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# BIOACCUMULATION OF 18 TRACE METALS IN MUSCLE AND EXOSKELETON IN THE NOBLE CRAYFISH (*ASTACUS ASTACUS* L.) IN THE RIVER ZETA (MONTENEGRO)

## SUMMARY

This study assessed the level of heavy metals accumulation in abdominal muscle and exoskeleton on of *Astacus astacus* L. collected from Zeta River in July, with the aim of predicting health risk effect on human consumers. Noble crayfish samples were collected from four different sites along the river. The concentration of metals in the muscle and chitin of the Noble crayfish was determined using atomic absorption spectrophotometer. The exoskeleton showed a decidedly higher content of most analysed trace elements with respect to muscles (two- to fourfold higher for Al, Ca; two-to eightfold for Fe and ten times higher for Sn and Mn). Hg, K, Mg and Zn were found in higher concentrations in the abdominal muscle in Noble crayfish from the River Zeta. Nonsignificant differences were found between muscles and exoskeleton for As, Cu, K, Na, Ni, and Si (except first locality). Muscle content analysis had indicated that sampling point I (first) had the lowest concentration and sampling point IV (the last) had the highest concentration of accumulated heavy metals. It was believed that domestic activities around the river the major contributing factor to the accumulation of toxic heavy metals in muscle and carapace sample analysed, it is recommended that intervention relevant authorities are needed to curtail chemical degradation of the aquatic biota over a period of time. The most of trace element concentrations found in crayfish tissues were in the range considered harmful to human health (except for Na, Ni, Sn and Cr). We hypothesize that *A. astacus* in the Zeta River aquatic communities can act as a vector of pollutants, as crayfish can transfer their relatively high amount of heavy metals to higher trophic levels.

**Keywords**: Bioaccumulation, heavy metals, crayfish muscle Exoskeletonon, pollution, Montenegro.

## INTRODUCTION

The Noble crayfish (*Astacus astacus* L.) stands as an important food in many parts of the Europe, being a rich source of protein. It is a commercial

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species in Montenegro. In the past, it was hunted in larger quantities (Marić and Rajković, 2004), and lately in much smaller ones, but there is no reliable data on that. In general, commercial hunting of all freshwater species is in significant decline (Marić, 2018; Pešić *et al.* 2019), and the causes have not been studied in detail. The health safety of aquatic organisms used for commercial purposes has also not been well and comprehensively studied. Only some species of fish have been studied in more detail from the aspect of bioaccumulation of heavy metals in muscles (Rakočević *et al.* 2018). The Noble crayfish has not been studied from this aspect. The commercial aspect and the lack of data on the quality of meat of this type are the main reasons for the research of metals presented in this paper.

In the least hundred years, a significant increase in the concentration of metals in the environment has been recorded (Vamerali *et al.* 2010). Heavy metals of natural and anthropogenic sources are released into aquatic ecosystems, where they pose a serious threat because of their toxicity, long persistence, bioaccumulation and biomagnifications in the food chain (Zhou *et al.* 2008). Aquatic invertebrates are sensitive to metals in their environment and are mostly used to assess aquatic ecosystem quality (Bonada *et al.* 2006). They can accumulate metals directly from the sediments or by food and, based on their life cycle, some species can accumulate more elements than the others (Pourang 1996). Heavy metals affect living organisms in various ways; some have essential functions (e.g. Cu, Mn and Zn) contributing to the maintenance of a good health in humans and animals (Rainbow 2007) and are toxic only at high levels, whereas others are xenobiotic: non-essential metals as Pb and Hg do not play any role in metabolism and are toxic even at low concentrations (Kouba *et al.* 2010). The aim of present work was to characterize heavy metal content in selected tissues of noble crayfish samples collected from different sites of the River Zeta in order to assess bioaccumulation mechanisms, evaluate whether these concentrations could be considered harmful for human health and, finally, to identify possible sources of contamination in the study area. We exploited the selective metal bioaccumulation in two different tissues of *A. astacus*, exoskeletonon, and abdominal muscle, which is the edible part. There is very scarce information on heavy metal distribution in biota of this area, in spite of the presence of many industrial and agricultural activities.

## MATERIAL AND METHODS

### *Area and habitat study*

Nikšić field is situated in the western part of the Republic of Montenegro. It is situated between 18$^{o}$ 30' to 19$^{o}$ 10' of eastern geographical longitude and 42$^{o}$ 42' to 42 $^{o}$ 53' of northern geographical latitude on the altitude of around 600-630m (Figure 1). The main water course of Nikšićko polje (field) is the so coaled Gornja Zeta (the Upper Zeta). The length of the River Gornja Zeta is around 15 km counting from the confluence of the Rastovac and the Sušica, than it disappears under the ground and appears again on the spring Glava Zete on peak elevation of 50m. There are three artificial lakes Krupac, Slano and Liverovići. The Noble crayfish also live in them.

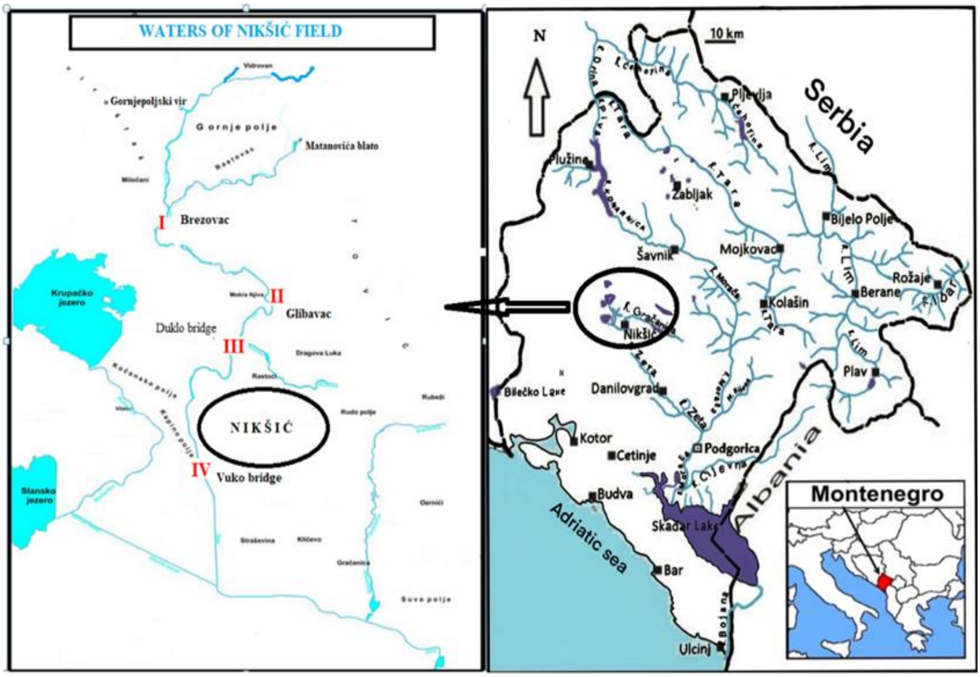


Figure 1. Map of water bodies in Nikšić field (sampling sites marked with Roman numerals)

The width of the riverbed is 7-25 m, and the water depth is 0.2 to 5 m. Different trees and shrubs grow on the banks of the river, mostly willows (*Salix* spp.) and poplars (*Populous* spp.). The bank of the river is in some places overgrown with reeds - *Phragmites* sp. The riverbed has different water depths, the lotic and lentic areas alternate. The bottom of the river is covered with aquatic vegetation (70%) which consists mainly of species of the genus Potamogeton, to a lesser extent the species Miriofilum sp. and Ranunculus sp. The fauna of the bottom of the river Zeta consists of representatives of Gastropods, Oligochaeta and insect larvae: Ephemeroptera, Plecoptera, Trichoptera, Chironomidae, Simulidae, etc. (Marić and Rajković, 2004). According to Marić (2019) were found six species of fishes: *Thimallus thimallus*; *Salmo farioides; Salmo labrax; Oncorhynchus mykiss Squalius plaryceps; Phoxinus* sp.

Noble crayfish were collected from four localities on the Zeta River (Figure 1) the first locality, Gornje Polje, is located in the immediate vicinity where two rivers, Rastovac and Bistrica, join and form the river Zeta. There are no industrial pollutants in this area. The 2nd locality (II), Mokra Njiva, 2-3 km away from the main road Nikšić - Plužine. The Brezovik hospital located upstream 2 km from this site, and it has a collector for water purification. The locality Duklov most (III), is located in the city zone. Upstream from this locality, the river Bistrica flows into the river Zeta. It flows through the industrial zone of the city and brings waste of different origin. The fourth locality (IV), Vukov most (bridge), is just below the city. All industrial waters and household waters upstream reach in the river.

***Crayfish collection and measurements and statistical analysis***

Our study was conducted in the summer (July) 2013 using a commercial fishing gears – the traps or fyke nets in the area of the upper course of the Zeta River, Montenegro. Researching area included 4 localities between which the distance is about 3 km. A total of 99 specimens of the Noble Crayfish (*Astacus astacus* L.) in the River Zeta were caught. All specimens (fresh) were measured for total length (TL, in cm) and weight (W, total wet weight in g) to the nearest 0.01g by an electronic balance. The total length (TL) of each specimens (measured using digital caliper to the nearest 0.01 mm) was taken from the beginning of the rostrum to the end of the telson. All specimens (sexually mature) of the Noble Crayfish caught at four site were larger than 9.5 cm. Differences between 4 sites and samples were analysed by Analysis of Variance (ANOVA). The statistical analyses were considered at a significance level of 1% ($P<0.01$.

Tissue samples of the caudal (tail) muscle and exoskeleton (from carapace) were taken, than milled using an electric grinder and stored in airtight polyethylene bottles at -18 °C until chemical analyses. All chemical analyses were performed in laboratory of Center for Eco-toxicological Research (CETI, Podgorica). Three parallel determinations were carried out for each parameter. Concentrations of analysed trace elements were expressed on a wet weight basis, as mg $kg^{-1}$. The limits of quantification (LOQ) were: 0.1 mg $kg^{-1}$ for Pb; 0.01 mg kg-$^{1}$ for Cd; 0.06 mg $kg^{-1}$ for As; 0.025 mg $kg^{-1}$ for Co and Cr; 0.02 mg $kg^{-1}$ for Ni; 0.2 mg $kg^{-1}$ for Fe, Mn, Cu and Zn; 0.001 mg $kg^{-1}$ for Hg; and 5 mg kg-$^{1}$ for Ca and Mg. Detailed procedure was described by Rakocevic et al (2018). Data analyses were performed with using Statistica 8.0 software (StatSoft, USA). Data are given as Mean±SD (standard deviation).

## RESULTS

The Noble crayfish caught ranged in total length from 9.50 to 14.60 cm and in weight from 23.50 to 125.90 g. No significant differences were found between the average length and weight of the investigated Noble crayfish from four localities (($P> 0.05$).

The concentrations of the relevant metals (15 metals) analysed in the muscle and exoskeleton - carapace from four localities in the Zeta River are presented in Table 1. Cadmium, Pb and Mo, if present, were in concentrations below the detection limit. A wide range of concentrations of some metals were found in the analysed tissues in the muscle: As < 0.025-16.0, Ca 25.2-116.1, Hg 33.1–97.1, Si 15.6-43.1 and in the exoskeleton Al 9.6-68.9, Ca 68.9-141.1, Fe 26.7-58.5, Mn 37.4-84.4.

A narrow range of concentrations was detected for each of the other trace metals in the muscle Al 3.7-13.1, Cr undetected-1.1, Cu 8.6-21.7, Fe 6.0-17.9, K 2.3-8.2, Mn 2.2-14.2, Mg 200.1-253.5, Na 0.8-2.2, Ni 0.04-0.14, Sn 0.2-1.6, Zn 17.6-19.8 and in the exoskeleton As 7.8-15.6, Cu 17.5-22.2, Hg 6.2–15.1, K 1.9-3.3, Mg 1.0-2.5, Na 1.9-2.7, Ni 0.03-0.06, Sn 27.9-40.8, Si 40.6-46.8, Zn 11.3-18.8. Cr was found only in the second locality in both carapace and abdominal muscle but was higher in the latter.

As shown in table 1, Al, Cu, Ca, Fe, Mn, Sn and Si concentrations were significantly higher in carapace of *A. astacus* than abdominal muscle. Mg, Hg, K and Zn mainly accumulated in crayfish muscle. Only two metals (Hg and Mg) were significantly higher in abdominal muscle of *A. astacus* than carapace. Mg primarily accumulated in crayfish abdominal muscle at similar levels for all sites (Tables 1). No significant differences were found for the other trace elements content among the sampled tissues.

Table 1. Concentrations of 15 metals in the muscle and chitin- exsosceleton samples from different locations. Data are given as Mean±SD.

| Locality | 1. | | 2. | | 3. | | 4 | |
|---|---|---|---|---|---|---|---|---|
| Metals/in | muscle | chitin | muscle | chitin | muscle | chitin | muscle | chitin |
| Al# | 3.7±2.01 | 9.6±5.50 | 13.1±6.33 | 68.9±9.82 | 11.4±6.23 | 20.6±6.05 | 8.5±5.88 | 49.8±7.54 |
| As | < 0.025 | 7.8±4.82 | 16.0±7.45 | 10.4±7.23 | 12.8±7.03 | 10.8±6.24 | 12.2±6.06 | 15.6±6.81 |
| Ca# | 25.2±5.87 | 68.9±12.34 | 36.7±8.79 | 141.1±15.38 | 37.3±7.54 | 56.2±10.16 | 116.1±12.89 | 49.9±10.84 |
| Cr | / | / | 1.1±0.32 | <0,02 | / | / | / | / |
| Cu # | 8.6±2.88 | 17.5±3.61 | 12.9±3.98 | 22.2±5.04 | 21.7±6.32 | 17.9±3.76 | 15.4±4.13 | 20.7±4.87 |
| Hg# | 85.9±43.58 | 10.7±4.65 | 97.1±34.12 | 12.5±6.72 | 59.3±9.58 | 6.2±3.61 | 33.1±8.62 | 15.1±6.81 |
| Fe# | 6.0±5.21 | 58.5±41.86 | 13.2±10.29 | 48.8±40.34 | 14.4±12.65 | 26.7±20.43 | 17.9±16.82 | 38.4±28.21 |
| K | 2.3±1.11 | 2.1±0.93 | 4.0±1.74 | 3.3±1.23 | 4.0±1.65 | 2.0±0.90 | 8.2±4.66 | 1.9±0.96 |
| Mn# | 2.2±0.55 | 60.9±20.11 | 3.7±1.08 | 37.4±6.24 | 6.8±2.45 | 63.1±8.33 | 14.2±4.56 | 84.4±25.66 |
| Mg# | 200.1±43.76 | 1.6±0.22 | 222.8±38.93 | 2.5±0.72 | 242.0±44.37 | 1.6±0.75 | 253.5±56.88 | 1.0±0.0.20 |
| Na | 0.8±0,21 | 1.9±0.28 | 1.7±0.31 | 2.7±0.33 | 2.1±0.32 | 2.0±0.24 | 2.2±0.43 | 2.3±0.36 |
| Ni | 0.04±0.02 | 0,05±0.4 | 0.10±0.09 | 0.05±0.03 | 0.14±0.12 | 0.06±0.03 | 0.05±0.03 | 0.03±0.02 |
| Sn# | 0.2±0.09 | 27.9±18.65 | 0.31±0.08 | 38.6±25.82 | 0.4±0.09 | 39.7±22.41 | 1.6±0.02 | 40.8±19.94 |
| Si# | 15.6±10.17 | 46.8±12.03 | 43.1±20.65 | 40.6±10.56 | 24.4±10.28 | 43.0±11.62 | 34.9±12.88 | 40.9±14.53 |
| Zn | 18.0±9,78 | 13.3±5.49 | 19.8±8.76 | 15.7±6.38 | 17.6±7.34 | 11.3±5.11 | 18.9±10.56 | 18.8±7.68 |

The concentrations of fifteen elements in the noble crayfish were examined and the results showed the wide variations for the concentration of the trace of almost all elements among four localities (Table 1). The concentration of heavy metals in muscle showed significant differences between sampling sites (one-way ANOVA, all $p < 0.01$), and was higher at Vukovog mosta (4 sites), while first sites exhibited a lower metal pollution scenario. As shown in table 1, the smallest amount were found for twelve metals (80%): As; Al; Cu; Ca; Fe; Mn; K; Ni; Na; Sn; Si and Mg in the muscles, while the highest values were not found at the first locality. The concentrations of Fe and Si in exosceleton of crayfish from first locality was highest comparing to the other three localities, and at the fourth only for Hg and Mn.

The concentrations of four essential elements (Mn, Fe, Cu and Zn) in the noble crayfish showed a similar range of concentrations on the longitudinal profile. Observed on the longitudinal profile, Mn (and Sn) have a slight increase in both tissues, and Mg only in the muscles, but all nonsignificant. Fe and Ca in abdominal muscle increase going downstream, and in chitin the values are higher for both elements in the first two localities. In contrast, the values for Cu in muscle are higher in the other two localities downstream. Only the concentration of Hg in the muscles decreases significantly downstream, but there is no regularity in the exoskeleton.

At the second locality - Mokra Njiva, the lowest values were not detected in the meat of river crayfish, and the largest amount was registered for two metals: Hg and Si. In the chitin cuticle at site II, Al, Fe and Ca have the highest values. Chromium was detected only at this site. At site III, Duklov most, the highest amount was found only for Ni in abdominal muscle of noble crayfish. Potassium and Manganese increase downstream, properly in muscle, while in chitin they do not increase properly.The concentrations of Fe, Mn, Mg and K in abdominal muscle of crayfish from last sites was highest comparing to the other three regions.

## DISCUSSION

To assess the health risk to the crayfish-consuming population, we investigated metals (18) in crayfish tissues (exoskeleton, abdominal muscle) in the upper part of the Zeta River. The Zeta River receives domestic and industrial untreated wastewaters from Nikšić city and surrounding villages during the last decades. In this study, the degree of metal accumulation in the tissues of crayfish samples collected from the Zeta River was investigated, because Crayfish may be used as an environmental indicator because their tissues tend to accumulate metals, including heavy ones (Anderson *et al.* 1997b). Almost all studies on the distribution of metals in crustacean tissues have shown that the hepatopancreas is the most important storage organ for trace elements and then gills or exoskeleton (Pourang and Dennis 2005, Alcorlo *et al.* 2006., Mistri et al 2020, Fikirdesici-Ergen *et al.* 2019). The abdominal muscle has consistently been found in the literature to be the tissue containing the lowest concentration of metals (Anderson *et al.* 1997; Mackevièienë 2002, Naghshbandi *et al.* 2007, Kouba *et al.* 2010 Protosowicki et al 2013), and our results also agree with this. According to Naghshbandi et al (2007) only Zn concentrations were significantly higher in abdominal muscle of A. leptodactylus than carapace. Our analyses indicated that seven metals was accumulated in the greatest amounts in the exoskeleton (Table 1). Several metals were several times larger in the exoskeleton than in the muscles, e.g. Al, Fe, Sn. According by Fikirdesica-Ergen *et al.* (2019) Cu, Zn, Mn, Fe and Al are almost 10 times higher in exoskeleton than in muscle. Al is a metal abundant in nature and there was common belief that it does not have a negative effect on human health (Ranau *et al.* 2001). High levels of metals in the exoskeletonon may be due to the absorption of metals from water (Anderson *et al.* 1997a, b) or the fact that it is the route for both absorption and excretion of metals from the organism (Mackevièienë 2002). The opposite results for Cu, Zn and As were denoted by Stanek *et al.* (2017) for spiny-cheek crayfish and Mistri *et al.* (2020) for *Procambarus clarkii*. It is generally known that the chitin cuticle has a protective role, it is logical to expect that it contains the most heavy metals, which means that the cuticle, in addition to mechanical or physical protection (from injuries), protects internal organs and meat from harmful metals. Therefore, it has a special role in young crabs because they change their clothes five or six times during the first year (Reynolds, 2002).

Only two metals (Hg and Mg) were significantly higher in abdominal muscule of *A. astacus* from the River Zeta, than exoskeleton. In crayfish, mercury is accumulated largely in muscle (Simon *et al.* 2000; Loukola-Ruskeeniemi *et al.* 2003; Kouba *et al.* 2010) and according to Wiener *et al.* (2003) the mercury predominantly accumulates and in fish muscle. According to Kouba *et al.* (2010) and nickel accumulated largely in muscles and exoskeleton, respectively. Nickel accumulated approximately equally in both tissues of noble crayfish from Zeta river. The values of Hg and Mn were very high at each site studied (Hg - 97.1 to 33.1 mg, Mg - 253.5 to 200.1 mg) in muscle. Mercury is explicitly toxic substances, although their high concentrations are tolerated by organism. However, decapods are resistant organisms to environmental contamination, and even relatively high concentrations of metals (in this example Hg) are usually not responsible for their mortality (Kouba *et al.* 2010). High level some metals in the Noble crayfish from the river Zeta suggesting the pollution of the environment by heavy metals. The accumulation of metals in crayfish tissues is dose- and time-dependent, and therefore may be reflective of the levels of metals in the environment (Antón *et al.* 2000; Sánchez-López *et al.* 2004; Alcorlo *et al.* 2006; Allert *et al.* (2009). However, the measured values for Pb, Cd and Cr were below the threshold, thus suggesting limited contamination in the Nikšić area. No significant differences were found for the other trace elements content among the sampled tissues. With regard to the concentrations, the metals formed the following order in muscle: Mg > Hg > Ca > Si > Zn > Cu > Fe > As > Al > Mn > K > Na > Sn > Ni > Cr >, and in exoskeleton: Ca > Mn > Fe > Si > Sn > Al > Cu > Zn > As > Hg > K > Na > Mg > Ni > Cr.

The observed order of increasing metal concentrations in crayfish organs is no consistent with their content in the lithosphere and hydrosphere provided by Information on the state of the environment in Montenegro for 2010, Ministry of Sustainable Development and Tourism 2010, Environmental Protection Agency of Montenegro). This shows that these arrays of metals are a consequence of pollution in this area. However, Iron, Mn and Al (in the Zeta river basin) are naturally abundant in sediments in Montenegro. Contamination can originate from a wide variety of anthropogenic sources such as disposal of high metal wastes in improperly protected landfills, land application of fertilizer, animal manures, sewage sludge, compost, pesticides and coal combustion residues located in the Zeta river basin. According to Wuana and Okieimen (2011). most commonly found at contaminated sites are: Pb, Cr, As, Zn, Cd, Cu, Hg, and Ni. According to Bagatto and Alikhan (1987) the content of zinc in the body of a crayfish is naturally high. The concentrations of As, Cr, Hg and Ni in the present study were higher than the maximum permissible limits for human consumption established by Montenegrin legislation. Although no EU threshold limit for human consumption of crayfish muscle is established for Cu and Zn, other national regulations set maximum limits, e.g. for Cu at 20 mg kg−1 w.w. in edible mass (Spain: Boletin Oficial del Estado 195, 15/8/1991), for Zn at 70 mg kg−1 w.w. (U.S. Food and Drugs Administration), as reported by Alcorlo *et al.* (2006) and our results also agree with this. The content of these metals in crayfish organs varied depending on where they were caught. The highest metals content was detected in the downstream samples (3 and 4) close to main wastewater discharge point suggesting an anthropogenic contribution to total metals concentrations in muscle of crayfish from River Zeta. Only one metals - Hg were significantly higher on first and second sites in abdominal muscle of *A. astacus*, while crayfish collected upstream showed lower values for all heavy metals. The Zeta River receives domestic and industrial untreated wastewaters from Nikšić city and surrounding villages during the last decades. Noble crayfish does not have a large home range, hence migrations do not influence the level of metals accumulated in its tissues (Bohl 1999). Specimens are therefore representative of the locations in which they are caught.

Mercury concentrations were similar among sites. Based on a comparison, we found higher Hg concentrations with respect to those found by Finerty *et al.* (1990), Hothem *et al.* (2007) etc. from highly industrialized areas. We assume that the source of Hg pollution comes from medical waste that is uncontrolled discharged near the riverbed, or is derived from pesticides. The pollution of the environment by heavy metals is a result of various industrial activities and is also a multi-element problem in many areas of the world (Waisberg *et al.* 2003., John *et al.* 2008). The highest Al, As, Ni and Si concentrations in crayfish were found near the contaminated sediments at Zeta River. The high values draw attention to the land-based domestic and industrial inputs. The Zeta River receives domestic and industrial untreated wastewaters from Nikšić city and surrounding villages during the last decades. Agriculture takes important place for the economic value of the study area. Conservative fungicides that contain Cu are mainly used for fruits plantations and green-houses around the study area and composed fertilizer enriched with Zn and microelements fertilizer contains Mn are also widely used for farming around study area. Measuring Hg, Pb, Cr, As, Ni and Sn levels in crayfish from the Zeta river is important to demonstrate the potential risk to humans if the crayfish are consumed.

Content of some toxic metals in the muscle of Noble crayfish from River Zeta exceed the statutory limits for fish and crayfish intended for human consumption. Therefore, the crayfish from this region, in general, are not safe for human consumption. The average Hg values were higher than the limits of

Montenegro Official Gazette (2009) and according to EU limits (European Union Regulation). Because contamination of crayfish by heavy metals may pose a real risk to consumer, therefore, it is important to have knowledge on heavy metals levels in the tissues of crayfish used for food, and further investigation should be continued in future studies. Although other studies have shown that muscle tissue contains the lowest heavy metal levels compared with other tissues, muscle tissue is an important measure from a health view point since it is the most edible part of the crayfish (Anderson *et al.*,1997; Alcorlo et al.,2006). Environmental pollution by heavy metals is an escalating problem worldwide.

## CONCLUSIONS

This study was carried out to provide information on heavy metal concentrations in noble crayfish which were consumed by local people and have commercially importance. Crayfish accumulated higher levels of heavy metals in the exosceleton than in the muscle, consistently with previous studies. The levels of certain metals accumulated in the abdominal muscle exceeded the threshold values established by the EU (e.g. Hg) legislation. Measuring The accumulation of metals in crayfish tissues from the Zeta river is important to demonstrate the potential risk to humans if the crayfish are consumed. Our results also evidence that Noble crayfish living in the River Zeta are not suitable for human consumption. Because contamination of crayfish by heavy metals may pose a real risk to consumer, therefore, it is important to have knowledge on heavy metals levels in the tissues of crayfish used for food, and further investigation should be continued in future studies. To the legislators, we propose A. astacus for use as a bio indicator of heavy metals due to its ability to accumulate these environmental pollutants.

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