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THE ACUTE TOXICITY OF COPPER AND ZINC ON THE PROTECTED STELLATE STURGEON JUVENILES (*ACIPENSER STELLATUS* PALLAS, 1771)

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Abstract: Pollution represents one of the main reasons for the decline of sturgeons in the Danube River, together with overfishing, damming, poaching, habitat lost, lack of effective management. Although water pollution by heavy metals in the Danube is considered to be very high, there is no specific data available on its impact on sturgeons *Acipenser stellatus* (stellate sturgeon), one of the three anadromous sturgeons existing presently in the Danube, which is critically endangered under IUCN Red List 2010 (International Union for Conservation of Nature) and listened in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Nowadays it is believed to be extinct from the upper and middle Danube. The aim of this study was to assess the acute toxicity of copper and zinc on stellate sturgeon juveniles by semi-static renewal test, according to OECD (Organisation for Economic Cooperation and Development) guidance, using dechlorinated tap water with a hardness of 340 mg/l $CaCO_3$. The LC50 values for copper and zinc ions, were determined to be 0.547 mg/l for Cu^{2+} and 34.22 mg/l for Zn^{2+} respectively, according to the probit method. Even if the calcium ions offer a clear protection against copper and zinc toxicity in the conditions of high water hardness, *Acipenser stellatus* species, remains very sensitive to the toxicity of these two metallic ions, with LC_{50} values lower than for other fish species.

Keywords: acute toxicity, copper, zinc, *Acipenser stellatus***,** pollution, Danube River

1. INTRODUCTION

Marine migratory sturgeons form one of the most valuable fish populations of the Lower Danube River ecosystem and are also important for the economy of Romania and some other riverside countries and freshwater fisheries (Ciolac & Patriche, 2005). The Danube River is important for sturgeon populations, because it still provides access to spawning habitats and enables their successful reproduction (Lenhardt et al., 2006).

Pollution represents one of the main reasons for the decline of sturgeons in the Danube River, together with overfishing, damming, poaching, habitat lost, and lack of effective management (Ciolac 2005; Lenhardt et al., 2008; Jaric et al., 2011) despite the fact that these populations are presumably managed and protected by fishery regulations (Williot et al., 2002).

Although water pollution by heavy metals in this river is considered to be very high, there is no specific data available on its impact on sturgeons (Bacalbasa-Dobrovici, 1997; Poleksic et al., 2010).

Acipenser stellatus, one of the three anadromous sturgeons existing presently in the Danube, is critically endangered under IUCN Red List (2010) and listened in Appendix II of CITES. It is now believed to be extinct from the upper and middle Danube and spawning migrations in the lower Danube had greatly reduced (Vecsei et al., 2007).

Although metals are naturally occurring and in many cases play essential roles in biochemical processes, human-related activities increase the entry, bioavailability, and toxicity of metals.

Different environmental conditions (pH, salinity, water hardness, dissolved organics) can influence metal toxicity, speciation and bioavailability (Van Veld & Nacci, 2008). It has been recognized that the toxicity of some heavy metals over freshwater fish is reduced in hard water compared with soft water (Pascoe et al., 1986).

The purpose of this study was to evaluate the acute toxicity of copper and zinc ions (two of the most encountered metals in the Danube) on stellate sturgeon juveniles. Both heavy metals were included in the list II of Directive 2006/11/EC approved by European Parliament and by the Council on pollution caused by certain dangerous substances discharged into the aquatic environment of the Community.

Even though all 27 sturgeon species have been the subject of scientific research, a few studies have been performed in the field of sturgeon toxicology (2% of the articles published over the past 15 years) (Jaric & Gessner, 2012).

Although acute toxicity data on endangered, sensitive species can be obtained from extrapolation of acute toxicity values across different taxonomic groups (Buckler et al. 2005), and from standard test procedure and common species, consideration should be given to direct testing, when captive and abundant local populations of listened species are available (Dwyer et al., 2005).

2. MATERIALS AND METHODS

2.1. Chemicals

Copper sulphate pentahydrate p.a. (CAS no.7758-99-8) and zinc sulphate heptahydrate p.a. (CAS no.7446-19-7) supplied by Chemical Company Iasi, were used as test substances and dissolved in distilled water.

2.2 Experimental fish

Juveniles of *Acipenser stellatus* (126-133 dph) with an average weight of 21.52 ± 4.54 g and a total length of.19.83 ± 3.17 cm, were obtained from Brates fish farm of the Institute of Research and Development for Aquatic Ecology, Fishing and Aquaculture (IRDAEFA) and brought to the IRDAEFA laboratory.

The juveniles were obtained by artificial reproduction at Brates fish farm, from wild parents caught in the Danube River, at Cotul Pisicii. The reproduction took place on May 19th and larvae started to hatch after five days.

The fish were kept in aquariums made of chemically inert material (glass) and of a suitable capacity in relation to the recommended loading (100 x 40 x 35 cm). The water was aerated and filtered using Eheim 2010 filters and was renewed daily (50% of the water). The fish were fed daily with 2% body weight, with granulated food until 24 hours before the start of test and exposed to 12 h light: 12 h dark natural photoperiod.

The fish were allowed to acclimatize to the laboratory conditions for seven days in dechlorinated tap water with a hardness of 340 mg/l $CaCO_3$. The same quality of water was used during the test.

2.3. Water chemistry analysis

Dissolved oxygen, temperature and pH were measured daily during the experimental period, using Hach HQ40d portable meter and Consort C532 pH-meter. The mean values for dissolved oxygen, temperature and pH where 8.33±0.32 mg/l O_2, 23.4±0.19°C, respectively 8.40±0.05.

2.4. Exposure method

The acute toxicity test was conducted following the OECD 203 guidance (Organization for Economic Cooperation and Development), under a semi-static test, with 50% batchwise renewal of test solution every 24 hours, with mortality as the measurement endpoint.

Due to lack of information in the open literature regarding the acute toxicity of copper and zinc, on stellate sturgeon juveniles, a range finding test for the determination of appropriate concentrations was conducted before the definitive test. Ten concentrations were used for copper and zinc and aquariums were stocked with a group of 3 individuals per each concentration.

The concentrations for the definitive test were chosen between concentrations that produce 100% mortality and those which produced no effect. Five concentrations in a geometric series were used for each metal. Accuracy was attained by a closer spacing between concentration with a factor of 1.46 for copper and 1.18 for zinc (OECD recommending a factor of ≤2.2). The definitive test was performed in two replicate (n=2) for each metal ion.

For the definitive test groups of 10 individuals selected randomly were placed in each aquarium. The fish were exposed to the test substances for a period of 96 hours. Mortality values were recorded at 24, 48, 72 and 96 hours and the concentration which produced 50% mortality rate (LC_{50}) was determined.

2.5. Data analysis

Statistical analysis was performed with SPSS 18 computer program and probit method was used as a statistical procedure for estimating the LC_{50} and the associated 95% confidence interval (Gerber & Finn, 2005).

3. RESULTS

The results of the range finding test for copper showed that the lowest concentration with effect of 100% mortality was 750μg/l and the highest concentration with 0% mortality was 100 μg/l (Table 1). Regarding the range finding test for zinc, the lowest concentration with 100% mortality was 50000μg/l (50 mg/l) and the highest concentration with 0% mortality was 25000 μg/l (25 mg/l) (Table 2). No mortality was recorded in control aquariums during the experiments.

Table 1. Results of copper range finding test

Concentration Cu μg/l	Mortality 96 h
0 (Control)	0
0.01	0
0.1	0
1	0
10	0
100	0 (0% mortality)
250	1
500	1
750	3 (100% mortality)
1000	3 (100% mortality)

Table 2. Results of copper range finding test

Concentration Zn μg/l	Mortality 96 h
0 (Control)	0
0.5	0
5	0
50	0
500	0
5000	0
10000	0
25000	0 (0% mortality)
40000	1
50000	3 (100% mortality)

In both copper and zinc exposure experiments, the highest mortalities were recorded in the first 24 hours. No mortality occurred after 72 hours for copper and 48 hours for zinc, respectively. At the highest zinc concentration (49.5 mg/l), all fish died within the first 24 hours (table 3 and 4).

The results showed that both copper and zinc are very toxic to stellate sturgeon, *Acipenser stellatus*, but hard water could offer a protective effect. The estimated LC_{50} (96 h) for copper was 0.547 mg/l (Table 5) while for zinc LC_{50} (96 h) it was 34.225 mg/l (Table 6).

Table 3. Cumulative mortality of stellate sturgeon recorded in copper experiments (n=20 in two replicates)

	No.mortality			
Concentration (μg/l)	24 h	48 h	72 h	96 h
150	0	0	0	0
220.46	2	3	3	3
324.03	2	4	4	4
476.26	2	4	5	5
700	7	13	15	15

Table 4. Cumulative mortality of stellate sturgeon recorded in zinc experiments (n=20 in two replicates)

	No.mortality			
Concentration (μg/l)	24 h	48 h	72 h	96 h
25500	0	0	0	0
30099.30	5	5	5	5
35528.16	8	9	9	9
41936.20	19	20	20	20
49500	20	20	20	20

Table 5. Lethal concentration of copper (LC_{10-90}) at 96 hours

Lethal Concentration (μg/l)	95% Confidence Limits for Concentration		
	Estimate	Lower Bound	Upper Bound
LC10	244.43	158.31	305.48
LC20	322.33	242.16	389.75
LC30	393.49	317.37	481.61
LC40	466.62	385.74	598.24
LC50	547.22	450.67	752.54
LC60	641.73	517.83	962.54
LC70	760.99	594.56	1265.67
LC80	929.00	693.69	1756.82
LC90	1225.09	853.14	2787.69

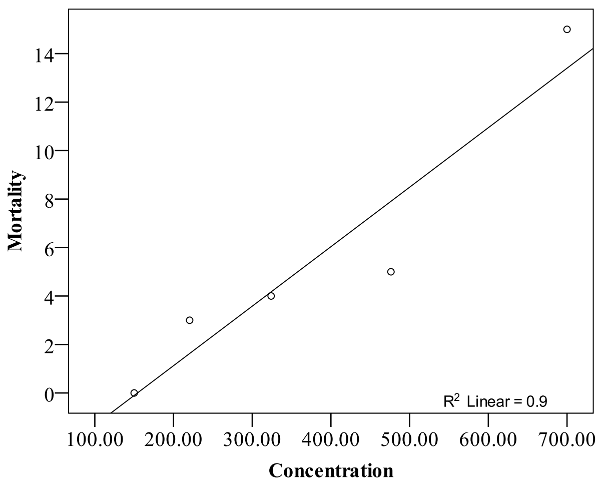

Figure 1. Scatter plot with regression line for copper

Table 6.Lethal concentration of zinc (LC_{10-90}) at 96 hours

Lethal Concentration (µg/l)	95% Confidence Limits for Concentration		
	Estimate	Lower Bound	Upper Bound
LC_{10}	29136.00	26310.54	30872.30
LC_{20}	30791.44	28474.73	32366.78
LC_{30}	32043.19	30059.69	33584.04
LC_{40}	33153.00	31390.68	34763.04
LC_{50}	34225.04	32584.55	36016.36
LC_{60}	35331.75	33714.31	37436.07
LC_{70}	36555.46	34856.59	39139.99
LC_{80}	38041.53	36132.64	41358.44
LC_{90}	40202.96	37852.65	44794.84

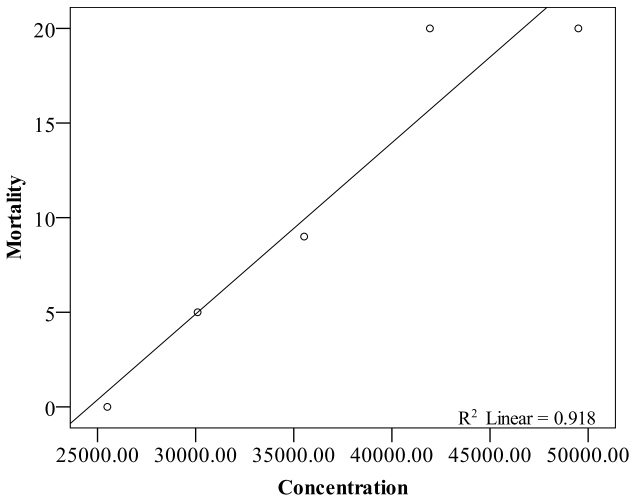

Figure 2.Scatter plot with regression line for zinc

Table7. Correlation coefficient of metals exposure and mortality

		Concentration	
		Copper	**Zinc**
Mortality	Pearson Correlation	.949*	.958*
	Sig. (2-tailed)	.014	.010
	N	5	5

*. Correlation is significant at the 0.05 level (2-tailed).

The square of the correlation R^2 was 0.90 for copper (Fig. 1) and 0.918 for zinc (Fig. 2) respectively, which indicated that 90.00% of the variation in mortality caused by copper and 91.80% by zinc could be due to concentration variation. The correlation between copper concentration and mortality was +0.949, and between zinc one and mortality was +0.958 (Table 7) suggesting that higher level of concentration were associated with higher levels of mortality. For both concentrations, the correlation was statistically significant ($p<0.05$ according to (2 -tailed) analysis).

4. DISCUSSIONS

The assessment of risk generated by metals to fish is often difficult to be measured due to large and complex effects of water characteristics on metal toxicity. The nature and magnitude of these effects can differ among metals, toxicity endpoints, fish species, and routes of exposure. According to Pascoe et al., 1986, several mechanisms may be considered in attempting to explain the effect of water hardness on heavy metals toxicity and these include: i) chemical mechanisms (competition for absorption site or complexation; ii) physiological mechanisms such as decreased gill membrane permeability, increased excretion of metal by various methods including chloride cell proliferation in hard water; iii) inactivation of absorbed metal by sequestration in granules or binding to proteins.

In aquariums with zinc sulphate solutions, the mortality stopped after 48 hours, except the 49.5mg/l Zn dose at which 100% mortality was recorded in the first 24 hours. On the other hand, in copper chloride aquariums mortality stopped after 72 hours at the highest two concentrations (476.26 µg/l and 700 µg/l). This confirms Hogstrand's affirmation (2012) that if the fish is able to survive the initial period of zinc exposure it is often able to make biochemical and physiological adjustments to restore homeostasis in a process that is known as acclimation.

At the highest two zinc ion concentrations, we observed white depositions on dead fish and aquariums, probably due to the formation of insoluble complexes in hard water and changes in metal speciation. These changes in speciation could not be attributed only to high water hardness, but also to the high pH level, that determined the dissolved zinc to be replaced by zinc precipitate, a product which is of very low toxicity to fish (Bradley & Sprague, 1985). In our experiments, these complexes remained lethal to all fish although they were able to reduce the zinc toxicity.

Previous studies proved that there are two principal mechanisms of Zn^{2+} acute toxicity: at very high industrial concentrations, the fish dies from impaired gas exchange, hypoxia, caused by gross morphological damage to the gills - edema, inflammation, cell sloughing, and fusion. The second mechanism, which operates at lower Zn^{2+} concentrations is an impairment of the calcium uptake across the apical membrane of the gill epithelial cells with consequential hypocalcaemia (Hogstrand & Wood, 1996, Hogstrand 2012).

Both zinc and calcium ions competitively inhibit the uptake of each other across the gills and compete for the same uptake sites (Barron & Albeke, 2000). The mortality induced by copper acute toxicity exposure is caused by osmoregulatory disturbance, where the balance Na^+ and Cl^- is impaired. It has been demonstrated that copper ions reduce sodium uptake and increase its loss in fish gills, which generates decreased blood sodium concentration and finally death.

Ca^{2+}, which offers clear protection against Cu toxicity assessed as acute mortality is assumed to compete with Cu^{2+} for the uptake by the gills of freshwater fish (Grosell, 2012). On the other hand, it seems that it keeps binding sites maximally saturated, preventing heavy metals ions interaction with proteins and the interference with normal protein function (Wurts & Perschbacher, 1994). Calcium is known to be important in the control of the integrity and permeability of the branchial epithelium in fishes (Taylor et al., 1996).

Comparing our results with recent ones (Vardy et al., 2013) concerning copper acute toxicity data for 100 days post hatch (dph) *Acipenser transmontanus* in a test water with a hardness of 65 mg/l $CaCO_3$, the importance of water hardness on the acute toxicity appeared to be crucial. In our experiment LC_{50} (0.547 mg/) was 10 times higher compared to that noticed by Vardy D. (0.054 mg/l). Also Vardy study proved that white sturgeon was more sensitive to Cu than rainbow trout (*Oncorhynchus mykisss*) and fathead minnow (*Pimephales promelas*) at all comparable life stages tested.

A similar study regarding copper acute toxicity was conducted by Little et al., 2012, on the white sturgeons in early life stages compared with rainbow trout (*Oncorhynchus mykiss*). White sturgeons at 123-167 dph were less sensitive to copper than rainbow trout (water hardness of 81-119 mg/l) with LC_{50} values ranging from 103.7 to 268.9 μg/l.

The LC_{50} (96h) for acute toxicity of Cu^{2+} to juvenile Chinese sturgeon *Acipenser sinensis* fingerlings was found to be 0.07 mg/ (Du Hao et al., 2007), wheareas for *Acipenser oxyrhynchus, Acipenser brevirostrum, Scaphirhynchus platorynchus* were 0.06 mg/l, 0.08 mg/l and 0.16 mg/l respectively, in water hardness of 160 to 180 mg/l $CaCO_3$. Also Zahedi et al., (2011), determined the 96h LC_{50} of copper, on Persian sturgeon, *Acipenser persicus*, to be 0.502 mg/l.

On the other hand, Mohammadnezhad et al. (2005), found that the LC_{50} of zinc in 96 hours is 21.58mg/l, in a study on the acute toxicity of zinc on *Acipenser nudiventris* fingerlings.

Many authors determined the acute toxicity of metals at different fish species with different water hardness, and had shown that both toxicity of copper and zinc is decreasing with increasing water hardness. So, Ebrahimpour et al., 2010 determined LC_{50} values for 96-hour acute toxicity of copper and zinc for *Capoeta fusca* at soft (40 mg/l $CaCO_3$) and high water hardness (380 mg/l $CaCO_3$) which were 1.1 mg/l respectively 7.5 mg/L for copper and 13.7 mg/l respectively 102.9 mg/L for zinc. Also, Kiyani et al. 2013, investigating the acute toxicity of copper and zinc on *Gambusia holbrooki* at different water hardness values showed that LC_{50} 96h at high water hardness (350 mg/l) was 0.65 mg/l for copper and 121.6 mg/l for zinc.

5. CONCLUSIONS

Taking into account that nowadays only few studies regarding the effects of copper and zinc on sturgeons, and especially on stellate sturgeon have been published out results could be considered a starting point for future researches in this field. Thus, further investigations are needed to assess the relative sensitivity of this species to heavy metals at different early stage.

Even if the calcium ions offer a clear protection against copper and zinc toxicity, *Acipenser stellatus*, remains a very sensitive species to the toxicity of the two metals, with LC_{50} values lower than for other freshwater fish species.

These pieces of information will be critical for the efforts of Danube sturgeon stock repopulation, and more studies on heavy metals acute and chronic toxicity in sturgeons are needed, due to poor water quality effect on survival of juveniles.

Understanding heavy metals acute toxicity in sturgeons, and the protective effect of hardness, could represent a milestone in prospective establishment of Danube water quality guidelines and criteria based upon lethal concentration (LC_{50}). Better knowledge of heavy metal toxicity on sturgeons is necessary for a proper management and conservation of sturgeon.

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