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MAPPING DIFFERENTIAL ELEMENTAL ACCUMULATION IN FISH TISSUES: IMPORTANCE OF FISH TISSUE SAMPLING STANDARDIZATION

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Abstract: The concentrations of As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se and Zn in the muscle, gills, liver and intestine of the wels catfish (*Silurus glanis*) from the Danube River were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The aim of the study was to determine whether in complex muscle/skin, gill filament/gill arch, proximal/distal liver and proximal/median/distal intestine samples, particular components differ in concentrations of the analyzed elements. Results indicated that there were no differences in the accumulation of different elements between the proximal and distal liver segments and between the proximal and median intestine sections. Conversely, elemental accumulation patterns in muscle and skin differed significantly. Significant differences were also observed between the gill arch and filaments, as well as between the distal and the two upper intestine sections. Findings indicated the importance of detailed reporting of tissue sampling, i.e. whether the skin was included in the muscle sample, as well as if the gill arch and filaments were analyzed together. Due to a potential bias that can be produced by different muscle/skin or gill arch/filament ratios included in the sample, we strongly recommend that they should not be analyzed together. Results of the present study might be of interest to the scientific community and stakeholders involved in aquatic ecosystem monitoring programs.

Key words: metal; Danube River; wels catfish; *Silurus glanis*; ICP-MS

INTRODUCTION

Metals are considered to be critical contaminants of the aquatic environment [1]. Fish are among the aquatic organisms most susceptible to water and sediment pollution [2]. At the same time, as species positioned at the top of the food chain, they can accumulate high metal levels [3]. The presence of metals in fish tissues is therefore of considerable importance to environmental and food safety, as well as for public health [4]. Metal pollution in fish has become a worldwide concern, and numerous studies and monitoring programs for metal accumulation in fish have been conducted [5-7].

Studies related to metal pollution in fish have been mainly focused on muscle tissue as the main fish part consumed by humans, as well as on the gills, liver, kidneys, and intestine, which represent either major accumulation centers in fish or the main metal uptake routes [5,8,9].

However, there is an apparent lack of a standardized approach regarding fish tissue sampling. To assess this issue in greater detail, we conducted a small literature survey. Analysis of a randomly selected sample of 100 articles dealing with metal accumulation in fish tissues indicated that authors rarely provide informa-

tion on the fish tissue-sampling procedure. As much as 77% of papers failed to report whether the skin was separated from the muscle tissue, or if both tissues were assessed together. A minority of authors either emphasized that they removed the skin from the muscle or that they assessed each tissue separately, while a single author reported that muscle and skin were assessed together. As stated by Crafford and Avenant-Oldewage [10], authors either include or remove the skin from the muscle sample, although they often fail to report which approach was employed. Furthermore, only 5% of the authors reported that they separated the gill arch and the filaments, while a single author stated that both gill segments were combined in a sample. None of the authors specified which part of the liver was included in the sample. Only a single author provided details on the intestine section that was sampled.

In the present study, we assessed elemental concentrations in different segments of muscle, gills, liver, and intestine of the wels catfish (*Silurus glanis*) from the Danube River in order to determine possible differences between them. Such information could indicate the importance of sampling procedure standardization and reporting in studies dealing with elemental accumulation in fish. This issue has not received proper attention so far, and the results of the present study might therefore be of interest to both the scientific community and the stakeholders involved in aquatic ecosystem monitoring programs.

MATERIALS AND METHODS

Sample collection

Wels catfish specimens (n=13) were collected by professional fishermen during March 2013 from the Danube River (1169 river km) in the vicinity of the city of Belgrade, Serbia (44º 49' 54.48" N, 20º 27' 23.68" E). The same sample was previously used to assess elemental accumulation in different tissues of the studied species [11]. Specimens were killed with a quick blow to the head, measured for their total body length (cm) and total body weight (g), checked for their sex and maturity by inspection of the gonads, and subsequently dissected. Samples of the muscle (right dorsal muscle), skin, gill filaments, gill arch, liver and intestine were collected. Each liver sample was separated into two sections, proximal and distal. Given that the intestine of catfish species is clearly differentiated into three principal regions – proximal, median and distal [12], samples from each region were sectioned. All samples were washed with distilled water and stored at -20°C prior to analysis.

Sample preparation and analysis

The samples were freeze-dried using a Christ rotary vacuum concentrator, model GAMMA 1-16LSC (Osterode am Harz, Germany). Analytical portions of approximately 0.3 g (dry weight) were accurately weighed and subsequently processed in a microwave digestion system. Samples were mineralized by adding 6 mL of 65% HNO_3 and 4 mL of 30% H_2O_2 (Merck, Darmstadt, Germany). Microwave assisted digestion was performed in a Speedwave™ MWS3+ oven (Berghof, GmbH, Eningen, Germany). The following temperature program was used (default food program): 5 min at 160°C; 15 min at 190°C; 20 min at 100°C. After cooling, digested samples were transferred into 100-mL polypropylene volumetric flasks and diluted to volume with ultrapure water. In order to assess the possible presence of trace elements in reagents or carry-over effects of digestion vessels, five reagent blank samples were prepared as well, one per each session, according to the described procedure. These samples were analyzed in each analytical batch.

Analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) using the instrument "iCap Q" (Thermo Scientific, Bremen, Germany), equipped with a collision cell and operating in kinetic energy discrimination (KED) mode. The following isotopes were measured: chromium (^{52}Cr), manganese (^{55}Mn), iron (^{57}Fe), cobalt (^{59}Co), nickel (^{60}Ni), copper (^{63}Cu), zinc (^{66}Zn), arsenic (^{75}As), selenium (^{77}Se), cadmium (^{111}Cd), and lead (^{208}Pb). Basic operating conditions of the instrument are shown in Table 1.

Torch position, ion optics and detector settings were adjusted daily using a tuning solution (Thermo

Table 1. Operating conditions for ICP-MS.

RF power	1550 W
Cooling gas flow	14 L/min
Nebulizer flow	1 L/min
Collision gas flow	1 mL/min
Operating mode	KED
Dwell time	10 ms (Cr, Mn, Fe, Co, Ni, Cu, Zn, Se) 100 ms (As, Cd, Pb)
Sampling cone	Platinum, 1 mm orifice diameter
Skimmer cone	Platinum, 0.75 mm orifice diameter

Table 2. Assigned and measured concentrations of the SRM 1577c and BCR-186 reference material used for quality control. Values are given with the standard uncertainties and with the 95% confidence interval.

Elements	**Assigned values (NIST 1577c)±U**	**Measured value ±U**
^{75}As, µg/kg	19.6±1.4	20.5±1.1
^{111}Cd, µg/kg	97±1.4	97.9±2.6
^{208}Pb, µg/kg	62.8±1.0-1.6%	63.3±2.6
^{63}Cu, mg/kg	275.2±4.6	271.9±5.7
^{57}Fe, mg/kg	197.94±0.65	197.43±5.21
^{66}Zn, mg/kg	181.1±1.0	180.9±1.8
^{55}Mn, mg/kg	10.46±0.47	10.55±0.25
^{52}Cr, µg/kg	53±14	51±2.8
^{59}Co, mg/kg	0.3±0.018	0.31±0.016
^{60}Ni, µg/kg	44.5±9.2	52.7±4.3
^{77}Se, mg/kg	2.031±0.045	2.055±0.066
Element	**Assigned value (BCR-186)±U**	**Measured value±U**
Hg (total), µg/g	1.97±0.04	2.02±0.07

Scientific Tune B) in order to optimize measurements and to minimize possible interferences. For quantitative analysis of the samples, a five-point calibration curve (including zero) was constructed for each isotope in the concentration range of 0.1-2.0 µg/L for ^{75}As, ^{111}Cd and ^{208}Pb, and 0.1-2.0 mg/L for ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn and ^{77}Se. An additional line of the peristaltic pump was used for an online introduction of a multi-element internal standard (^{6}Li, ^{45}Sc – 10 ng/mL; ^{71}Ga, ^{89}Y, ^{209}Bi – 2 ng/mL), covering a wide mass range. Concentrations of each measured isotope were corrected for response factors of both higher and lower mass internal standards using the interpolation method.

The quality of the analytical process with respect to accuracy and precision was assessed by analysis of the standard reference material SRM 1577c (NIST, Gaithersburg, MD, USA). Reference material was prepared in a random manner during microwave digestion of each sample batch and run at the beginning, in the middle and at the end of each sample list. Measured concentrations were within the range of the certified values for all isotopes (Table 2).

Mercury (Hg) was measured using cold vapor technique by atomic absorption spectrometer SpectrAA 220 (Varian, Palo Alto, USA) with a VGA 77 hydride system and $SnCl_2$ in HCl as a reductant. Calibration was performed in five points; standard concentration range was 0.5-15.0 ng/mL. Absorption was measured at 257.3 nm. The quality of the analytical process was controlled using BCR-186 certified reference material (IRMM, Geel, Belgium). Reference material preparation and analysis were conducted in the same manner as described previously. Obtained Hg concentrations corresponded to the certified value (Table 2). All concentrations were expressed as µg g^{-1} dry weight (dw).

Statistical analysis

The statistical analysis included comparisons of metal and trace element concentrations between skin and muscle, gill arch and filaments, proximal and distal liver sections, and the three intestine sections (proximal, median and distal). The normality of distribution of the analyzed samples was tested by the Kolmogorov-Smirnov test. Since the variables lacked normality of distribution, nonparametric tests were applied. Groups were compared by the Mann-Whitney U test ($p<0.05$).

RESULTS

The average body length and weight of the analyzed wels catfish specimens were 64.2±4.5 cm (55.5-69.0 cm) and 1773±327 g (1190-2390 g), respectively. All specimens were immature, with males representing the majority of specimens (85%).

Elemental accumulation in the muscle and the skin significantly differed ($p<0.05$) for the majority of elements (Table 3). Significantly higher As, Co, Cu,

Table 3. Metal and trace element concentrations in muscle and skin of the wels catfish (mean, standard deviation, minimum and maximum values). Concentrations are expressed as μg g^{-1} dry weight.

		As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Zn
Muscle	Mean	0.131[a]	0.004	0.018[a]	0.138	0.949[a]	19.46[a]	1.598[a]	0.674[a]	0.120	0.006	0.905	19.62[a]
	S. dev.	0.097	0.007	0.024	0.325	0.656	15.08	0.536	0.401	0.211	0.012	0.333	10.20
	Min	0.040	0.000	0.000	0.000	0.210	7.29	0.656	0.400	0.000	0.000	0.340	10.28
	Max	0.394	0.024	0.090	1.190	1.980	63.62	2.841	1.910	0.680	0.035	1.420	40.03
Skin	Mean	0.360[b]	0.005	0.038[b]	0.154	1.902[b]	25.86[b]	0.657[b]	0.872[b]	0.244	0.009	0.866	58.95[b]
	S. dev.	0.322	0.006	0.010	0.206	0.736	8.81	0.185	0.233	0.286	0.012	0.405	11.72
	Min	0.055	0.000	0.020	0.000	0.990	17.09	0.274	0.470	0.000	0.000	0.370	42.69
	Max	1.062	0.019	0.060	0.580	3.010	44.36	0.949	1.240	1.020	0.040	1.620	79.19

[a, b] The value with a different letter in the same column is different (Mann-Whitney U test, $p<0.05$)

Table 4. Metal and trace element concentrations in gill filaments and gill arch of the wels catfish (mean, standard deviation, minimum and maximum values). Concentrations are expressed as μg g^{-1} dry weight.

		As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Zn
Gill filaments	Mean	0.088	0.036[a]	0.135[a]	0.271[a]	4.462[a]	162.98[a]	0.328[a]	14.982[a]	0.166	0.236	2.122[a]	80.42
	S. dev.	0.024	0.014	0.059	0.116	1.784	38.31	0.082	10.357	0.161	0.188	0.528	20.84
	Min	0.045	0.021	0.060	0.110	2.580	125.60	0.221	5.990	0.000	0.100	1.250	63.15
	Max	0.129	0.074	0.240	0.510	8.640	258.27	0.490	44.060	0.450	0.788	3.120	138.71
Gill arch	Mean	0.117	0.005[b]	0.054[b]	0.090[b]	0.412[b]	43.98[b]	0.071[b]	34.666[b]	0.211	0.387	0.715[b]	69.81
	S. dev.	0.097	0.004	0.019	0.117	0.194	19.70	0.054	20.245	0.312	0.407	0.275	5.33
	Min	0.036	0.000	0.030	0.002	0.150	23.16	0.034	15.920	0.000	0.192	0.280	60.72
	Max	0.386	0.013	0.100	0.450	0.760	77.82	0.216	84.230	0.860	1.718	1.240	79.18

[a, b] The value with a different letter in the same column is different (Mann-Whitney U test, $p<0.05$)

Table 5. Metal and trace element concentrations in two liver sections (proximal and distal) of the wels catfish (mean, standard deviation, minimum and maximum values). Concentrations are expressed as μg g^{-1} dry weight.

		As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Zn
Proximal section	Mean	0.106	0.217	0.363	0.090	18.905	577.04	0.598	4.722	0.088	0.067	7.518	91.20
	S. dev.	0.049	0.113	0.154	0.186	5.539	431.16	0.290	1.390	0.115	0.034	2.090	22.25
	Min	0.043	0.043	0.080	0.000	7.810	132.22	0.173	2.250	0.000	0.008	3.890	45.04
	Max	0.199	0.361	0.650	0.670	28.230	1542.84	1.458	7.820	0.370	0.122	12.060	125.28
Distal section	Mean	0.096	0.177	0.355	0.012	17.770	745.68	0.639	4.568	0.059	0.067	7.935	93.14
	S. dev.	0.046	0.104	0.151	0.018	5.095	468.09	0.301	1.275	0.086	0.032	2.203	22.19
	Min	0.042	0.033	0.100	0.000	7.600	151.62	0.264	2.400	0.000	0.013	4.650	48.59
	Max	0.195	0.347	0.600	0.040	24.340	1852.70	1.562	7.070	0.290	0.112	12.940	124.84

Fe, Mn and Zn concentrations were detected in the skin, while the muscle had significantly higher Hg concentrations. There were no differences between the two tissues regarding Cr, Cd, Ni, Pb and Se concentrations.

The gill filaments and gill arch also differed significantly ($p<0.05$) for most of the studied elements (Table 4). Gill filaments had significantly higher Cd, Co, Cr, Cu, Fe, Se and Hg concentrations and significantly lower Mn concentrations than the gill arch. There were no differences with regard to As, Ni, Pb and Zn between the two gill segments.

There were no differences ($p>0.05$) between elemental concentrations in the two studied liver sections (Table 5). The proximal and median intestine segments had the same elemental accumulation levels,

Table 6. Metal and trace element concentrations in three intestine sections (proximal, median, and distal) of the wels catfish (mean, standard deviation, minimum and maximum values). Concentrations are expressed as μg g^{-1} dry weight.

		As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Zn
Proximal section	Mean	0.216	0.364	0.118[a]	0.097	5.588	117.05	0.721	4.538[a]	0.114	0.121	2.877	72.45[a]
	S. dev.	0.336	0.284	0.040	0.162	2.114	74.07	0.264	3.011	0.139	0.199	1.091	18.42
	Min	0.047	0.000	0.070	0.000	0.790	28.42	0.324	0.190	0.000	0.000	0.880	33.47
	Max	1.321	0.986	0.200	0.580	8.930	278.05	1.256	12.670	0.420	0.769	4.360	94.19
Median section	Mean	0.174	0.495	0.116[a]	0.060	5.123	98.07	0.557	4.320[a]	0.134	0.064	2.497	70.01[a]
	S. dev.	0.223	0.383	0.039	0.054	1.086	81.23	0.118	2.494	0.165	0.051	0.613	11.27
	Min	0.035	0.146	0.060	0.000	3.210	40.29	0.317	2.240	0.000	0.000	1.400	41.03
	Max	0.876	1.472	0.180	0.180	7.720	351.62	0.770	12.200	0.580	0.171	3.470	85.09
Distal section	Mean	0.157	0.315	0.351[b]	0.084	5.156	86.56	0.642	3.198[b]	0.074	0.054	2.128	94.36[b]
	S. dev.	0.136	0.310	0.192	0.070	0.970	53.36	0.172	2.659	0.058	0.045	0.531	10.88
	Min	0.044	0.059	0.130	0.000	2.740	41.80	0.433	1.500	0.000	0.000	1.100	75.26
	Max	0.480	1.197	0.760	0.260	6.340	224.27	0.893	11.270	0.190	0.149	2.860	112.64

[a, b] The value with a different letter in the same column is different (Mann-Whitney U test, $p<0.05$)

while they both had significantly higher Co and Zn concentrations and lower Mn concentrations than the posterior segment (Table 6).

DISCUSSION

No differences in elemental accumulation were observed between the two studied liver segments, as well as between the two upper intestine sections. On the other hand, the muscle and skin significantly differed in their elemental accumulation patterns. Significant differences were also observed between the gill arch and the filaments, as well as between the distal and two upper intestine sections.

Higher accumulation of As, Co, Cu, Fe, Mn and Zn in the skin than in the muscle was also observed by other authors [9,13-15]. Higher concentrations in the skin could be the result of metal complexion with the mucus [14]. Metal ions from water are able to bind to the mucus layer present on the body surface, which can lead to a higher uptake and absorption in the skin [16]. This is particularly the case with fishes without scales, such as wels catfish, where the mucus layer serves as a shield against permeation of environmental chemicals [17,18]. On the other hand, the muscle has a weak accumulation potential and often represents the tissue with the lowest elemental concentrations in fish [6,19,20]. Uysal et al. [21] observed a lack of clear accumulation patterns between the two tissues, since different species had maximum concentrations in either muscle or skin. In the present study, higher Hg concentrations were detected in the muscle (Table 3), while Storelli et al. [9] did not observe any differences between these two tissues. According to Fu et al. [22], skin is not an active tissue for Hg bioaccumulation. The inclusion of skin in the sample can actually reduce resulting concentrations detected in the muscle sample [23], and consequently present a false finding of the acceptable metal levels in fish meat. Other authors also found differences between the two tissues as regards Cd, Cr, Ni, Pb and Se accumulation [9,10-15,21], which was not observed in the present study. Removal of skin from the muscle sample for metal analyses is commonly recommended by fish sampling protocols [23-25].

Potential differences between the gill arch and filaments with regard to elemental accumulation have been rarely assessed. Crafford and Avenant-Oldewage [10] reported higher Ni and Pb accumulation in the gill arch, which was not observed in the present study. However, bony tissues are considered to be a major Pb accumulation center, where it accumulates due to its similarity to calcium [26,27]. Our results indicated a higher Mn concentration in the gill arch, while most of the other studied elements had lower concentrations than those found in the gill filaments (Table 4).

Mn tends to accumulate at the highest levels in bony tissues and it also represents a normal constituent of vertebrate skeletal tissues [26,27]. Higher Cd, Co, Cr, Cu, Fe, Hg and Se accumulation levels in the gill filaments are probably a result of the direct uptake from water, since gills represent the main accumulation route of waterborne pollution [8,9]. Some metals also tend to accumulate at higher concentrations in gills due to their slow excretion rate [28].

Our findings indicated that there were no differences in elemental accumulation between the two studied liver sections (Table 5). To our knowledge, this issue was not assessed in any of the previous studies.

Assessment of metal accumulation in the intestine indicated that Co, Mn and Zn concentrations in the distal section differed from those in the two upper intestine sections, while there were no differences observed between the latter two. The observed differential accumulation among the studied intestine sections could be caused by differences in their activity. According to the literature survey, the present study was the first to address this issue.

Findings of the present study emphasize the necessity of a detailed reporting of how fish tissue is sampled. It is especially important to report whether the skin was included with the muscle sample, as well as if the gill arch and filaments were analyzed together. Moreover, information on the exact intestine section sampled should also be provided, especially if the study is focused on the elements for which differences in accumulation level have been observed in the present study. On the other hand, our findings indicate that such information is not necessary when the liver is used for analysis.

It is important to note that a potential bias can occur if different ratios of muscle and skin are included in a sample, and the same holds true for the gill arch and filaments. Therefore, we strongly recommend that the skin should not be analyzed together with the muscle, nor the gill arch with the gill filaments. We believe that the presented findings will be of importance to a wider scientific community, particularly regarding implications for human consumption when assessing metal levels in edible fish, as well as implications for biological monitoring practices.

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