Metallic Elements (Cu, Zn, Ni and Mn) Toxicity Effects Determination on a Fresh Water Fish *Cyprinus Carpio* (Common Carp) Laboratory Acclimatized

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Metallic elements copper (Cu), zinc (Zn), nickel (Ni) and manganese (Mn) are some of the most commonly found in water and sediment samples collected from the Danube - Danube Delta. These elements are important as essential micronutrients, being normally present at low concentrations in biological organisms, but in high concentrations they become toxic with immediate and delayed effects. The role of this metals is still controversial, that's why bioconcentration potential is so important. In this non-clinical study, we tested in vitro effect of heavy metals on carp, Cyprinus carpio, reproducing in vivo presence of Cu, Zn, Ni and Mn in the Romanian's surface water. The toxicity tests were performed according to OECD 203 by detecting the average (50%) lethal concentration - LC50 on aquatic organisms (freshwater fish) at 96h. The results pointed out that, copper value for LC 50 at 96h was estimated as 3.4 mg/L (concentrations tested in the range of 0.1 - 4.75 mg/L). Zinc value for LC 50 at 96h was estimated as 20.8 mg/L (concentrations tested in the range of 0.028 – 29.6 mg/L). Nickel value for LC 50 at 96h was estimated as 40.1 mg/L (concentrations tested in the range of 0.008 - 84.5 mg/L). For manganese the mortality effects has recorded at LC 50 at 96h at estimated value higher than 53 mg/L (concentrations tested in the range of 0.04 - 53.9 mg/L). The accuracy of the testing metals concentration was insured by the screening of the dilution water, as well as food and control fish, acclimated in laboratory conditions.

Keywords: aquatic organisms, Cyprinus carpio, LC50, metals, toxicity effects

The metals are continuously eliminated in aquatic systems from various natural or anthropogenic sources. At the same time these elements are essential micronutrients, playing important roles in the metabolism of a (micro) organisms. The presence of metals in higher than natural concentrations may influence or modify the natural functions of ecosystems due they toxic effects, long-term persistence, bioaccumulation and biomagnification potential in the trophic chain [1]. Thus, the role of these metals is still controversial and very important, as long as they have a different toxicity impact on different group of aquatic organisms (e.g. algae, crustaceans, fish), the bioconcentration potential depending on concentration and period of the exposure.

Aquatic organisms may be exposed to elevated or low concentrations of metals due to accidental or continuous emissions, maintaining the level of concentration over long periods of time. The process of up-taking water compounds by aquatic organism can be developed through several mechanisms, depending on the nature of the compounds and the specific conditions in the aqueous medium. The primary route of metals up-take from water is directly through skin and gills [2, 3]. Elimination processes result in reduction of the chemical compounds concentration in the body and can occur through passive or active mechanisms, similar to environmental xenobiotic take-up processes. Many aquatic organisms could excrete lipophilic compounds through water passive diffusion or through feces. Pisces can do it through gills. The chemical compounds usually pass metabolic products into more hydrophilic products that are eliminated through the urine. The metabolic products are eliminated back in the water, so that bioconcentration/bioaccumulation is the next step of the processes of up-taking and removing chemical compounds from the body [4, 5]. Heavy metals poisoning of fish could lead to histological changes such as gill necrosis or degeneration of fatty liver tissues [6, 7].

Acute toxicity tests provide a measurement of pollutant effect on a target species, and under specific environmental conditions can quickly determine a strong response. The acute and chronic toxicity tests help to monitor and understand the effects of accidental metal discharges in the nature followed up by metal accumulation in sediments or organisms [8].

Our seasonal monitoring studies during the period 2003-2013 in the Danube Delta – Sfantu-Gheorghe Branch revealed higher metal concentrations (Hg, Cu, Ni, Cd, Cr, Pb, Hg, As) in the sediment than water, due to sediment storage capacity, but all together their concentrations were within the admissible limits. Moreover, metals Cu, Pb, Zn, Cr, Ni, Cd, Mn and Fe were the most abundant in sediments from Danube Delta [9-11]. This data has been used as a basic criterion of the metal selection [12-14].

The most important rules regulating bioaccumulation are: EU Regulation no. 253/2011 [15], The European Act COM (2011)-876 final [16] and European Water Framework Directive [17], establishing a framework for Community action in the field of water policy (EQS – Environmental Quality Standards, Directive 2013/39/EU) in aquatic biota (especially fish, crustaceans and mollusks) for some of the hazardous chemicals [18].

In present study, we assessed the acute lethal toxicity effect at 96h and determined the mean lethal concentration - LC 50 of selected metals (*Cu, Zn, Mn, and Ni*) on *Ciprinus carpio* fish in accordance with OECD test method 203 [19].

Experimental part

Ecotoxicity tests

The metal selection (*Mn, Cu, Zn and Ni*) was based on their abundance in the surface water and sediment from

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Table 1
THE EXPERIMENTAL DESIGN OF THE ACUTE MORTALITY/BEHAVIOR TESTS

Metal	Stock solution / pH	Tested conc. –0h (mg/L)	Tested conc. – 96h (mg/L)	Fish No. / 5 liters of tested solution	Monitored Parameters					
					0h			96h		
					pH	T° C	O_2 dissolved (mg/L)	pH	T° C	O_2 dissolved (mg/L)
Mn	260 mg/L 6-6.5 pH	0.044	0.028	5	8.48	22.7	8.50	8.16	22.1	8.43
		0.29	0.28	5	8.48	22.6	8.46	8.47	22.1	8.40
		2.58	2.86	5	8.43	22.7	8.45	8.40	22.0	8.39
		24.4	22.8	5	8.22	22.7	8.48	8.11	22.0	8.40
		53.9	53.1	5	8.22	22.6	8.46	8.21	21.6	8.37
Cu	330 mg/L 5-5.5 pH	0.1	0.06	5	8.01	24.2	8.07	7.93	24.1	8.01
		0.4	0.13	5	8.09	24.1	7.41	8.02	23.9	7.52
		0.99	0.16	5	7.99	24.1	7.20	7.82	24.0	7.31
		3.24	1.88	5	7.77	23.7	8.03	7.71	23.8	8.03
		4.75	3.19	5	7.36	23.6	7.38	7.21	23.4	7.42
Ni	220 mg/L 6-6.5 pH	0.008	0.004	5	8.51	22.6	8.50	8.43	21.8	8.43
		0.11	0.10	5	8.50	22.6	8.50	8.48	21.6	8.47
		1.09	1.04	5	8.48	22.7	8.51	8.10	22.0	8.40
		9.89	9.35	5	8.40	22.5	8.43	8.03	21.8	8.37
		84.5	79.4	5	7.87	22.6	8.39	7.89	21.9	8.36
Zn	420 mg/L 6-6.5 pH	0.028	0.01	5	7.88	23.6	7.04	7.76	23.6	7.14
		1.48	0.6	5	7.91	23.5	7.76	7.84	23.4	7.66
		11.95	9.55	5	7.55	23.6	7.80	7.52	23.6	7.82
		18.6	8.85	5	7.50	23.7	7.82	7.51	23.5	7.74
		29.6	20.1	5	7.41	23.6	7.98	7.32	23.6	7.90
Contro 1 1	Dilution water	No metal	No metal	5	8.44	22.5	8.55	8.04	21.6	8.50
Contro 1 2	Dilution water	No metal	No metal	5	8.31	23.6	7.84	8.14	22.6	8.02

previously analyzed Danube - Danube Delta samples [9 - 11]. Five concentrations of the above mentioned metals were selected according to their toxicity and environmental values previously identified (table 1).

The ecotoxicity tests of metals on *Cyprinus carpio* were carried out for 96h by the static acute lethal toxicity method (without replacing the test solution) according to OECD test method 203 [19]. The biological material, 1+ years carp population (*Cyprinus carpio*) was purchased with a certificate of origin and health from the specialized fish-breeding station. They were acclimatized for one year in the laboratory prior to the ecotoxicity tests. The concentrations of the selected metals from the aquatic tanks as well as other physicochemical parameters were monitored at the beginning (0h) and at the end of the test (96h).

Metal screening

Metal determinations were performed both for stock solutions and experimental solutions (at 0h and 96h) using inductively coupled plasma atomic emission spectrometry (ICP-EOS) technique. The performance parameters of the methods applied are presented in table 2.

Analytical method for determination of metals from biological samples

The control samples (untreated fish with metals) were sacrificed and fish organs such as liver, white and red muscles, gonads, kidneys, heart, gills, brain, intestine and skin were harvest in order to be analyzed for their metal content. The metals analysis from the control fish (uncontaminated) was performed from dehydrated samples at approx. 50° C in drying stove or from frozen samples at -20° C. The sample digestion step (both for dehydrated and as such) was performed using High performance microwave digestion system, Milestone Ethos Up Equipment [20]. Briefly, the bio samples were mixed with ultrapure nitric acid and hydrogen peroxide then the samples were subjected to an increasing

Parameter	LOQ (µg/L)	Precision (%)	Recovery (%)	Uncertainty (%)
Mn	0.35	1.85	101.3	10.5
Cu	1.00	2.57	102.9	9.05
Ni	1.20	3.47	96.01	10.0
Zn	2.10	4.15	100.8	9.20

Table 2
PRECISION, QUANTIFICATION LIMIT, RECOVERY, UNCERTAINTY VALUES FOR METAL DETERMINATION

Steps	Power (W)	Inside vessel Temperature (°C)	Outside vessel Temperature (°C)	Time (minutes)
1	1800	200	145	15
2	1800	200	145	15
Cooling	-	-		20

Table 3
FISH TISSUE DIGESTION METHOD

temperature of up to 200°C. The microwave program applied to Milestone Ethos Up System is presented in table 3. Analytical determinations were performed according to SR EN ISO 11885:2009 standard [21].

Results and discussions

Toxicological characterization

According to the literature the acute toxicity concentrations of metals were dependent on the tested species, exposure time, type of toxicity test, laboratory conditions [22- 24].

According to PAN Pesticide Database – Chemical Toxicity Studies on Aquatic Organisms [18], the acute toxic concentrations (LC50 - fish) of the selected metals are within the following ranges 1.3 to 10.4 mg/L for *Ni*; 0.45 to 30 mg/L for *Zn*; 0.1 to 15.61 mg/L for *Mn*.

Our in-house toxicity data of metals for fish (*Cyprinus carpio*) pointed out that *Cu, Zn, Mn* and *Ni* showed toxic effects and LC 50-96h values were at (corresponding): 2.17 ± 0.50 mg/L, 12.23 ± 5.0 mg/L, >53 ± 8 mg/L and 65.77 ± 20 mg/L [12-13].

Other studies highlighted different toxic concentration intervals: 6.16 - 47.58 mg/L for *Ni*; 0.15 - 21.4 mg/L for *Zn*; 0.28 - 34.5 mg/L for *Cu* [25-27].

Fish exposure for 60 days at different metal concentrations revealed different chronic effects and MATC values (the concentration at which the contaminated organisms no longer show signs of behavioral, morphological or biochemical damage) were established [28]. The monitored physiological parameters from chronic test revealed that *Cu* was nontoxic at the concentration of 0.05 mg/L, *Ni* at 0.1 mg/L, *Zn* at 0.6 mg/L, compared with non-intoxicated fish (control). Acute toxicity tests performed within this study showed that *Manganese* concentration ranged between 0.04 and 53.9 mg/L did not induce any mortality effects during 96h. This result confirmed the previous data obtained in-house, where the LC 50-96h value was estimated to be higher than 53 mg/L.

Copper was tested in the concentration range between 1 to 4.75 mg/L and it induced 80% mortality at 4.75 mg/L and 40% mortality at of 3.24 mg/L. There was no mortality at the other concentrations (0.1, 0.4 and 0.99 mg/L) and the LC 50-96h value was estimated at 3.4 mg/L, a value close to the LC 50 previously detected in our in-house studies.

Nickel concentrations ranged from 0.008 to 84.5 mg/L were tested and 100% mortality of tested fish was induced at 84.5 mg/L, but only 20% at 9.89 mg/L.

There was no mortality at the other concentrations (0.008, 0.11 and 1.09 mg/L). The LC 50-96h value was estimated to be 40.1 mg/L, a value close to the LC 50 previously detected in our in-house tests and also similar with the values published in literature.

Zinc tested in the concentration range 0.028 - 29.6 mg/L induced 80% mortality of the tested fish at 29.6 mg/L and 20% mortality at the concentration of 18.6 mg/L. There were no mortality at the other concentrations (0.028, 1.48 and 11.95 mg/L). The LC 50-96h value was estimated to be 20.8 mg/L, a value close to other LC 50 data published in literature.

No changes in fish behavior, such as agitation, and no pathological external changes for all tested metals at non-lethal concentrations have been observed.

Bioaccumulation

The metal screening of control fish tissues and organs showed some abnormalities.

Thus, *copper* exceeded the admissible limit (30 mg/kg dry weight - dw) in the liver, in the other organs and tissues, it was present within acceptable limits (tables 4, 5). M*anganese* exceeded the admissible limit (1 mg/kg dw) in muscle, gills, skin (table 4), gonads, intestine, kidney, brain and heart (table 5). *Zinc* showed exceedances over the admitted norms (30-100 mg/kg dw) in all analyzed parts. *Nickel* exceeded 10 mg/kg dw concentrations in gonad, kidney, brain and heart (table 5). It was also present in white muscles, gills (table 4), gonads, intestines and kidneys (table 5). The differences in metal concentration among individuals (e.g. in the liver) depended on sex, weight, dimensions, suggesting the specificity of each organism to metabolize metals. The values of the metal concentrations determined directly from the wet sample (2) were higher compared to those obtained from the dehydrated samples (1).

Specifying multiple concentrations for the same tissues or organ indicates individual analysis. Bold values indicate exceedances above the specified values in the international rules on metal content in fish muscles. Values in blue indicate the presence of metals in significant not normalized concentrations.

Metal inputs from dilution water and food

The accuracy of metal concentration and subsequently of the toxicity results were insured by a metals screening input of the dilution water, food and control fish. Fish food have been also analyzed for the metal content, establishing the amount of additional metal contamination during bio concentration test.

The quality of dilution water used in the preparation of the test solutions and in the control test was checked before the experimental part. The results of dilution water analyses showed that metal concentrations did not exceed

Table 4
PROFILE OF METALS BY TYPE OF TISSUES, mg/kg DW

Metal	White muscles		Red muscles		Gills		Skin	
	1	2	1	2	1	2	2	2
Cu	1.79 1.69	5.40 5.05	5.52	9.14	4.33	6.13	3.98	3.15
Mn	0.88 0.79	**1.35** **1.35**	0.77	**1.63**	**5.18**	**5.21**	**1.88**	0.86
Zn	23.5 **30.45**	**37.1** **37**	**48.7**	**56.9**	**674**	**872**	**263**	**485**
Ni	1.57 <0.03	2.70 2.69	<0.03	<0.03	<0.03	9.50	<0.03	**12.9**

Note: 1 - dehydrated samples at 50° C, dry matter approximately 96%;
2 - wet samples, dry matter approximately 20%.

Table 5
PROFILE OF METALS BY TYPE OF ORGANS, mg/kg DW

Metal	The female gonad		Male gonad	Liver	Intestine	Kidney	Brain	Heart
	2	2	2	2	2	2	2	2
Cu	14	13	4.90	**73.3 110 89.9**	13.5	18.5	11	15
Mn	**1.72**	**2.17**	**1.05**	**5.41 1.71 5.75**	**4.50**	**2.06**	**1.23**	**1.07**
Zn	**892**	**871**	**79.1**	**450 243 189**	**644**	**1237**	**87.2**	**207**
Ni	<0.03	3.26	**40.6**	4.41 8.17 2.46	0.90	**4.11**	**10.7**	**24.6**

Table 6
CHARACTERISTICS OF THE DILUTION WATER

Indicator analyzed	M.U.	Determined values	Maximum admissible values	Indicator analyzed	M.U.	Determined values
pH	pH unit	8.2	6.0 – 8.5	Mo	µg/L	0.8
Total hardness	mg/L $CaCO_3$	166.61	10 – 250	V	µg/L	4.8
CCO-Cr	mgO_2/L	6.30	15	Zn	µg/L	4.4
Suspensions	mg/L	4,8	20	Ni	µg/L	<1.2
Filterable residue	mg/L	230	500	Pb	µg/L	<0.15
Ammonium	mg/L	0.11	0.1	As	µg/L	<0.6
Free residual chlorine	mg/L	<0.03	0.005	Cd	µg/L	<0.4
Dissolved oxygen	mg/L	8.14	higher than 4	Cr	µg/L	<1.3
Temperature	^{0}C	19.6	18 – 25^{0}C	Sr	µg/L	279
Cu	µg/L	3	-	Se	µg/L	<0.34
Fe	µg/L	4.4	-	Sb	µg/L	<0.9
Mn	µg/L	0.6	-	Mg	mg/ L	5.3

Table 7
CHARACTERISTICS OF METALS - FISH FEED (mg/kg DW)

Analyzed indicator	Value	Daily intake*	Analyzed indicator	Value	Daily intake*
Cu	4.97	0.0497	Cd	0.04	0.0004
Fe	89.4	0.894	Cr	0.26	0.0026
Mn	35.8	0.358	Sr	3.28	0.0328
Mo	0.72	0.0072	Se	0.56	0.0056
V	1.18	0.0118	Sb	<0.15	0.0015
Zn	57.8	0.578	Mg	903	9.03
Ni	0.46	0.0046	Ca	1233	12.33
Pb	<0.15	0.0015	Ti	2.66	0.0266
As	<0.13	0.0013			

*1% for a group of fish weighing approximately 1 kg

the limits allowed by National Law 458 of 08.07.2002 on the quality of drinking water (table 6).

All the analyzed metals determined in fish were also present in the dilution water (table 6) and in the food (table 7).

Thus, *Cu, Mn and Zn* were found in water and food. *Cu* was 3 µg/L in dilution water and 4.97 mg/kg in food, *Mn* was 0.6 µg/L in dilution water and 35.8 mg/kg in food, *Zn* was 4.4 µg/L in dilution water and 57.8 mg/kg in food. *Ni* was present in low quantities in food (0.46 mg/kg).

As it can see the highest metal concentrations come from food. It was appreciated that the steady supply of food for three years led to the imitation of bioaccumulation conditions.

Conclusions

The results pointed out that our concentration values for tested metals confirmed the literature data. In the meantime the metal's screening of control fish showed that Mn and Zn were found above the permissible limits in all analyzed organs and tissues from. Ni is mainly presents in skin, gonads, kidneys, brain and heart, and Cu in the liver.

The significant concentrations of Mn, Zn and Cu found in the environment could lead to bioaccumulation of these

elements over time. The order of overtaking of the metals by organs and tissues was: liver> kidneys> gills> gonads> red muscles> white muscles> skin> brain = heart> intestines.

Thus we concluded that differences in metal concentration between individuals (e.g. liver) are highlighted, suggesting the specificity of each organism to metabolize metals.

Also, the other important fact is that all metals found in fish were also present in the dilution water and food. *Cu, Mn and Zn* are found in the highest concentrations in food. We have appreciated that a steady supply of food for three years led to the imitation of bioaccumulation conditions in fish organisms. These facts lead as to the conclusion that, measuring the impact of the accidental metalic pollutants, eliminated in aquatic systems from various natural or anthropogenic sources, we have to maximize them effects due to the bioaccumulation processes of this metals, happened day by day through feeding of the animals. It's hard to estimate the precise level of the concentration at each the particular organism may die (e.g. LC 50 at 96h). Our laboratory results only point out on the average concentrations on each an average half of the population will not survive. It does mean that damages can be metabolically irreversible, depending on biological resistance of each organism. Thereby we intend to use the data presented here to develop the next more depth researches.

Acknowledgements: This research was financed by the financed by the Ministry of Education and Research of Romania, Program Nucleu code PN 16-25.02.04.

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Manuscript received: 15.01.2017