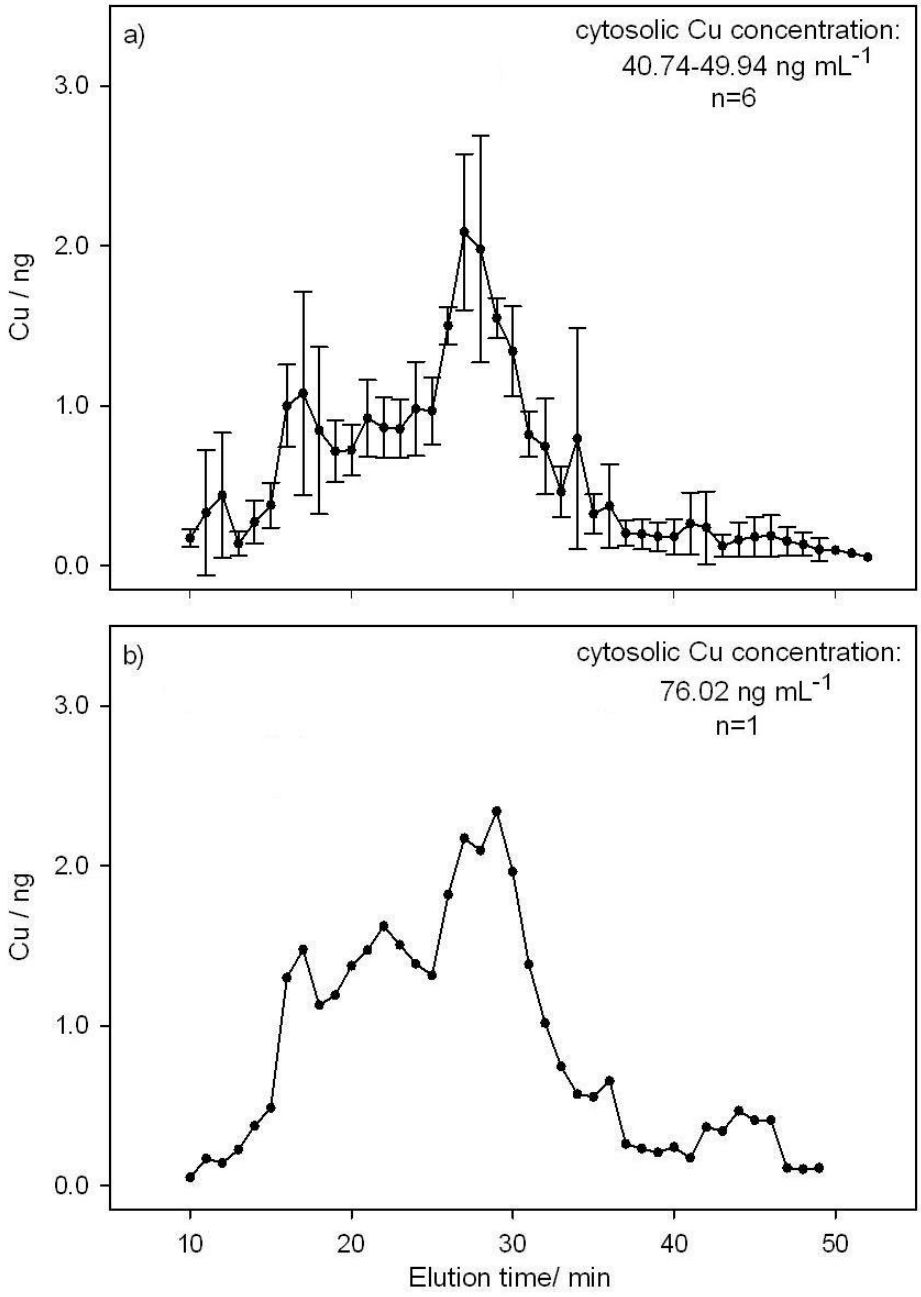
Slika 9. Raspodjele Pb među biomolekulama različitih molekulskih masa u citosolima jetara klenova (*S. cephalus*) dobivene SEC-HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Oznaka slike u radu **ESI IX**.

7.2. Dodatne informacije uz rad:

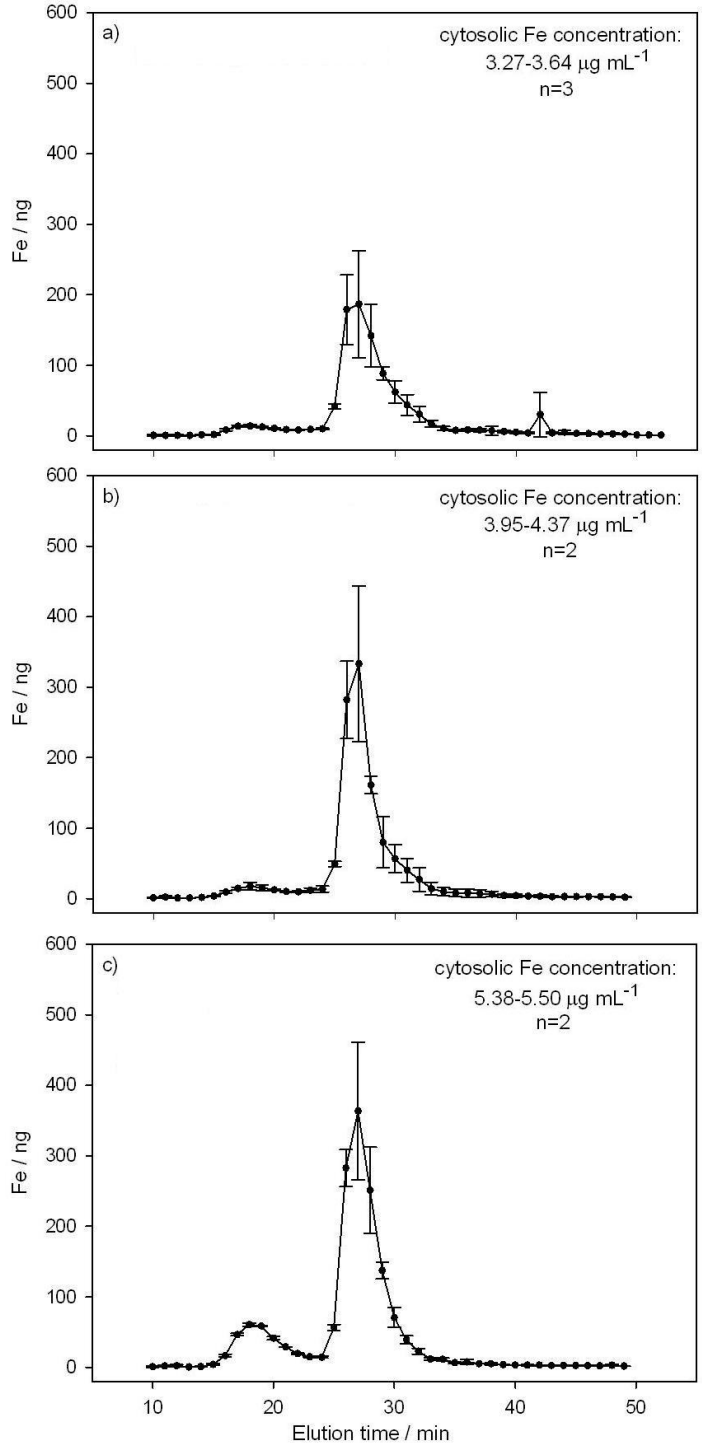
Krasnići N, Dragun Z, Erk M, Raspor B: Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (*Squalius cephalus* L.). Environmental Science and Pollution Research 21 (2014), 23: 13512-13521. doi:10.1007/s11356-014-3274-0



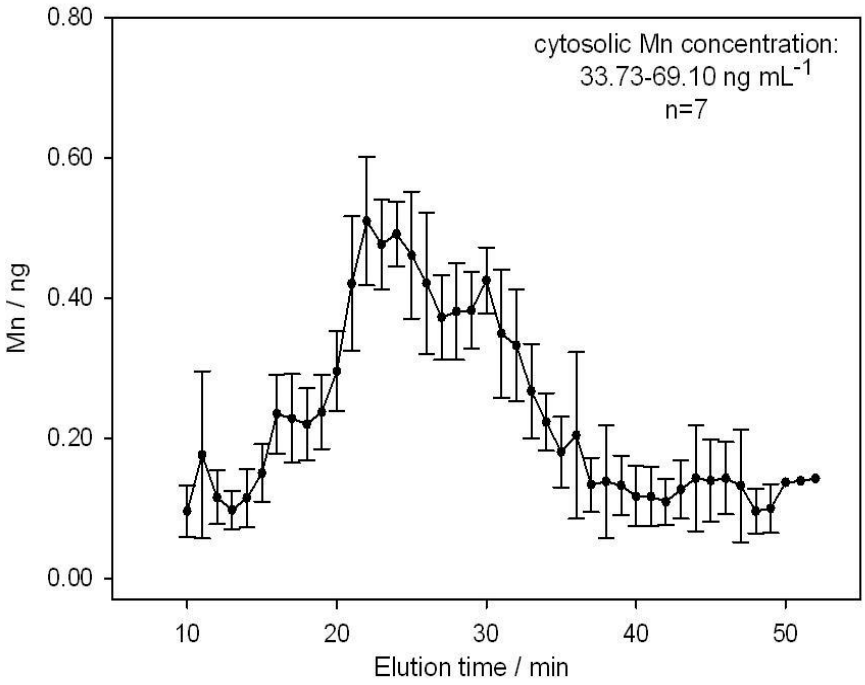
Slika 10. Profili raspodjele Co među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u dvije skupine, prema citosolskim koncentracijama Co (ng mL^{-1}) u škrgama klenova. Oznaka slike u radu **SI-1**.



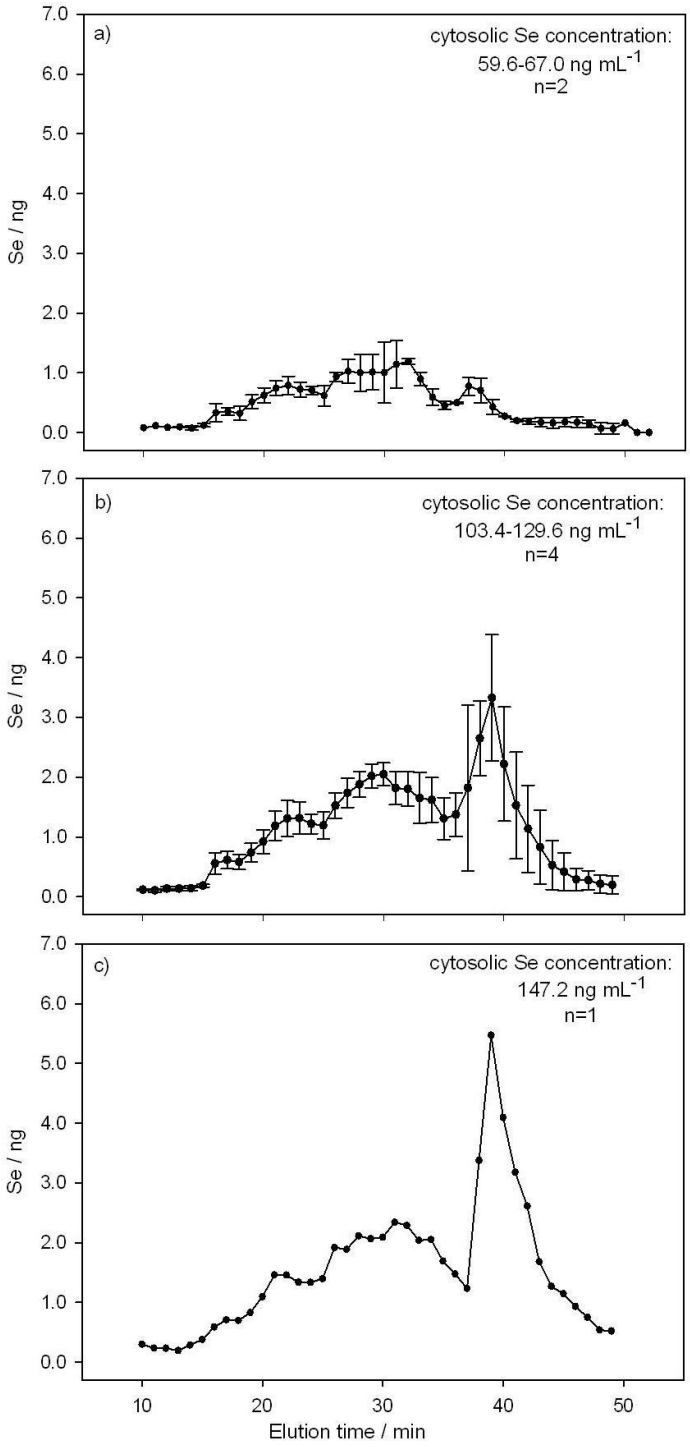
Slika 11. Profili raspodjele Cu među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u dvije skupine, prema citosolskim koncentracijama Cu (ng mL^{-1}) u škrgama klenova. Oznaka slike u radu **SI-2**.



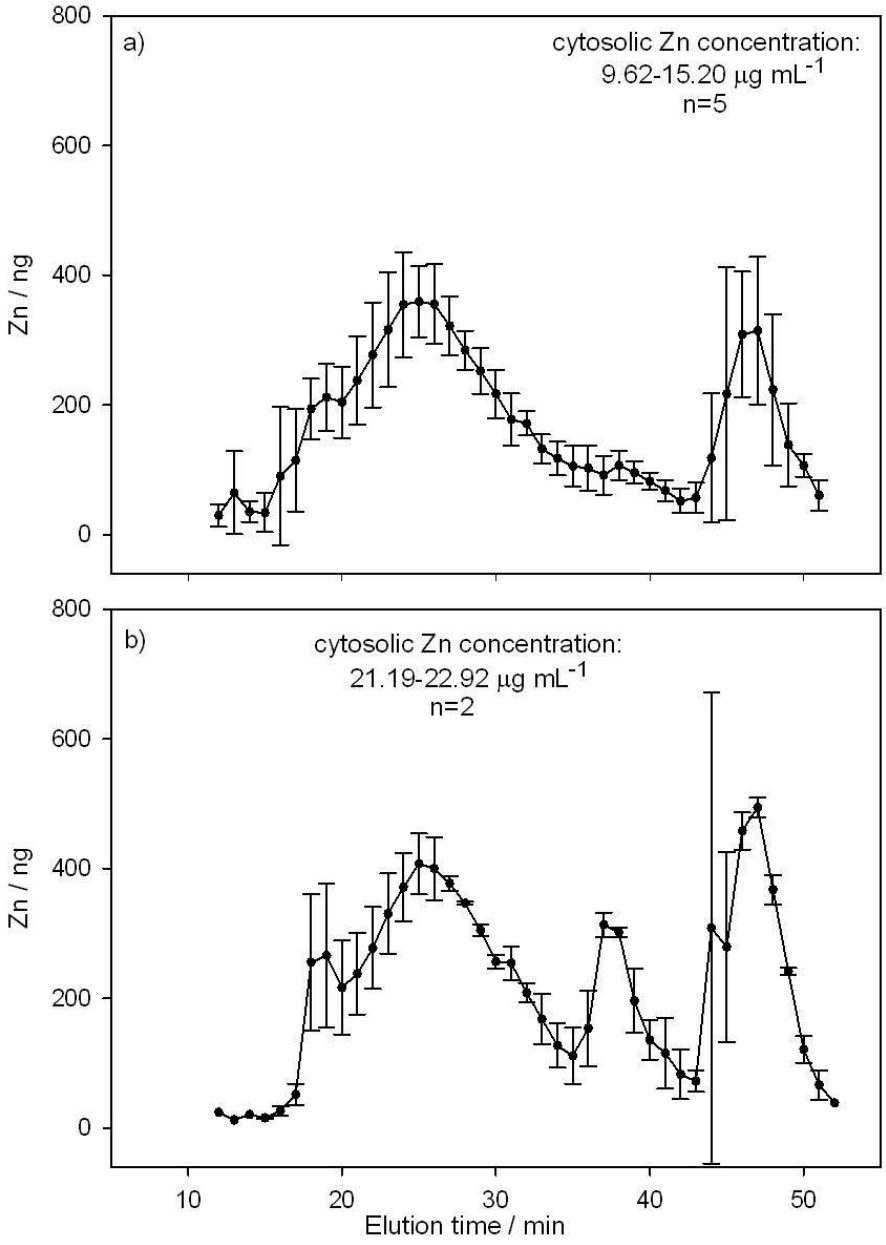
Slika 12. Profili raspodjele Fe među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u tri skupine, prema citosolskim koncentracijama Fe (μg mL^{-1}) u škrgama klenova. Oznaka slike u radu **SI-3**.



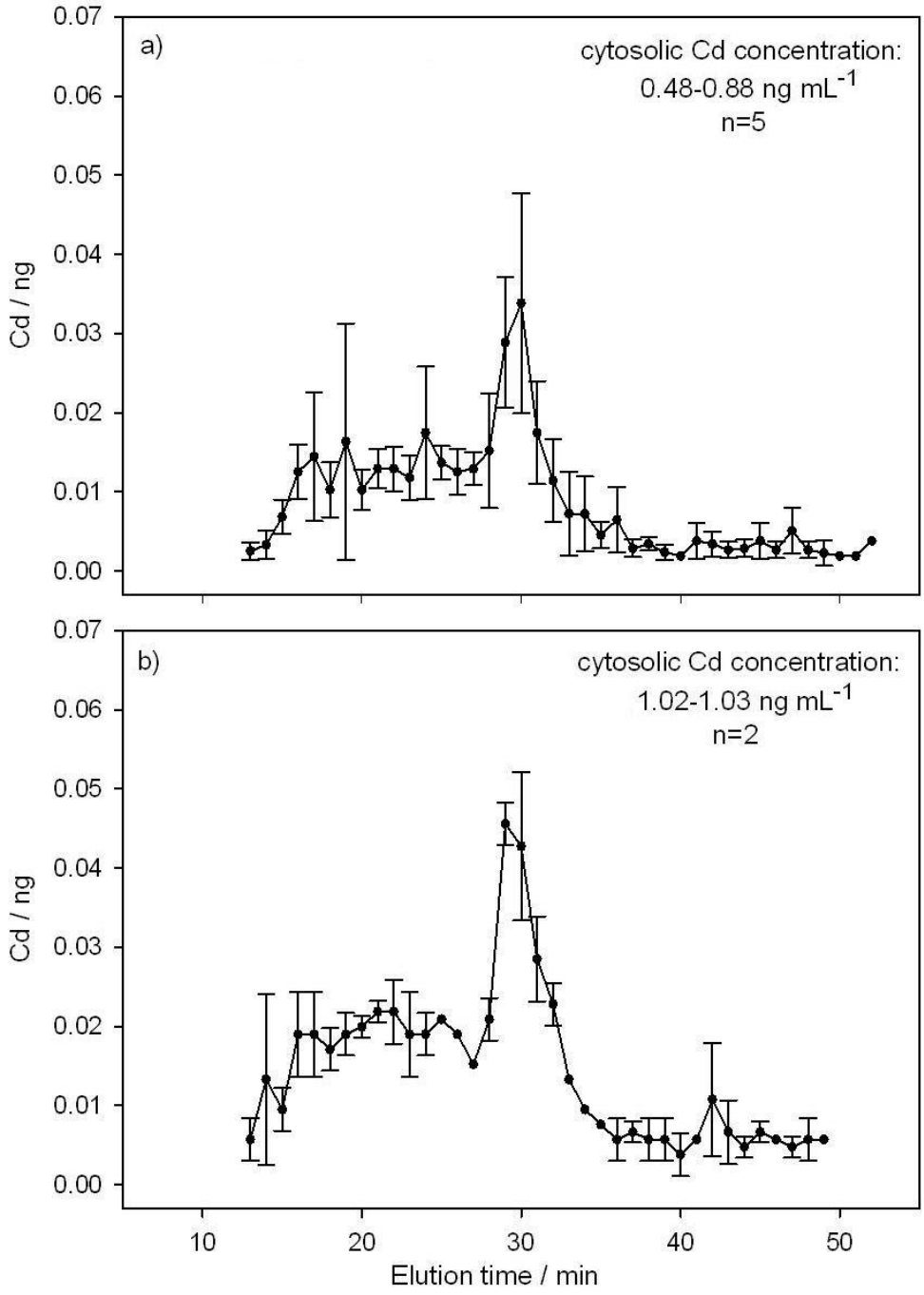
Slika 13. Profili raspodjele Mn među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Oznaka slike u radu **SI-4**.



Slika 14. Profili raspodjele Se među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u tri skupine, prema citosolskim koncentracijama Se (ng mL^{-1}) u škrgama klenova. Oznaka slike u radu **SI-5**.



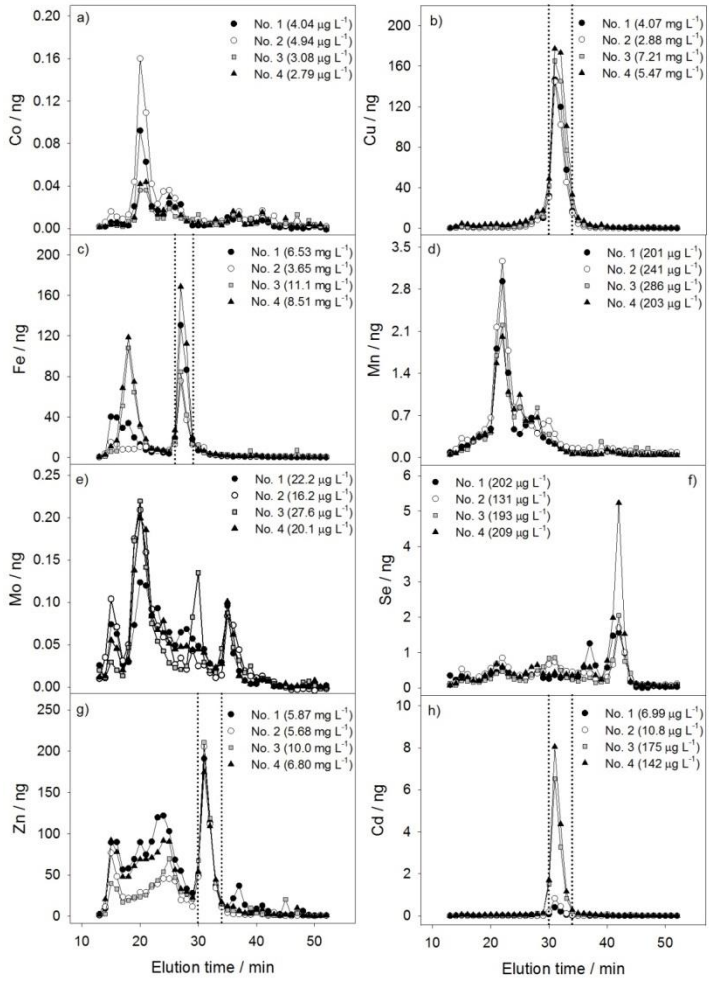
Slika 15. Profili raspodjele Zn među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u dvije skupine, prema citosolskim koncentracijama Zn (µg mL^{-1}) u škrgama klenova. Oznaka slike u radu **SI-6**.



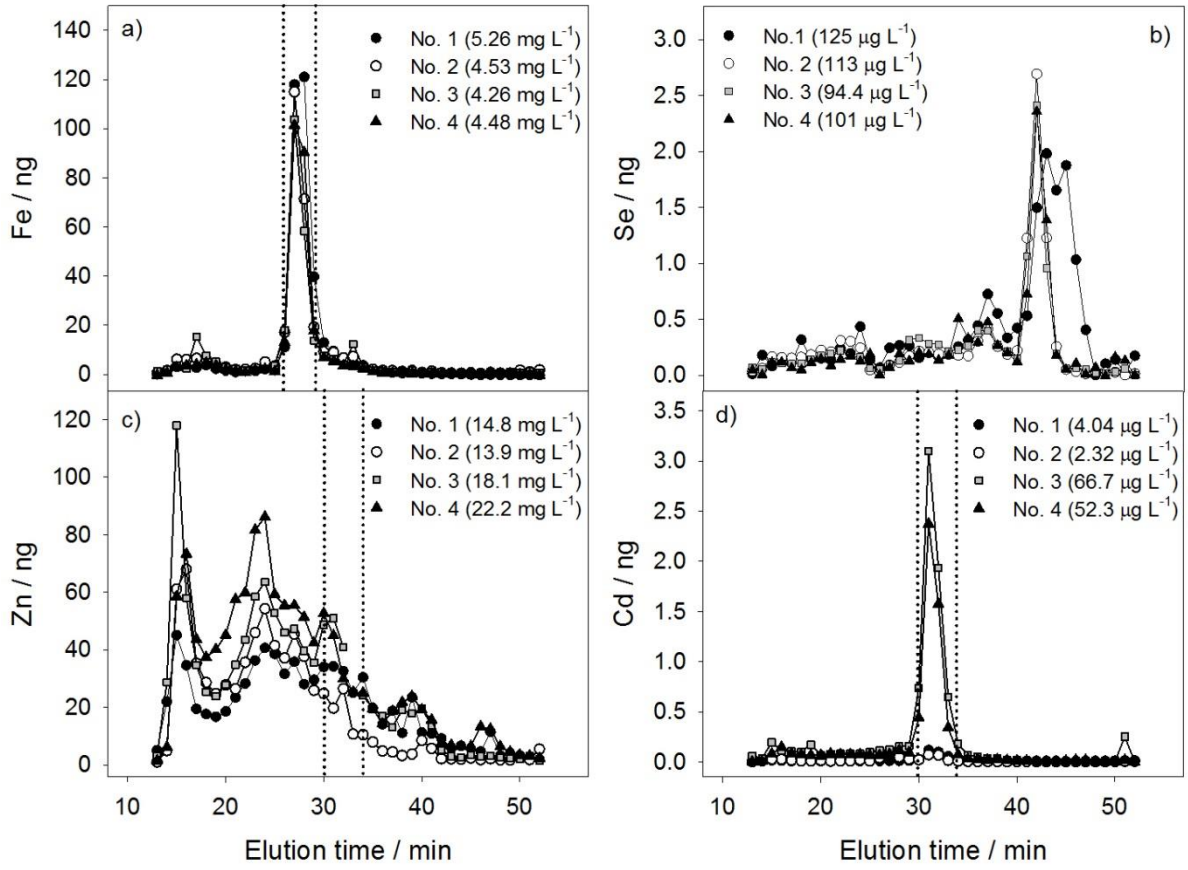
Slika 16. Profili raspodjele Cd među biomolekulama različitih molekulskih masa u citosolima škrga klenova (*S. cephalus*) dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. Analizirani uzorci su podijeljeni u dvije skupine, prema citosolskim koncentracijama Cd (ng mL^{-1}) u škrgama klenova. Oznaka slike u radu **SI-7**.

7.3. Dodatne informacije uz rad:

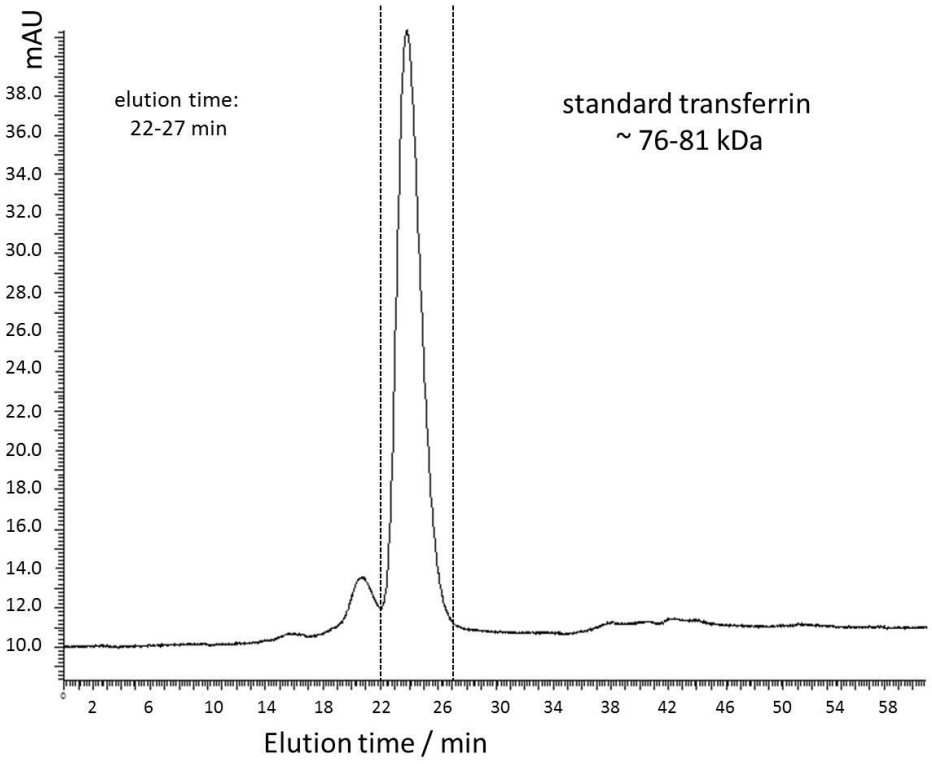
Krasnići N, Dragun Z, Kazazić S, Muharemović H, Erk M, Jordanova M, Rebok K, Kostov V: Characterization and identification of selected metal-binding biomolecules from hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928) by various techniques of liquid chromatography and mass spectrometry. Metallomics (2019), 11: 1060-1078. doi:10.1039/C9MT00036D.



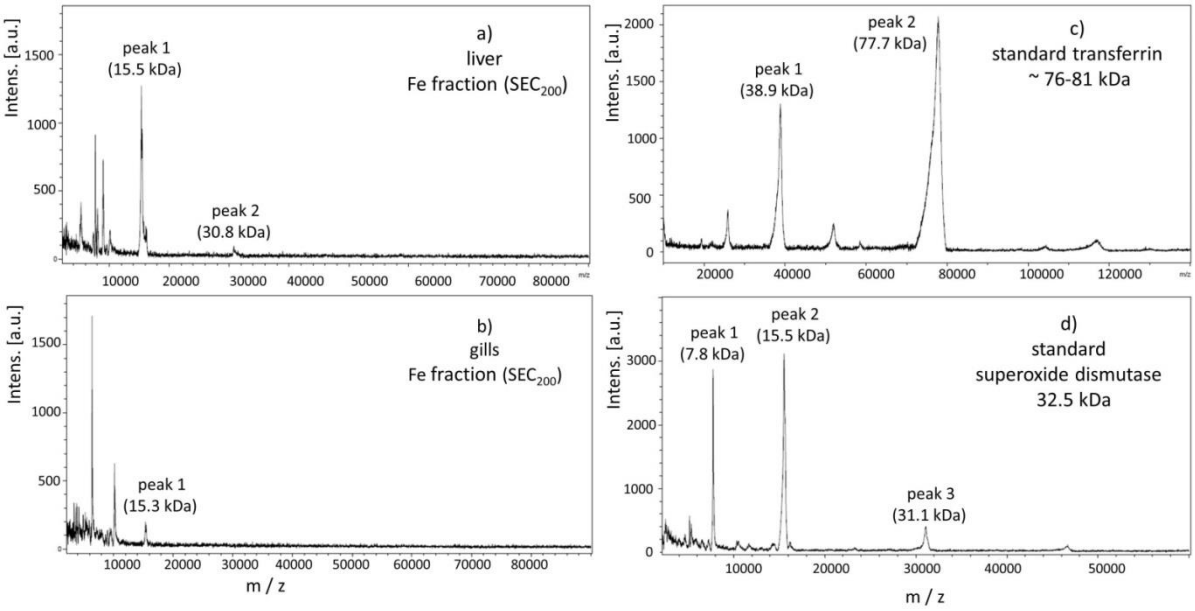
Slika 17. Raspodjele osam odabranih metala (a - Co, b - Cu, c - Fe, d - Mn, e - Mo, f - Se, g - Zn i h - Cd) među biomolekulama različitih molekulskih masa u citosolima jetara četiriju vardarskih klenova (*S. vardarensis*) uzorkovanih u dvjema makedonskim rijekama (Bregalnica i Zletovska) (uzorci br. 1, 2, 3 i 4). Profili su dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. SMM pikovi koji sadrže pretežno Fe (t_e 26-29 min) i NMM-pikovi koji sadrže pretežno Cd, Cu i Zn (t_e 30-34 min), a koji su označeni točkastim linijama, prikupljeni su za daljnje AEC-HPLC analize. Oznaka slike u radu **SI-1**.



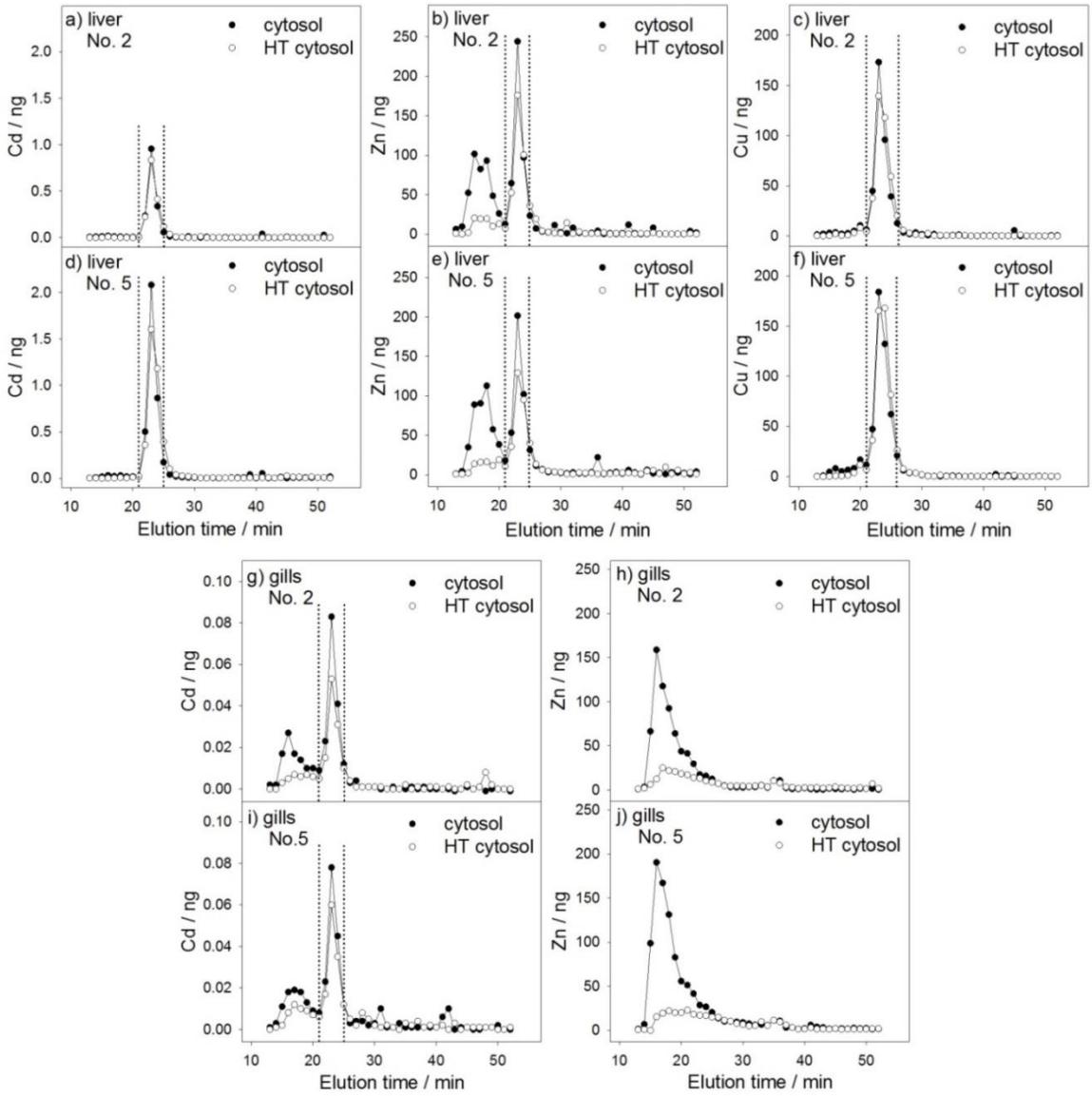
Slika 18. Raspodjele četiri odabrana elementa (a - Fe, b - Se, c - Zn, d - Cd) među biomolekulama različitih molekulskih masa u citosolima škrga četiriju vardarskih klenova (*S. vardarensis*) uzorkovanih u dvjema makedonskim rijekama (Bregalnica i Zletovska) (uzorci br. 1, 2, 3 i 4). Profili su dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 200 10/300 GL i mjerenjem na HR ICP-MS-u. SMM pikovi koji sadrže pretežno Fe (t_e 26-29 min) i NMM-pikove koji sadrže pretežno Cd i Zn (t_e 30-34 min), a koji su označeni točkastim linijama, prikupljeni su za daljnje AEC-HPLC analize. Oznaka slike u radu **SI-2**.



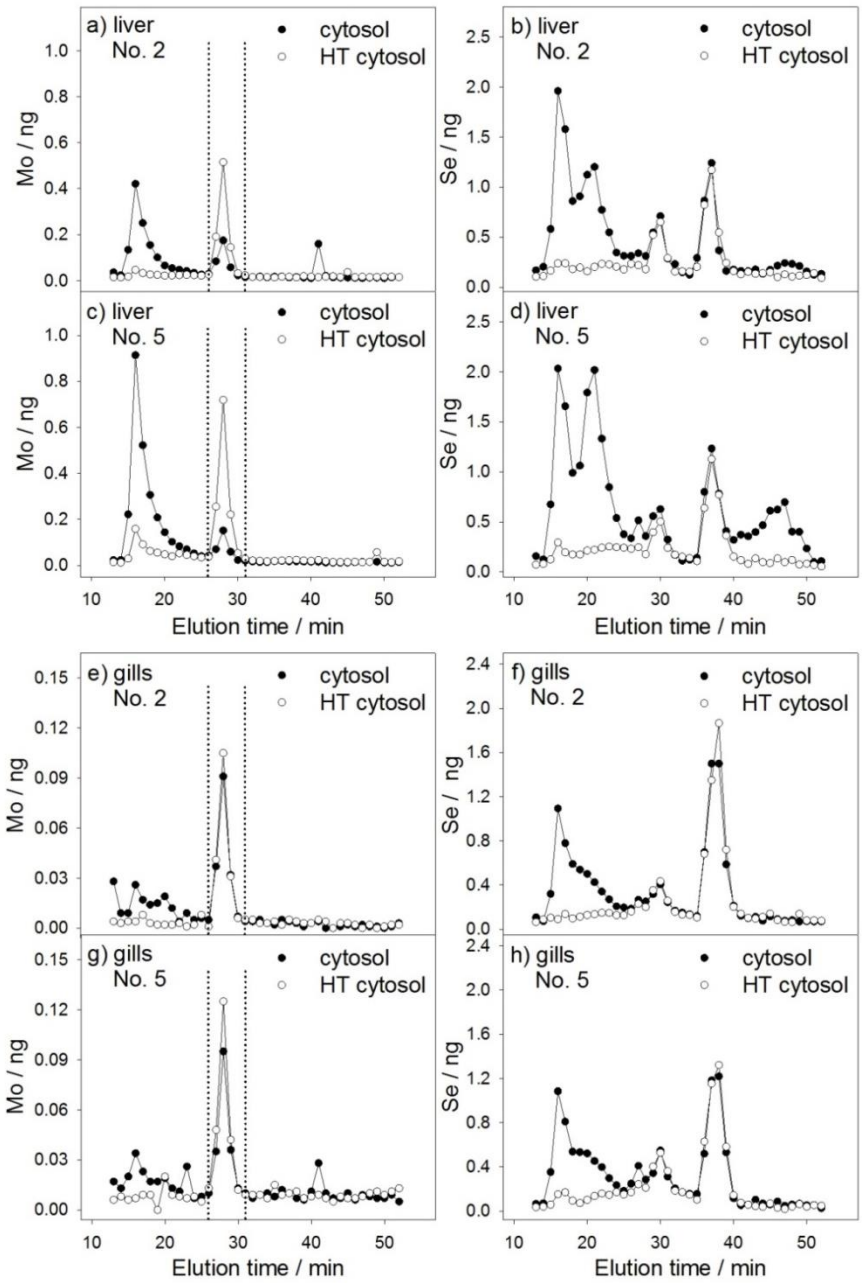
Slika 19. SEC_{200}-HPLC kromatografski profil standarda transferina (humani, koncentracija 2 mg mL^{-1}) dobiven snimanjem UV detektorom na 280 nm. Oznaka slike u radu **SI-3**.



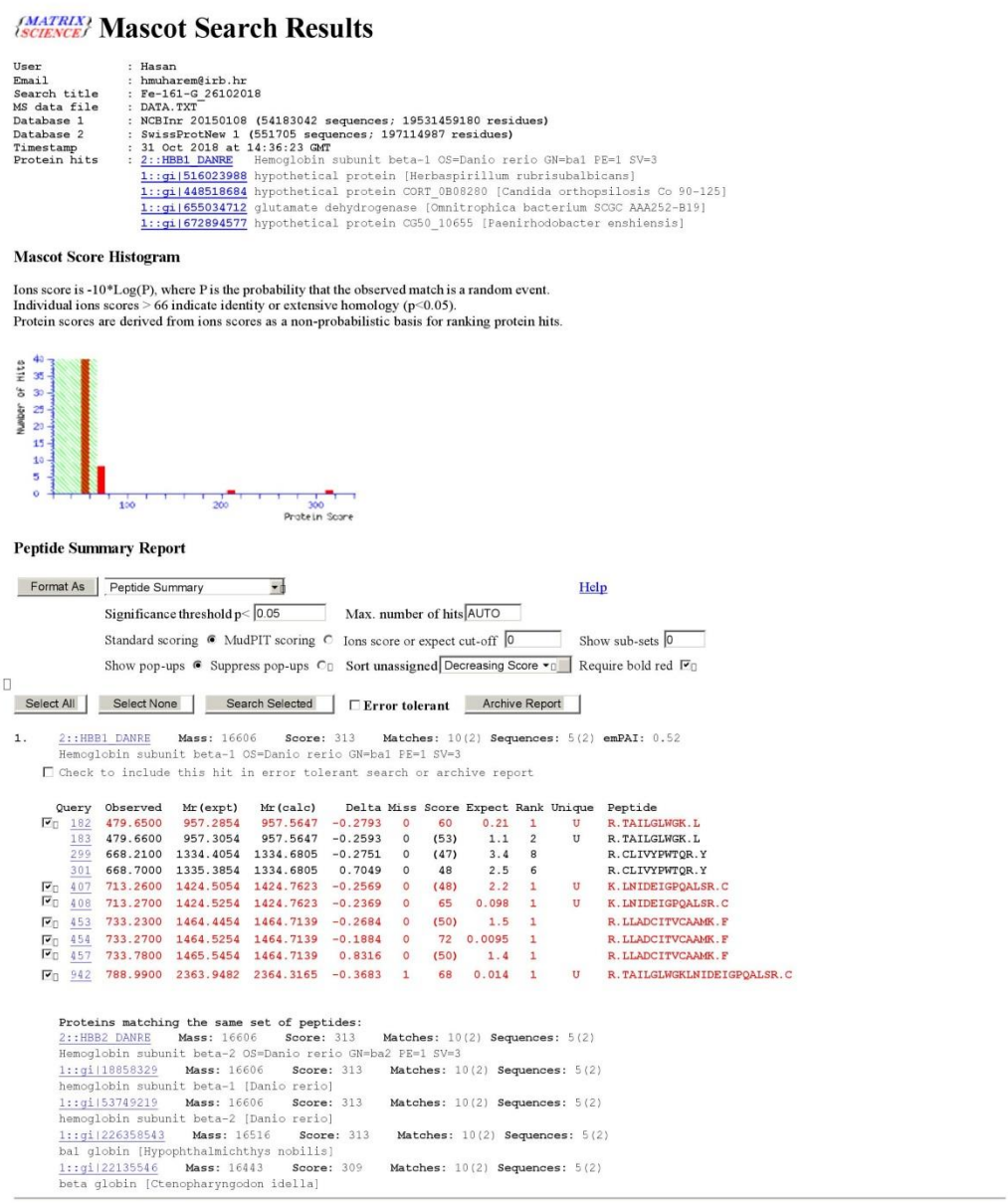
Slika 20. Spektri masa dobiveni pomoću MALDI-TOF-MS-a za SMM biomolekule (~20-50 kDa) koje vežu Fe u citosolima jetara (a) i škrga (b) vardarskih klenova razdvojene pomoću SEC_{200}-HPLC-a, te spektri masa standarda proteina transferina (c) i standarda enzima superoksid dismutaze (d). Oznaka slike u radu **SI-4**.



Slika 21. Raspodjele Cd (a, d), Zn (b, e) i Cu (c, f) u jetrima te raspodjele Cd (g, i) i Zn (h, j) u škrgama među biomolekulama različitih molekulskih masa iz citosola i toplinski obrađenih citosola dvaju vardarskih klenova (*S. vardarensis*) uzorkovanih u dvjema makedonskim rijekama (Bregalnica i Zletovska) (uzorci br. 2 i 5). Profili su dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 75 10/300 GL i mjerenjem na HR ICP-MS-u. NMM pikovi toplinski obrađenih jetrenih citosola (L-Cd-pikovi), koji su sadržavali Cd, Cu i Zn (t_e 21-25 min), a koji su označeni točkastim linijama, prikupljeni su za daljnje AEC-HPLC analize. NMM pikovi toplinski obrađenih citosola škrga (G-Cd-pikovi), koji su sadržavali Cd (t_e 21-25 min), a koji su označeni točkastim linijama, prikupljeni su za izravne MS analize. Oznaka slike u radu **SI-5**.



Slika 22. Raspodjele Mo (a, c) i Se (b, d) u jetrima te Mo (e, g) i Se (f, h) u škrgama među biomolekulama različitih molekulskih masa iz citosola i toplinski obrađenih citosola dvaju vardarskih klenova (*S. vardarensis*) uzorkovanih u dvjema makedonskim rijekama (Bregalnica i Zletovska) (uzorci br. 2 i 5). Profili su dobiveni HPLC razdvajanjem na koloni Superdex^{TM} 75 10/300 GL i mjerenjem na HR ICP-MS-u. JNMM pikovi toplinski obrađenih citosola jetara i škrga (t_e 26-30 min), koji su sadržavali pretežno Mo (~5-10 kDa) te tragove Se, a koji su označeni točkastim linijama, prikupljeni su za izravne MS analize. Oznaka slike u radu **SI-6**.



MATRIX SCIENCE **Mascot Search Results**

User : Hasan
Email : hmuharem@irb.hr
Search title : Fe-161-G_26102018
MS data file : DATA.TXT
Database 1 : NCBInr 20150108 (54183042 sequences; 19531459180 residues)
Database 2 : SwissProtNew 1 (551705 sequences; 197114987 residues)
Timestamp : 31 Oct 2018 at 14:36:23 GMT
Protein hits : 2::HBB1_DANRE Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
1::gi|516023988 hypothetical protein [Herbaspirillum rubrisubalbicans]
1::gi|448518684 hypothetical protein CORT_0B08280 [Candida orthopsilosis Co 90-125]
1::gi|655034712 glutamate dehydrogenase [Omnitrophica bacterium SCGC AAA252-B19]
1::gi|672894577 hypothetical protein CG50_10655 [Paenirhodobacter enshiensis]

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 66 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Number of Hits
Protein Score

Peptide Summary Report

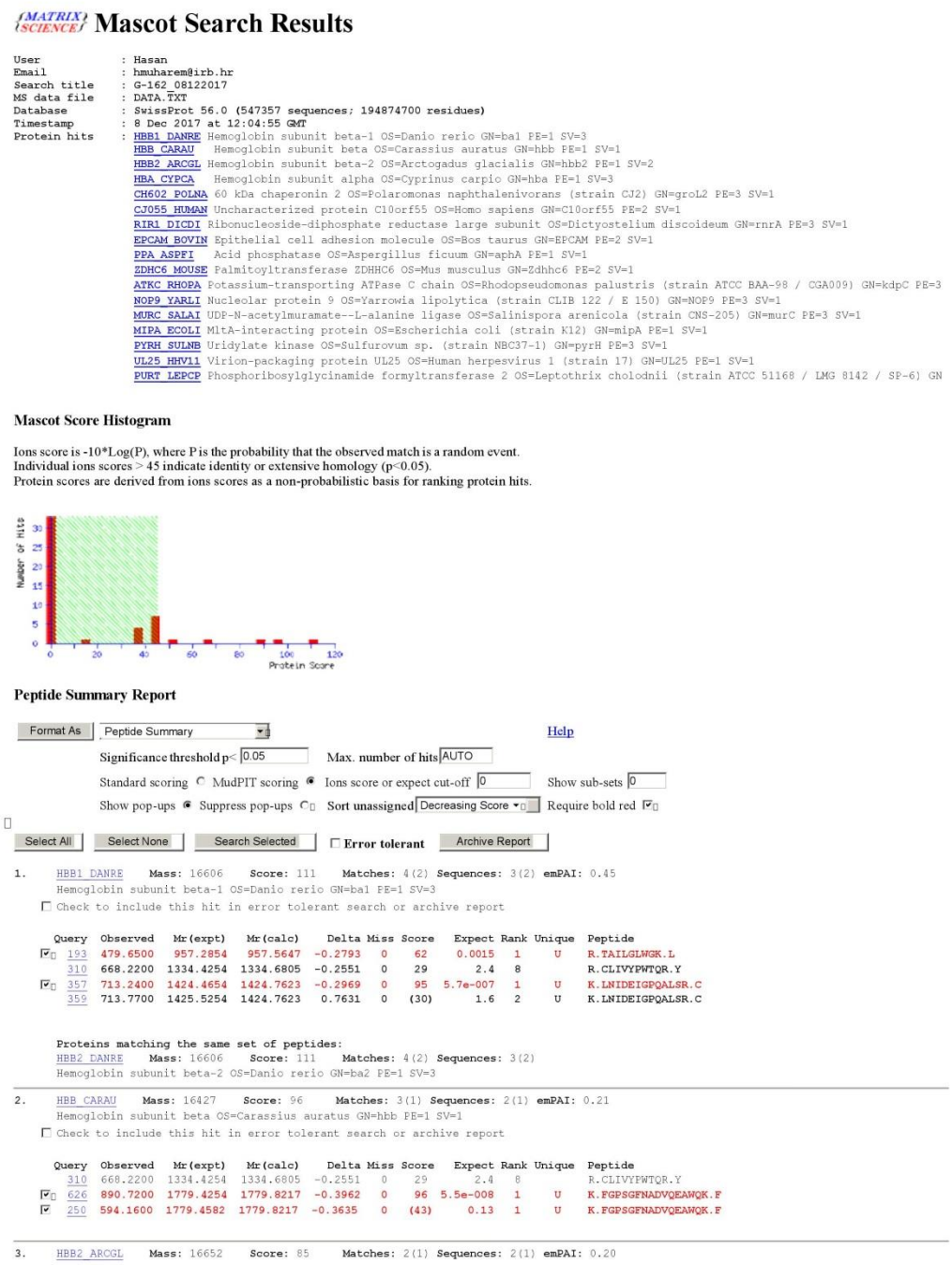
Format As | Peptide Summary | Help
Significance threshold p< 0.05 Max. number of hits AUTO
Standard scoring MudPIT scoring Ions score or expect cut-off 0 Show sub-sets 0
Show pop-ups Suppress pop-ups Sort unassigned Decreasing Score Require bold red
Select All | Select None | Search Selected | Error tolerant | Archive Report

1. 2::HBB1_DANRE Mass: 16606 Score: 313 Matches: 10(2) Sequences: 5(2) emPAI: 0.52
Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
182	479.6500	957.2854	957.5647	-0.2793	0	60	0.21	1	U	R.TAILGLWGK.L
183	479.6600	957.3054	957.5647	-0.2593	0	(53)	1.1	2	U	R.TAILGLWGK.L
299	668.2100	1334.4054	1334.6805	-0.2751	0	(47)	3.4	8		R.CLIVYPWTQR.Y
301	668.7000	1335.3854	1334.6805	0.7049	0	48	2.5	6		R.CLIVYPWTQR.Y
407	713.2600	1424.5054	1424.7623	-0.2569	0	(48)	2.2	1	U	K.LNIDEIGPQALSR.C
408	713.2700	1424.5254	1424.7623	-0.2369	0	65	0.098	1	U	K.LNIDEIGPQALSR.C
453	733.2300	1464.4454	1464.7139	-0.2684	0	(50)	1.5	1		R.LLADCITVCAAMK.F
454	733.2700	1464.5254	1464.7139	-0.1884	0	72	0.0095	1		R.LLADCITVCAAMK.F
457	733.7800	1465.5454	1464.7139	0.8316	0	(50)	1.4	1		R.LLADCITVCAAMK.F
942	788.9900	2363.9482	2364.3165	-0.3683	1	68	0.014	1	U	R.TAILGLWGKLNIDEIGPQALSR.C

Proteins matching the same set of peptides:
2::HBB2_DANRE Mass: 16606 Score: 313 Matches: 10(2) Sequences: 5(2)
Hemoglobin subunit beta-2 OS=Danio rerio GN=ba2 PE=1 SV=3
1::gi|18858329 Mass: 16606 Score: 313 Matches: 10(2) Sequences: 5(2)
hemoglobin subunit beta-1 [Danio rerio]
1::gi|53749219 Mass: 16606 Score: 313 Matches: 10(2) Sequences: 5(2)
hemoglobin subunit beta-2 [Danio rerio]
1::gi|226358543 Mass: 16516 Score: 313 Matches: 10(2) Sequences: 5(2)
ba1 globin [Hypophthalmichthys nobilis]
1::gi|22135546 Mass: 16443 Score: 309 Matches: 10(2) Sequences: 5(2)
beta globin [Ctenopharyngodon idella]

Slika 23. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu škrga vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.



MATRIX SCIENCE **Mascot Search Results**

User : Hasan
Email : hmuharem@irb.hr
Search title : G-162_08122017
MS data file : DATA.TXT
Database : SwissProt 56.0 (547357 sequences; 194874700 residues)
Timestamp : 8 Dec 2017 at 12:04:55 GMT
Protein hits :
HBB1_DANRE Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
HBB_CARAU Hemoglobin subunit beta OS=Carassius auratus GN=hbb PE=1 SV=1
HBB2_ARCGL Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
HBA_CYPCA Hemoglobin subunit alpha OS=Cyprinus carpio GN=hba PE=1 SV=3
CH602_POLNA 60 kDa chaperonin 2 OS=Polaromonas naphthalenivorans (strain CJ2) GN=groL2 PE=3 SV=1
CJ055_HUMAN Uncharacterized protein C10orf55 OS=Homo sapiens GN=C10orf55 PE=2 SV=1
RIR1_DICDI Ribonucleoside-diphosphate reductase large subunit OS=Dictyostelium discoideum GN=rnrA PE=3 SV=1
EPCAM_BOVIN Epithelial cell adhesion molecule OS=Bos taurus GN=EPCAM PE=2 SV=1
PPA_ASPFI Acid phosphatase OS=Aspergillus ficuum GN=aphA PE=1 SV=1
ZDHC6_MOUSE Palmitoyltransferase ZDHHC6 OS=Mus musculus GN=Zdhhc6 PE=2 SV=1
ATKC_RHOPA Potassium-transporting ATPase C chain OS=Rhodopseudomonas palustris (strain ATCC BAA-98 / CGA009) GN=kdpC PE=3
NOP9_YARLI Nucleolar protein 9 OS=Yarrowia lipolytica (strain CLIB 122 / E 150) GN=NOP9 PE=3 SV=1
MURC_SALAI UDP-N-acetylmuramate--L-alanine ligase OS=Salinispora arenicola (strain CNS-205) GN=murC PE=3 SV=1
MIPA_ECOLI MltA-interacting protein OS=Escherichia coli (strain K12) GN=mipA PE=1 SV=1
PYRH_SULNB Uridylate kinase OS=Sulfurovum sp. (strain NBC37-1) GN=pyrH PE=3 SV=1
UL25_HHV11 Virion-packaging protein UL25 OS=Human herpesvirus 1 (strain 17) GN=UL25 PE=1 SV=1
PURT_LEPCP Phosphoribosylglycinamide formyltransferase 2 OS=Leptothrix cholodnii (strain ATCC 51168 / LMG 8142 / SP-6) GN

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 45 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Peptide Summary Report

Format As: Peptide Summary — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red
Select All | Select None | Search Selected | Error tolerant | Archive Report

1. HBB1_DANRE Mass: 16606 Score: 111 Matches: 4(2) Sequences: 3(2) emPAI: 0.45
Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
Check to include this hit in error tolerant search or archive report

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193	479.6500	957.2854	957.5647	-0.2793	0	62	0.0015	1	U	R.TAILGLWGK.L
310	668.2200	1334.4254	1334.6805	-0.2551	0	29	2.4	8		R.CLIVYPWTQR.Y
357	713.2400	1424.4654	1424.7623	-0.2969	0	95	5.7e-007	1	U	K.LNIDEIGPQALSR.C
359	713.7700	1425.5254	1424.7623	0.7631	0	(30)	1.6	2	U	K.LNIDEIGPQALSR.C

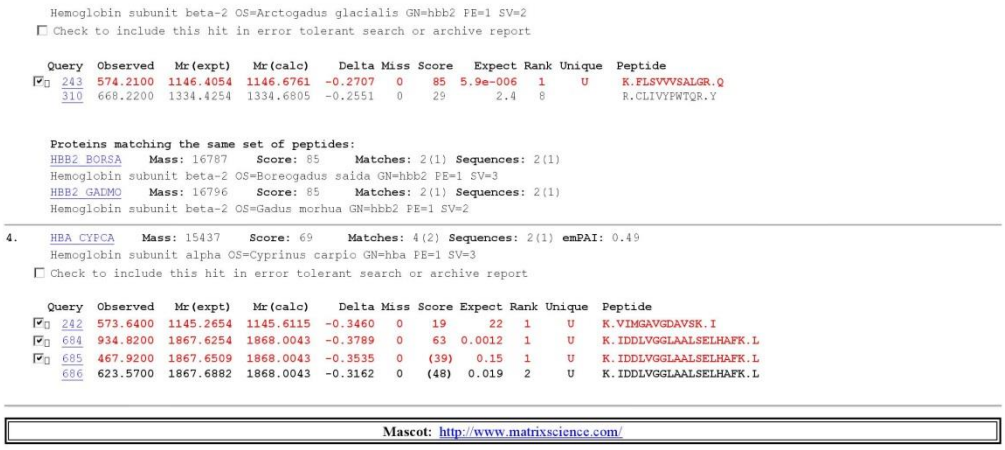
Proteins matching the same set of peptides:
HBB2_DANRE Mass: 16606 Score: 111 Matches: 4(2) Sequences: 3(2)
Hemoglobin subunit beta-2 OS=Danio rerio GN=ba2 PE=1 SV=3

2. HBB_CARAU Mass: 16427 Score: 96 Matches: 3(1) Sequences: 2(1) emPAI: 0.21
Hemoglobin subunit beta OS=Carassius auratus GN=hbb PE=1 SV=1
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
310	668.2200	1334.4254	1334.6805	-0.2551	0	29	2.4	8		R.CLIVYPWTQR.Y
626	890.7200	1779.4254	1779.8217	-0.3962	0	96	5.5e-008	1	U	K.FGPSGFNADVQEAWQK.F
250	594.1600	1779.4582	1779.8217	-0.3635	0	(43)	0.13	1	U	K.FGPSGFNADVQEAWQK.F

3. HBB2_ARCGL Mass: 16652 Score: 85 Matches: 2(1) Sequences: 2(1) emPAI: 0.20

Slika 24. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu škrga vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.



Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	243	574.2100	1146.4054	1146.6761	-0.2707	0	85	5.9e-006	1	U	K.FLSVVVSALGR.Q
	310	668.2200	1334.4254	1334.6805	-0.2551	0	29	2.4	8		R.CLIVYPWTQR.Y

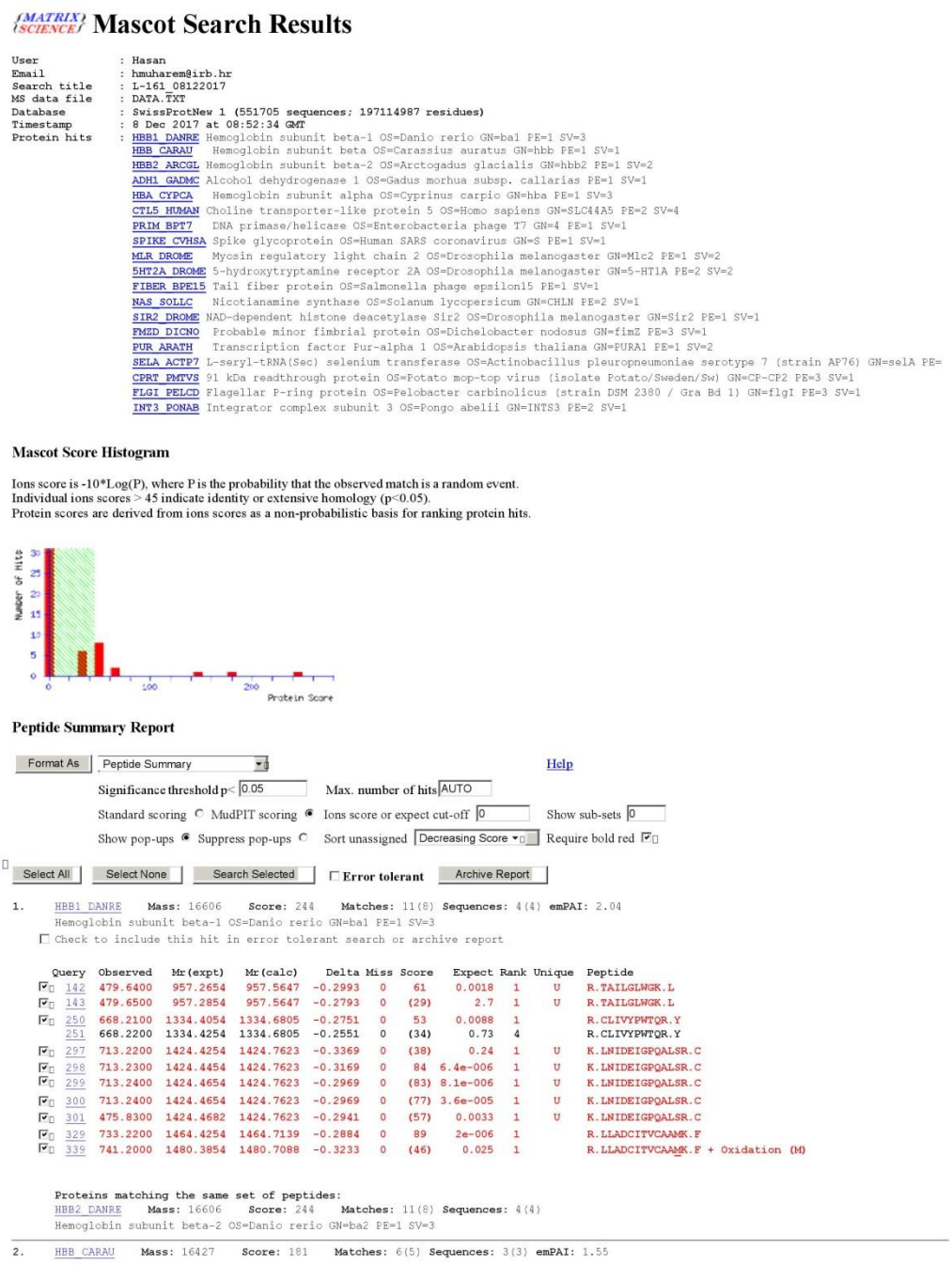
Proteins matching the same set of peptides:
HBB2_BORSA Mass: 16787 Score: 85 Matches: 2(1) Sequences: 2(1)
Hemoglobin subunit beta-2 OS=Boreogadus saida GN=hbb2 PE=1 SV=3
HBB2_GADMO Mass: 16796 Score: 85 Matches: 2(1) Sequences: 2(1)
Hemoglobin subunit beta-2 OS=Gadus morhua GN=hbb2 PE=1 SV=2

4. HBA_CYPCA Mass: 15437 Score: 69 Matches: 4(2) Sequences: 2(1) emPAI: 0.49
Hemoglobin subunit alpha OS=Cyprinus carpio GN=hba PE=1 SV=3
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	242	573.6400	1145.2654	1145.6115	-0.3460	0	19	22	1	U	K.VIMGAVGDAVSK.I
☑	684	934.8200	1867.6254	1868.0043	-0.3789	0	63	0.0012	1	U	K.IDDLVGGLAALSELHAFK.L
☑	685	467.9200	1867.6509	1868.0043	-0.3535	0	(39)	0.15	1	U	K.IDDLVGGLAALSELHAFK.L
	686	623.5700	1867.6882	1868.0043	-0.3162	0	(48)	0.019	2	U	K.IDDLVGGLAALSELHAFK.L

Mascot: http://www.matrixscience.com/

Slika 24 - nastavak. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu škrga vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.



MATRIX SCIENCE **Mascot Search Results**

User : Hasan
Email : hmuharem@irb.hr
Search title : L-161_08122017
MS data file : DATA.TXT
Database : SwissProtNew 1 (551705 sequences; 197114987 residues)
Timestamp : 8 Dec 2017 at 08:52:34 GMT
Protein hits :
HBB1_DANRE Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
HBB_CARAU Hemoglobin subunit beta OS=Carassius auratus GN=hbb PE=1 SV=1
HBB2_ARCGL Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
ADH1_GADMC Alcohol dehydrogenase 1 OS=Gadus morhua subsp. callarias PE=1 SV=1
HBA_CYPCA Hemoglobin subunit alpha OS=Cyprinus carpio GN=hba PE=1 SV=3
CTL5_HUMAN Choline transporter-like protein 5 OS=Homo sapiens GN=SLC44A5 PE=2 SV=4
PRIM_BPT7 DNA primase/helicase OS=Enterobacteria phage T7 GN=4 PE=1 SV=1
SPIKE_CVHSA Spike glycoprotein OS=Human SARS coronavirus GN=S PE=1 SV=1
MLR_DROME Mycsin regulatory light chain 2 OS=Drosophila melanogaster GN=Mlc2 PE=1 SV=2
5HT2A_DROME 5-hydroxytryptamine receptor 2A OS=Drosophila melanogaster GN=5-HT1A PE=2 SV=2
FIBER_BPE15 Tail fiber protein OS=Salmonella phage epsilon15 PE=1 SV=1
NAS_SOLLC Nicotianamine synthase OS=Solanum lycopersicum GN=CHLN PE=2 SV=1
SIR2_DROME NAD-dependent histone deacetylase Sir2 OS=Drosophila melanogaster GN=Sir2 PE=1 SV=1
FMZD_DICNO Probable minor fimbrial protein OS=Dichelobacter nodosus GN=fimZ PE=3 SV=1
PUR_ARATH Transcription factor Pur-alpha 1 OS=Arabidopsis thaliana GN=PURA1 PE=1 SV=2
SELA_ACTP7 L-seryl-tRNA(Sec) selenium transferase OS=Actinobacillus pleuropneumoniae serotype 7 (strain AP76) GN=selA PE=
CPRT_PMTVS 91 kDa readthrough protein OS=Potato mop-top virus (isolate Potato/Sweden/Sw) GN=CP-CP2 PE=3 SV=1
FLGI_PELCD Flagellar P-ring protein OS=Pelobacter carbinolicus (strain DSM 2380 / Gra Bd 1) GN=flgI PE=3 SV=1
INT3_PONAB Integrator complex subunit 3 OS=Pongo abelii GN=INTS3 PE=2 SV=1

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 45 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Peptide Summary Report

Format As: Peptide Summary — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red
Select All | Select None | Search Selected | Error tolerant | Archive Report

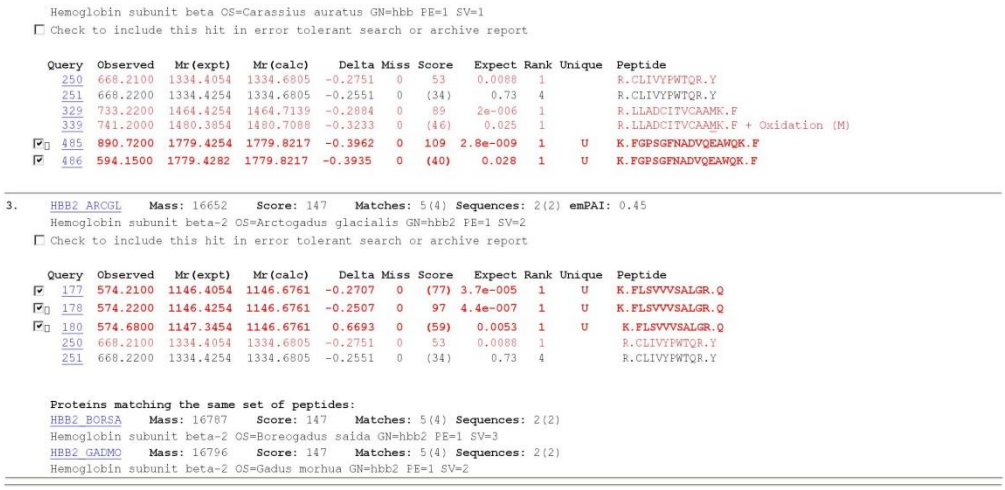
1. HBB1_DANRE Mass: 16606 Score: 244 Matches: 11(8) Sequences: 4(4) emPAI: 2.04
Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
142	479.6400	957.2654	957.5647	-0.2993	0	61	0.0018	1	U	R.TAILGLWGK.L
143	479.6500	957.2854	957.5647	-0.2793	0	(29)	2.7	1	U	R.TAILGLWGK.L
250	668.2100	1334.4054	1334.6805	-0.2751	0	53	0.0088	1		R.CLIVYPWTQR.Y
251	668.2200	1334.4254	1334.6805	-0.2551	0	(34)	0.73	4		R.CLIVYPWTQR.Y
297	713.2200	1424.4254	1424.7623	-0.3369	0	(38)	0.24	1	U	K.LNIDEIGPQALSR.C
298	713.2300	1424.4454	1424.7623	-0.3169	0	84	6.4e-006	1	U	K.LNIDEIGPQALSR.C
299	713.2400	1424.4654	1424.7623	-0.2969	0	(83)	8.1e-006	1	U	K.LNIDEIGPQALSR.C
300	713.2400	1424.4654	1424.7623	-0.2969	0	(77)	3.6e-005	1	U	K.LNIDEIGPQALSR.C
301	475.8300	1424.4682	1424.7623	-0.2941	0	(57)	0.0033	1	U	K.LNIDEIGPQALSR.C
329	733.2200	1464.4254	1464.7139	-0.2884	0	89	2e-006	1		R.LLADCITVCAAMK.F
339	741.2000	1480.3854	1480.7088	-0.3233	0	(46)	0.025	1		R.LLADCITVCAAMK.F + Oxidation (M)

Proteins matching the same set of peptides:
HBB2_DANRE Mass: 16606 Score: 244 Matches: 11(8) Sequences: 4(4)
Hemoglobin subunit beta-2 OS=Danio rerio GN=ba2 PE=1 SV=3

2. HBB_CARAU Mass: 16427 Score: 181 Matches: 6(5) Sequences: 3(3) emPAI: 1.55

Slika 25. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu jetara vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.



Hemoglobin subunit beta OS=Carassius auratus GN=hbb PE=1 SV=1
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
250	668.2100	1334.4054	1334.6805	-0.2751	0	53	0.0088	1		R.CLIVYPWTQR.Y
251	668.2200	1334.4254	1334.6805	-0.2551	0	(34)	0.73	4		R.CLIVYPWTQR.Y
329	733.2200	1464.4254	1464.7139	-0.2884	0	89	2e-006	1		R.LLADCITVCAAMK.F
339	741.2000	1480.3854	1480.7088	-0.3233	0	(46)	0.025	1		R.LLADCITVCAAMK.F + Oxidation (M)
485	890.7200	1779.4254	1779.8217	-0.3962	0	109	2.8e-009	1	U	K.FGPSGFNADVQEAWQK.F
486	594.1500	1779.4282	1779.8217	-0.3935	0	(40)	0.028	1	U	K.FGPSGFNADVQEAWQK.F

3. HBB2_ARCGL Mass: 16652 Score: 147 Matches: 5(4) Sequences: 2(2) emPAI: 0.45
Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
177	574.2100	1146.4054	1146.6761	-0.2707	0	(77)	3.7e-005	1	U	K.FLSVVVSALGR.Q
178	574.2200	1146.4254	1146.6761	-0.2507	0	97	4.4e-007	1	U	K.FLSVVVSALGR.Q
180	574.6800	1147.3454	1146.6761	0.6693	0	(59)	0.0053	1	U	K.FLSVVVSALGR.Q
250	668.2100	1334.4054	1334.6805	-0.2751	0	53	0.0088	1		R.CLIVYPWTQR.Y
251	668.2200	1334.4254	1334.6805	-0.2551	0	(34)	0.73	4		R.CLIVYPWTQR.Y

Proteins matching the same set of peptides:
HBB2_BORSA Mass: 16787 Score: 147 Matches: 5(4) Sequences: 2(2)
Hemoglobin subunit beta-2 OS=Boreogadus saida GN=hbb2 PE=1 SV=3
HBB2_GADMO Mass: 16796 Score: 147 Matches: 5(4) Sequences: 2(2)
Hemoglobin subunit beta-2 OS=Gadus morhua GN=hbb2 PE=1 SV=2

Slika 25 - nastavak. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu jetara vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.

MATRIX SCIENCE **Mascot Search Results**

User : Hasan
Email : hmuharem@irb.hr
Search title : L-162_08122017
MS data file : DATA.TXT
Database : SwissProt 56.0 (547357 sequences; 194874700 residues)
Timestamp : 8 Dec 2017 at 10:16:35 GMT
Protein hits :
HBB1_DANRE Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
HBB_CARAU Hemoglobin subunit beta OS=Carassius auratus GN=hbb PE=1 SV=1
HBB2_ARCGL Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
RNH_SYNAS Ribonuclease H OS=Syntrophus aciditrophicus (strain SB) GN=rnhA PE=3 SV=1
HXA2_BOVIN Homeobox protein Hox-A2 OS=Bos taurus GN=HOXA2 PE=2 SV=1
PGK_HELPJ Phosphoglycerate kinase OS=Helicobacter pylori (strain J99 / ATCC 700824) GN=pgk PE=3 SV=1
5HT2A_DROME 5-hydroxytryptamine receptor 2A OS=Drosophila melanogaster GN=5-HT1A PE=2 SV=2
SECA_CLOTE Protein translocase subunit SecA OS=Clostridium tetani (strain Massachusetts / E88) GN=secA PE=3 SV=2
DNAG_METBF DNA primase DnaG OS=Methanosarcina barkeri (strain Fusaro / DSM 804) GN=dnaG PE=3 SV=1
Y1127_ANASK UPF0173 metal-dependent hydrolase AnaeK_1127 OS=Anaeromyxobacter sp. (strain K) GN=AnaeK_1127 PE=3 SV=1
GCSPA_FLAPR Glycine dehydrogenase (decarboxylating) A, mitochondrial OS=Flaveria pringlei GN=GDCSPA PE=2 SV=1
B3G2P_DROME Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase P OS=Drosophila melanogaster GN=GlcAT-P PE=2
SYR_DESVH Arginine--tRNA ligase OS=Desulfovibrio vulgaris (strain Hildenborough / ATCC 29579 / NCIMB 8303) GN=argS PE=3
TBX39_CAEEL Putative T-box protein 39 OS=Caenorhabditis elegans GN=tbx-39 PE=1 SV=1
RS11_METST 30S ribosomal protein S11 OS=Methanosphaera stadtmanae (strain ATCC 43021 / DSM 3091 / JCM 11832 / MCB-3) GN=r
SDHD_BURTA Probable D-serine dehydratase OS=Burkholderia thailandensis (strain E264 / ATCC 700388 / DSM 13276 / CIP 10630

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 45 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Peptide Summary Report

Format As: Peptide Summary — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red
Select All | Select None | Search Selected | Error tolerant | Archive Report

1. HBB1_DANRE Mass: 16606 Score: 225 Matches: 8(5) Sequences: 4(3) emPAI: 0.74
Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
Check to include this hit in error tolerant search or archive report

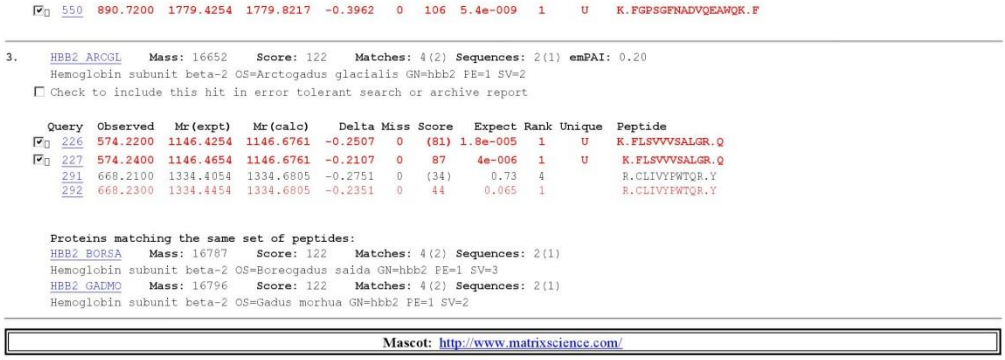
Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
185	479.6700	957.3254	957.5647	-0.2393	0	65	0.00064	1	U	R.TAILGLWGK.L
291	668.2100	1334.4054	1334.6805	-0.2751	0	(34)	0.73	4		R.CLIVYPWTQR.Y
292	668.2300	1334.4454	1334.6805	-0.2351	0	44	0.065	1		R.CLIVYPWTQR.Y
358	713.2300	1424.4454	1424.7623	-0.3169	0	(69)	0.00022	1	U	K.LNIDEIGPQALSR.C
359	713.2300	1424.4454	1424.7623	-0.3169	0	95	4.9e-007	1	U	K.LNIDEIGPQALSR.C
360	713.2300	1424.4454	1424.7623	-0.3169	0	(79)	3.2e-005	1	U	K.LNIDEIGPQALSR.C
361	713.2800	1424.5454	1424.7623	-0.2169	0	(48)	0.055	1	U	K.LNIDEIGPQALSR.C
383	733.2100	1464.4054	1464.7139	-0.3084	0	90	1.4e-006	1		R.LLADCITVCAAMK.F

Proteins matching the same set of peptides:
HBB2_DANRE Mass: 16606 Score: 225 Matches: 8(5) Sequences: 4(3)
Hemoglobin subunit beta-2 OS=Danio rerio GN=ba2 PE=1 SV=3

2. HBB_CARAU Mass: 16427 Score: 156 Matches: 4(2) Sequences: 3(2) emPAI: 0.45
Hemoglobin subunit beta OS=Carassius auratus GN=hbb PE=1 SV=1
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
291	668.2100	1334.4054	1334.6805	-0.2751	0	(34)	0.73	4		R.CLIVYPWTQR.Y
292	668.2300	1334.4454	1334.6805	-0.2351	0	44	0.065	1		R.CLIVYPWTQR.Y
383	733.2100	1464.4054	1464.7139	-0.3084	0	90	1.4e-006	1		R.LLADCITVCAAMK.F

Slika 26. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu jetara vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.



☑	550	890.7200	1779.4254	1779.8217	-0.3962	0	106	5.4e-009	1	U	K.FGPSGFNADVQEAWQK.F

3. HBB2_ARCGL Mass: 16652 Score: 122 Matches: 4(2) Sequences: 2(1) emPAI: 0.20
Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	226	574.2200	1146.4254	1146.6761	-0.2507	0	(81)	1.8e-005	1	U	K.FLSVVVSALGR.Q
☑	227	574.2400	1146.4654	1146.6761	-0.2107	0	87	4e-006	1	U	K.FLSVVVSALGR.Q
	291	668.2100	1334.4054	1334.6805	-0.2751	0	(34)	0.73	4		R.CLIVYPWTQR.Y
	292	668.2300	1334.4454	1334.6805	-0.2351	0	44	0.065	1		R.CLIVYPWTQR.Y

Proteins matching the same set of peptides:
HBB2_BORSA Mass: 16787 Score: 122 Matches: 4(2) Sequences: 2(1)
Hemoglobin subunit beta-2 OS=Boreogadus saida GN=hbb2 PE=1 SV=3
HBB2_GADMO Mass: 16796 Score: 122 Matches: 4(2) Sequences: 2(1)
Hemoglobin subunit beta-2 OS=Gadus morhua GN=hbb2 PE=1 SV=2

Mascot: http://www.matrixscience.com/

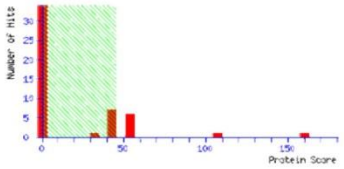
Slika 26 - nastavak. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu jetara vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.

Mascot Search Results

User : Hasan
Email : hmuharem@irb.hr
Search title : L-165_08122017
MS data file : DATA.TXT
Database : SwissProt 56.0 (547357 sequences; 194874700 residues)
Timestamp : 8 Dec 2017 at 11:08:08 GMT
Protein hits : HBB1_DANRE Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
HBB2_ARCGL Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
HXA2_BOVIN Homeobox protein Hox-A2 OS=Bos taurus GN=HOXA2 PE=2 SV=1
FHUD_BACSU Iron(3+)-hydroxamate-binding protein FhuD OS=Bacillus subtilis (strain 168) GN=fhuD PE=1 SV=1
RS20_BIFAA 30S ribosomal protein S20 OS=Bifidobacterium adolescentis (strain ATCC 15703 / DSM 20083 / NCTC 11814 / E194a)
HBA_CYPCA Hemoglobin subunit alpha OS=Cyprinus carpio GN=hba PE=1 SV=3
ACKA_CAMJ8 Acetate kinase OS=Campylobacter jejuni subsp. jejuni serotype O:6 (strain 81116 / NCTC 11828) GN=ackA PE=3 SV=
MKS3_MOUSE Meckelin OS=Mus musculus GN=Tmem67 PE=1 SV=2
SOCS1_HUMAN Suppressor of cytokine signaling 1 OS=Homo sapiens GN=SOCS1 PE=1 SV=1
NM111_ASPCL Pro-apoptotic serine protease nma111 OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3
TADA3_MOUSE Transcriptional adapter 3 OS=Mus musculus GN=Tada3 PE=1 SV=1
CH60_COXB1 60 kDa chaperonin OS=Coxiella burnetii (strain CbuK_Q154) GN=groL PE=3 SV=1
PYRD_RHOFT Dihydroorotate dehydrogenase (quinone) OS=Rhodoferax ferrireducens (strain ATCC BAA-621 / DSM 15236 / T118) GN
SYA_VIBPA Alanine--tRNA ligase OS=Vibrio parahaemolyticus serotype O3:K6 (strain RIMD 2210633) GN=alaS PE=3 SV=1
UBP36_DROVI Ubiquitin carboxyl-terminal hydrolase 36 OS=Drosophila virilis GN=Usp36 PE=3 SV=1
CSLF4_ORYSJ Mixed-linked glucan synthase 4 OS=Oryza sativa subsp. japonica GN=CSLF4 PE=3 SV=1

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 45 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary — Help
Significance threshold p< 0.05 Max. number of hits AUTO
Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 Show sub-sets 0
Show pop-ups / Suppress pop-ups Sort unassigned Decreasing Score Require bold red
Select All | Select None | Search Selected | Error tolerant | Archive Report

1. HBB1_DANRE Mass: 16606 Score: 160 Matches: 5(3) Sequences: 4(3) emPAI: 0.74
Hemoglobin subunit beta-1 OS=Danio rerio GN=ba1 PE=1 SV=3
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
209	479.6700	957.3254	957.5647	-0.2393	0	45	0.069	1	U	R.TAILGLWGK.L
210	479.7000	957.3854	957.5647	-0.1793	0	(29)	2.8	6	U	R.TAILGLWGK.L
361	668.2100	1334.4054	1334.6805	-0.2751	0	53	0.0082	1		R.CLIVYPWTQR.Y
427	713.2300	1424.4454	1424.7623	-0.3169	0	100	1.5e-007	1	U	K.LNIDEIGPQALSR.C
453	733.2000	1464.3854	1464.7139	-0.3284	0	95	4.3e-007	1		R.LLADCITVCAAMK.F

Proteins matching the same set of peptides:
HBB2_DANRE Mass: 16606 Score: 160 Matches: 5(3) Sequences: 4(3)
Hemoglobin subunit beta-2 OS=Danio rerio GN=ba2 PE=1 SV=3

2. HBB2_ARCGL Mass: 16652 Score: 106 Matches: 2(2) Sequences: 2(2) emPAI: 0.45
Hemoglobin subunit beta-2 OS=Arctogadus glacialis GN=hbb2 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
269	574.2300	1146.4454	1146.6761	-0.2307	0	98	3.5e-007	1	U	K.FLSVVVSALGR.Q
361	668.2100	1334.4054	1334.6805	-0.2751	0	53	0.0082	1		R.CLIVYPWTQR.Y

Proteins matching the same set of peptides:
HBB2_BORSA Mass: 16787 Score: 106 Matches: 2(2) Sequences: 2(2)

Hemoglobin subunit beta-2 OS=Boreogadus saida GN=hbb2 PE=1 SV=3
HBB2_GADMO Mass: 16796 Score: 106 Matches: 2(2) Sequences: 2(2)
Hemoglobin subunit beta-2 OS=Gadus morhua GN=hbb2 PE=1 SV=2

Mascot: http://www.matrixscience.com/

Slika 27. Rezultati pretraživanja Mascot baze: LC-MS/MS identifikacija SMM biomolekula koje vežu Fe u citosolu jetara vardarskog klena razdvojenih pomoću SEC_{200}-HPLC-a (~20-50 kDa) i pročišćenih pomoću AEC-HPLC-a.

POPIS KORIŠTENIH KRATICA

AEC-HPLC	anionsko izmjenjivačka tekućinska kromatografija visoke djelotvornosti
HR ICP-MS	spektrometrija masa visoke rezolucije s induktivno spregnutom plazmom
JNMM	biomolekule jako niskih molekulskih masa (<10 kDa)
LC-MS/MS	tekućinska kromatografija - tandemna spektrometrija masa
MALDI-TOF-MS	spektrometrija masa s matricom potpomognutom ionizacijom desorpcijom laserskog zračenja s analizatorom masa s vremenom leta
MS	spektrometrija masa
MT	metalotionein
NMM	biomolekule niskih molekulskih masa (10-30 kDa)
SEC-HPLC	tekućinska kromatografija visoke djelotvornosti s isključenjem po veličini
SMM	biomolekule srednjih molekulskih masa (30-100 kDa)
SOD	superoksid dismutaza
VMM	biomolekule visokih molekulskih masa (>100 kDa)

ŽIVOTOPIS

8. ŽIVOTOPIS

Nesrete Krasnići rođena je 11. studenog 1982. godine u mjestu Damjane na Kosovu, gdje je završila osnovnu školu. Nakon završene srednje škole u Zagrebu, 2008. godine je diplomirala na Prehrambeno-biotehnološkom fakultetu Sveučilišta u Zagrebu, smjer prehrambeno inženjerstvo. Od 2009. godine do danas zaposlena je prvo kao stručna suradnica te zatim kao viša stručna suradnica u Laboratoriju za biološke učinke metala Zavoda za istraživanje mora i okoliša Instituta Ruđer Bošković. Njezin stručni i znanstveni rad obuhvaćaju analize raspodjele metala među citosolskim biomolekulama u organima bioindikatorskih organizama pomoću raznih tehnika tekućinske kromatografije visoke djelotvornosti i masene spektrometrije visoke rezolucije s induktivno spregnutom plazmom, zatim određivanje ukupnih proteina spektrofotometrijskom metodom kao i određivanje metalotioneina elektrokemijskom metodom (diferencijalna pulsna polarografija). U akademskoj godini 2013./2014. upisala je poslijediplomski sveučilišni doktorski studij biologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu. Za znanstvene aktivnosti i dostignuća, dodijeljene su joj godišnje nagrade Instituta Ruđer Bošković za mlade znanstvenike bez doktorata za dva znanstvena rada objavljena u časopisima visokog čimbenika odjeka (2013. i 2014.). Dobitnica je stipendije Instituta Ruđer Bošković za kratkoročni boravak u Francuskoj (Bordeaux Imaging Center (BIC) u Bordeauxu i Université de Pau et des Pays de l'Adour/CNRS/Cameca u Pauu i Parizu) te stipendije ERASMUS+, radi tromjesečnog znanstveno-stručnog usavršavanja na Kemijskom fakultetu Sveučilišta u Oviedu u Španjolskoj. Dobitnica je i brojnih stipendija za sudjelovanje na znanstvenim skupovima u inozemstvu (u Sloveniji, Velikoj Britaniji i Estoniji). Sudjelovala je na brojnim domaćim i međunarodnim tečajevima i znanstvenim skupovima.

Do sada je autorica/suautorica 23 znanstvena rada, od čega je prva autorica na četiri znanstvena rada u časopisima citiranim u Web of Science (WoS) bazi podataka, te 46 sažetaka u zbornicima znanstvenih skupova.

Popis objavljenih znanstvenih radova

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