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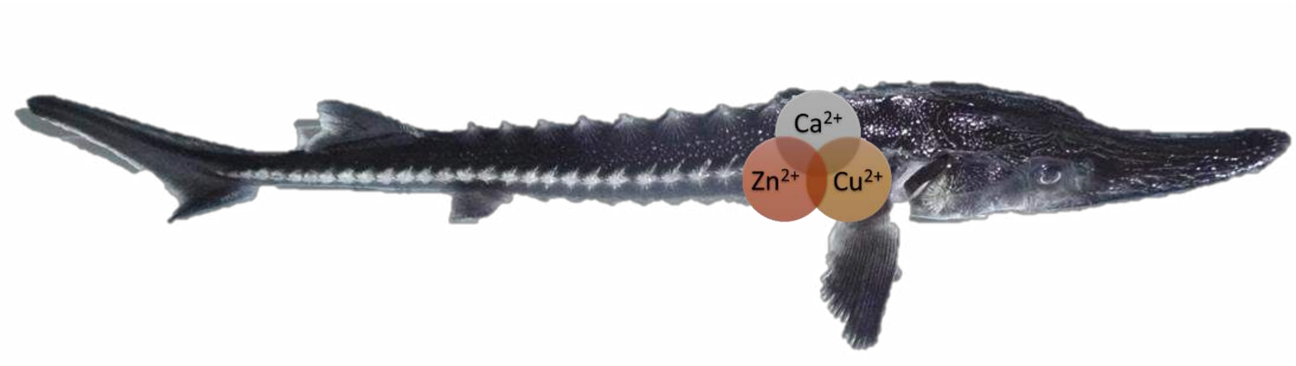
SUMMARY OF THE PhD THESIS

Research on the toxic effect of heavy metals on farmed stellate sturgeon (*Acipenser stellatus*, Pallas 1771)



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CONTENT

INTRODUCTION ... 7
PART I CURRENT STATE OF KNOWLEDGE ... 11
I. Actual protection status of sturgeons ... 12
II. Causes of sturgeon populations decline in Danube and possible threats ... 13
III. The current state on research of heavy metals effects on Danube sturgeons ... 16
IV. Copper and Zinc –chemical elements essential for the organisms but with a toxic potential ... 19
IV.1. Copper ... 20
IV.1.1. Physico-chemical characterization of copper and its functions in the organism ... 20
IV.1.2. Chemical speciation of copper ... 21
IV.1.3. Mechanisms of copper uptake and excretion ... 22
IV.1.4 Bioaccumulation of copper in tissues ... 24
IV.1.5. Mechanisms of toxicity ... 25
IV.2. Zinc ... 27
IV.2.1. Physico-chemical characterization of zinc and its functions in the organism ... 27
IV.2.2. Chemical speciation of zinc ... 29
IV.2.3. Mechanisms of zinc uptake and excretion ... 30
IV.2.4. Bioaccumulation of zinc in tissues ... 34
IV.2.5. Mechanism of toxicity ... 35
V. Factors influencing the toxicity of heavy metals ... 36
VI. Histopathology- an essential biomarker of heavy metal toxicity ... 46
PART II PERSONAL CONTRIBUTIONS ... 47
II.1. ACUTE TOXICITY TEST ... 48
II.1.1. INTRODUCTION ... 49
II.1.2. MATERIALS AND METHODS ... 50
II.1.3. RESULTS AND DISCUSSIONS ... 57
II.1.4. CONCLUSION ... 68
II.2 CHRONIC TOXICITY TEST ... 71
II.2.1. INTRODUCTION ... 72

II.2.2. MATERIALS AND METHODS 73
II.2.2.1 Determination of heavy metals accumulation by inductively coupled plasma mass spectrometry 76
II.2.2.2. Determination of histopathological changes induced by heavy metal exposure 80
II.2.2.2.1. Tissue preservation and processing for histological determination 80
II.2.2.2.2. Section staining by Hemalaun Meyer Eosin method 81
II.2.2.2.3. Semi-quantitative assessments of histopathological changes 82
II.2.3. RESULTS AND DISCUSSIONS 90
II.2.3.1 BIOACCUMULATION OF HEAVY METALS AND THEIR HISTOPATHOLOGICAL CHANGES INDUCED IN THE LIVER 92
II.2.3.1.1 BIOACCUMULATION OF THE HEAVY METALS IN THE LIVER 92
II.2.3.1.1.1.Bioaccumulation of copper and zinc in individual experiments 92
II.2.3.1.1.2.Bioaccumulation of copper and zinc in binary mixture 95
II.2.3.1.2.HISTOPATHOLOGICAL CHANGES INDUCED BY HEAVY METALS IN THE LIVER 97
II.2.3.1.2.1 Normal histological structure of the liver 97
II.2.3.1.2.2 Histopahtological chamges induced by copper and zinc in individual experiments 104
II.2.3.1.2.3 Histopathological changes induced by copper and zinc in binary mixture 106
II.2.3.1.2.4 Semi-quantitative assessments of histopathological changes....... 108
II.2.3.1.3 PARTIAL CONCLUSIONS 111
II.2.3.2. BIOACCUMULATION OF HEAVY METALS AND THEIR HISTOPATHOLOGICAL CHANGES INDUCED IN THE GILL 126
II.2.3.2.1. BIOACCUMULATION OF THE HEAVY METALS IN THE LIVER 126
II.2.3.2.1.1 Bioaccumulation of copper and zinc in individual experiments . 126
II.2.3.2.1.2 Bioaccumulation of copper and zinc in binary mixture 128
II.2.3.2.2. HISTOPATHOLOGICAL CHANGES INDUCED BY HEAVY METALS IN THE GILL 132
II.2.3.2.2.1. Normal histological structure of the gill..................................... 132
II.2.3.2.2.2. Histopahtological chamges induced by copper and zinc in individual experiments 134
II.2.3.2.2.3. Histopathological changes induced by copper and zinc in binary mixture 136
II.2.3.2.2.4. Semi-quantitative assessments of histopathological changes...... 143

II.2.3.2.3 PARTIAL CONCLUSIONS 146
II.2.3.3 HISTOPATHOLOGICAL CHANGES INDUCED BY HEAVY METALS IN THE INTESTINE 163
II.2.3.3.1 Normal histological structure of the digestive tube and its annex glands 163
II.2.3.3.2 Histopahtological chamges induced by copper and zinc in individual experiments 176
II.2.3.3.3 Histopathological changes induced by copper and zinc in binary mixture 177
II.2.3.3.4. PARTIAL CONCLUSIONS 182
II.2.4. GENERAL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS 193
BIBLIOGRAPHY 198

PLATES

PLATE I 114
PLATE II 120
PLATE III 124
PLATE IV 149
PLATE V 155
PLATE VI 160
PLATE VII 184
PLATE VIII 186
PLATE IX 189

INTRODUCTION

At present, all sturgeon species in the Danube River are critically endangered (with the exception of *Acipenser ruthenus* considered to be vulnerable), many of which are almost extinct despite their conservation status and protective measures.

The *Acipenser stellatus* species chosen as the subject of this research is one of the five sturgeon species still present in the Danube basin, being critically endangered, exposed to direct pollution with heavy metals from both water and sediment.

Although the heavy water pollution in the Lower Danube is very high, there is no available data on their impact on sturgeons (Bacalbasa-Dobrovici, 1997).

Both copper and zinc are two of the most common metals in Danube water and sediment (ICPDR, 2015), which justifies the need to determine their effects on aquatic organisms, in particular Acipenseridae species, and the need to establish water quality criteria. Although both metals are essential for different biochemical and physiological processes, they can become toxic at high concentrations.

Studies on the bioaccumulation of heavy metals in sturgeons in the Lower Danube (Poleksic et al., 2010; Onara et al., 2013) have highlighted the increase in their heavy metals concentration in different organs.

Due to the commercial importance of sturgeons, the emphasis was mainly on the study of bioaccumulation and the effects of heavy metals in the roe and muscle, many studies being published in this area. But it is known that muscle is not always a good indicator of contamination of the whole organism (Jaric et al., 2011). Thus, I considered it essential to analyze other tissues such as gills, intestines and liver (three of the most important organs in direct uptake and metabolism of xenobiotics).

The histopathological studies in sturgeons regarding the effects of heavy metals on normal tissue morphology are very few, with emphasis on the histology of digestive system development (Ostaszewska et al., 2011) and the effects of food deprivation (Gisbert and Doroshov, 2003; Furne et al., 2012), especially to improve the conditions for the growth of sturgeons in aquaculture.

The bibliometric analysis by Jaric and Gessner (2012) on ISI articles published in sturgeon research has shown that the field Toxicology is very little studied, with only 2% of the total of articles published between 1996 and 2010. All 27 species of sturgeon were the subject of the study, but the most frequently studied species were *Acipenser transmontanus, Acipenser bearii, Acipenser fulvescens, Polyodon spathula,* which underlines the need for studying also the native species. There is insufficient information on the sensitivity of *Acipenser stellatus* species to heavy metals.

There is much information in the literature on the effects of heavy metals, especially copper and zinc on fish, but there is very little information on the toxicity of these two metals, both individually and in a mixture on a valuable group such as sturgeons (Acipenseridae).

Thus, the **aim** of this PhD thesis was to determine the sensitivity of juvenile stellate sturgeon (*Acipenser stellatus*) to heavy metal contamination.

The paper is structured into **two main objectives**, as follows:

The first part of this paper aimed at studying the acute toxicity of copper and zinc in stellate sturgeon juvenile (*Acipenser stellatus*). Due to the lack of bibliographic data on the lethal concentration of copper and zinc in stellate sturgeon, we performed an acute toxicity test to determine the LC50 lethal concentration after 96 hours of exposure, respectively the concentration that kills 50% of the individuals in 96 hours.

The second part of the paper aimed at improving the knowledge of the chronic toxicity induced by the two metals in the stellate sturgeon (*Acipenser stellatus*), respectively the exposure for 7 and 14 days of stellate sturgeon juveniles to two different concentrations of copper and zinc (10 and 25% of the lethal concentration previously determined). At the same time, the juveniles were subjected under the same conditions of exposure to a binary mixture of metals, namely two different concentrations of metals, for 7 days.

To achieve this goal, three sub-objectives were set, namely:

- Determination of copper and zinc bioaccumulation, both in individual experiments and in binary mixture, in liver and gill;

Although the existence of a bioaccumulation model for copper and zinc has been demonstrated by various experiments, the present study is the first to be done for *Acipenser stellatus*, which compares the bioaccumulation patterns of the two metals under the same exposure conditions in both individual experiments and binary mixtures.

- Identification using optical microscopy of the histopathological changes induced by the two metals, in individual experiments and in the mixture on the three important organs in metal uptake and metabolism.

- The histological characterization of the three organs in the *Acipenser stellatus* as a result of the growth of the juveniles in the recirculating system. Since these juveniles obtained by artificial breeding can be used for the restocking of the Danube with sturgeons, the issue of juveniles capacity released to adapt to the wild is questioned.

Given the scientific and economic importance, but also the status of the endangered species, an understanding of the ability of *Acipenser stellatus* to deal with toxic substances in the environment, such as heavy metals, is essential.

Taking into consideration all these aspects, the theme of the doctoral thesis "Research on the toxic effect of heavy metals on stellate sturgeon (*Acipenser stellatus,* Pallas 1771)", is an important and topical topic, which aims to address the histopathological and bioaccumulation aspects of heavy metals in different organs of stellate sturgeon, confirming the sensitivity of the species *Acipenser stellatus* to heavy metal poisoning.

The novelty of this PhD thesis is the use of a less studied species of the Order Acipenseriformes, and the highlighting of histopathological changes. The novelty level raised a number of problems, regarding the lack of specialized literature in the field of histopathology in sturgeons. This, in conjunction with the sensitivity of the *Acipenser stellatus* species, confirms the need for research in order to obtain new data on the impact of heavy metals on sturgeon populations in general, but especially on stellate surgeon (*Acipenser stellatus*).

PART I.
CURRENT STATE OF KNOWLEDGE

Some of the most valuable fish species in economic and scientific terms are sturgeon species. Sturgeons and paddlefishes belong to the Actinopterygii Class, Acipenseriformes Order, with two families: Acipenseridae and Polyodontidae. The Acipenseridae family has two subfamilies: Acipenserinae and Scaphirhynchinae, with four genera: *Acipenser, Huso, Scaphirhynchus, Pseudoscaphirhynchus*. The Polyodontidae family comprises two genera: *Polyodon* and *Psephurus* (Bemis et al., 1997), each with one species.

Some of the most important sturgeon species in Europe, both scientifically and commercially, are the sturgeon species found in the Danube river basin.

In the first part of the PhD thesis, I presented the current protection status of sturgeons, the causes of the decline of the sturgeon populations in the Danube, as well as the possible threats. Since the Danube River is one of Europe's most important rivers, it is a source of water with different uses, and so the quality of the Danube basin is under great influence from a wide variety of anthropogenic activities.

Copper and zinc are two of the most common metals in Danube waters and sediments (Poleksic et al., 2010; Ilie et al., 2014; ICPDR, 2015). High copper concentrations (over 0.2

mg / l) were recorded in Danube waters while zinc concentrations are generally less than 0.1 mg / l (Tudor et al., 2016). Accidentally, the concentration of both metals may increase as a result of mining discharges along the Danube basin, where concentrations up to 5 mg / l for copper and 156 mg / l for zinc were recorded (Bird et al., 2010)

Few studies have been conducted on the contamination of Danube sturgeons by heavy metals, these studies indicating the bioaccumulation of heavy metals in different tissues.

Fish requires both copper and zinc as micronutrients, and they can get these metals either from water or from food. The copper and zinc requirements differ between species, even for different stages of the same species. Although both metals are essential for metabolic processes, at high concentrations they can become toxic. Studies conducted on different fish species have shown that both essential and non-essential metals can produce different toxic effects on fish

I also presented in the first part of the thesis, the physicochemical characterization of copper and zinc, chemical speciation, their functions in the organism, the mechanisms of branchial and intestinal uptake. Toxicity of heavy metals in water is not only a matter of concentration in the environment, because water chemistry influences considerably not only the bioavailability of the metal but also the physiology and thus its susceptibility (Grosell, 2012). The bioavailability of a chemical may be influenced by a number of abiotic parameters such as temperature and water chemistry, but also by a number of biological factors that have been presented.

PART II. PERSONAL CONTRIBUTIONS

II.1. ACUTE TOXICITY TEST

II.1.1. INTRODUCTION

The acute toxicity test is one of the many tools available in aquatic toxicology but which is a basic method for obtaining a rapid and reproducible estimate of the toxic effects of a test substance.

II.1.2. MATERIALS AND METHODS

Individuals belonging to the species *Acipenser stellatus* (stellate sturgeon), having a length of 19,83 ± 3,17 cm and a weight of 21,52 ± 4,54 g (Fig. II.1.1) were obtained from the Brates Fish Farm of the Institute of Research and Development for Aquatic Ecology, Fisheries and Aquaculture (ICDEAPA) Galati and transported for acclimatization in institute's laboratory.

The fish were allowed to acclimate to laboratory conditions for seven days prior to the start of the experiment in the institute basins, in tap water with a 19-degree hardness, equivalent to 340 mg / l $CaCO_3$.

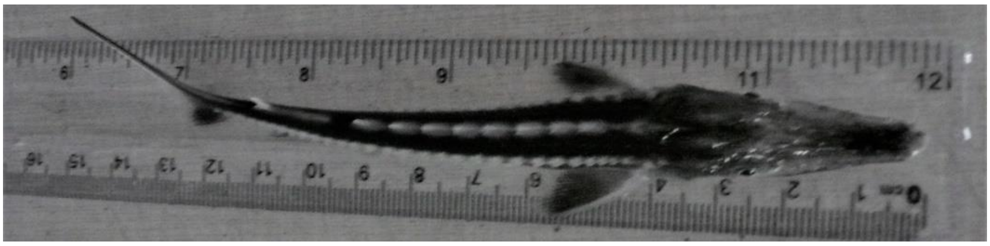

Fig. II.1.1 *Acipenser stellatus* (original foto)

The determination of the acute toxicity of copper and zinc in the stellate sturgeon was done according to OECD 203 guideline using the semi-static test with 50% water change every 24 hours. Mortality of juveniles was recorded every 24 hours.

The acute toxicity test was performed in duplicate (n = 20) for each metal, groups of 10 individuals randomly selected were placed in each aquarium and exposed to the test substance for a period of 96 hours.

Mortality was recorded every 24, 48, 72, 96 hours, and the concentration that killed 50% of individuals (LC50) after 96 hours of exposure was determined. No mortality was recorded in the control group throughout the experiment. The fish were collected as soon as their death was observed.

Statistical analysis

Statistical analysis and lethal concentration determination were performed using SPSS18 and the PROBIT method was used as a statistical procedure for determining lethal concentration and 95% confidence interval (Gerber and Finn, 2005).

II.1.3. RESULTS AND DISCUSSIONS

With regard to the acute toxicity test, for both copper and zinc, most mortalities were recorded in the first 24 hours. No mortality was recorded after 72 hours for copper and 48 hours for zinc. At the highest concentration of zinc (49.5 mg / l), all fish died within the first 24 hours.

It was observed that the mortality rate increased with the increase of copper and zinc ions (Fig. II.1.8, Fig. II.1.9), but also with the exposure period. The acute toxicity test showed that mortality was directly proportional to the concentration of heavy metals, but no mortality among the control group individuals was recorded.

The coefficient of determination (R^2) was estimated to be 0.90 (Fig. II.1.8) for copper and 0.918 for zinc respectively (Fig. II.1.9), indicating that 90% of mortality variations determined by copper and 91.80% of mortality variations determined by zinc are due to the change in metal concentration.

The LC50 lethal concentration of copper after 96 hours of exposure was estimated to be **0.547 mg / l.** The LC50 lethal concentration after 96 hours of exposure was estimated to be **34.225 mg/l.**

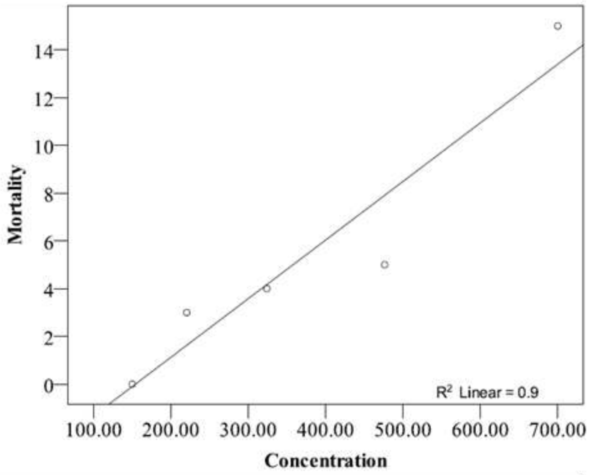

Fig. II.1.8 Mortalities of stellate sturgeon juveniles (*Acipenser stellatus*) (n = 20) after copper exposure (96 hours)

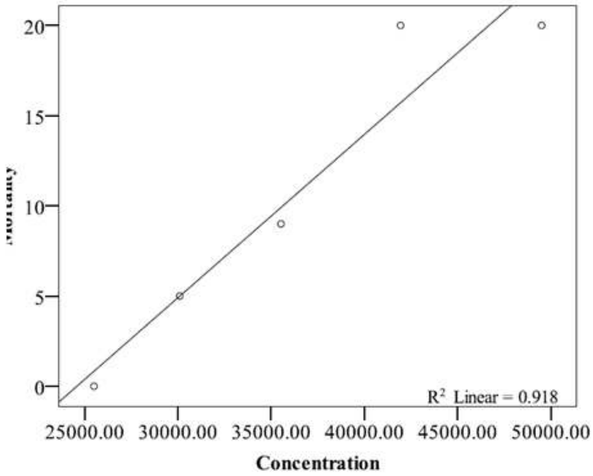

Fig.II.1.9 Mortalities of stellate sturgeon juveniles (*Acipenser stellatus*) (n = 20) after zinc exposure (96 hours)

Using the method of Awoyeni et al., 2014, we calculated a copper and zinc toxicity factor, based on lethal concentration LC50 96h, resulting a toxicity ratio (TF) of 62.56: 1, indicating the that copper ions are 62.5 times more toxic than zinc ions.

II.1.4. CONCLUSIONS

The present study is first one conducted to determine the acute toxicity of copper and zinc in the stellate sturgeon (*Acipenser stellatus*) in a water with a high hardness of 340 mg/l $CaCO_3$. The lethal concentration of copper was estimated to be 0.547 mg /l for copper and 34.22 mg /l for zinc.

Calculating a toxicity factor of copper and zinc, based on the lethal concentration CL50 96h, resulted in a toxicity ratio (TF) of 62.56: 1, indicating that copper ions are more toxic 62.5 times compared to ions. of zinc.

Although calcium provides a clear protection against copper and zinc toxicity, *Acipenser stellatus* remains a sensitive species susceptible to the toxicity of the two metals with low lethal concentrations (LC50 96 hours) compared to other fish species exposed to heavy metals in water hardness close to the one in this study.

Taking into account that very few studies have been published on the effects of copper and zinc on sturgeons, especially on stellate sturgeon (*Acipenser stellatus*), the present study can be considered a starting point for further research in the field, further studies being necessary on the toxicity of other heavy metals.

II.2. CHRONIC TOXICITY TEST

II.2.1. INTRODUCTION

In the second part of the Ph.D. thesis, I wanted to highlight the effects of chronic exposure of stellate sturgeon juveniles, to copper and zinc in both individual experiments and binary mixtures. The study was conducted according to OECD 204 "Fish, prolonged toxicity test: 14 days study" at sublethal concentrations of the two metals (10% and 25% of the lethal concentration LC50 96h previously determined) for a period of 7 and 14 days.

II.2.2. MATERIALS AND METHODS

The chronic toxicity test was conducted at the Institute of Research and Development for Aquatic Ecology, Fisheries and Aquaculture (ICDEAPA) Galati. Juveniles of *Acipenser stellatus* were brought from the Brateş farm, coming from the same lot, as the ones from the acute toxicity test, respectively from parents caught in the Danube River at Cotul Pisicii.

The fish were distributed in 12 groups of 10 individuals. The fish in the first two groups were kept in declined water as control groups, while the other groups were exposed to heavy metals. In order to achieve the objectives, juveniles of *Acipenser stellatus* were exposed to two different concentrations of copper and zinc.

Juveniles were exposed for 7 and 14 days to 10% and 25% of the lethal concentration (CL 50-96h) previously determined as 0.547 mg / l for Cu^{2+} and 34.225 mg / l Zn^{2+} in individual tests. After 7 and 14 days, juveniles were captured and anesthetized by immersion to sedation in 2-phenoxyethanol solution. They were sacrificed by evisceration, the liver, gill and intestine being immediately taken.

Livers and gills were divided into two sub-samples: for histopathological determinations, the samples were fixed in 10% formol and for metals bioaccumulation determination they were stored at -80 ° C until their analysis.

II.2.2.1 Determination of heavy metals bioaccumulation in tissues by inductively coupled plasma mass spectrometry

The tissue samples were analyzed in the Toxicology-Chemistry laboratory at the Institute of Diagnosis and Animal Health, Bucharest. Sample analysis was performed using inductively coupled plasma mass spectrometer (ICP-MS), Elan DRC II, manufactured by Perkin-Elmer, USA.

II.2.2.2. Determination of histopathological changes induced by heavy metals

II.2.2.2.1. Preservation and processing of tissues for histological analysis

The tissues were fixed in formol (10%) mono and disodium phosphate buffered (Chemical Company Iasi) in order to preserve the biological material and maintain the morphological characters of the cells and tissues. After 24 hours, the samples were subjected to dehydration in successive alcohol baths and then to clarification in toluene. The pieces were then included in paraffin, 2 paraffin baths at 60° for 3 hours/bath, with paraffin periodic replacement for the purpose of extracting the solvent from the tissues. The pieces thus processed were cut into a Lipshaw microtome at a thickness of 7 µm.

Sectional paraffin bands were put on pre-washed glass slides and covered with a fine layer of glycerinized albumin. In order to stretch the sections, a gelatin solution was dropped on their edges, and the slides were placed on a platinum heated to 50°C. After stretching, excess liquid was drained, and the slides were allowed to dry at room temperature in a stand for 2 weeks.

II.2.2.2.2. Sections staining by Hemalaun Meyer-Eosin method

For the staining of the sections, the Hemalaun Mayer-Eosin method was used which determined the coloration of nuclei in blue-violet and the cytoplasm in the red.

II.2.2.2.3. Semi-quantitative evaluations of histopathological changes

In the present study, I performed a semi-quantitative evaluation of histopathological changes induced by heavy metals at the level of the two studied organs. In order to achieve this goal, we used two evaluation methods: the method proposed by Bernet et al., 1999 and the method proposed by Poleksic and Mitrovic-Tutundzic (1994), applicable only to gills.

II.2.3. RESULTS AND DISCUSSIONS

II.2.3.1 BIOACCUMULATION OF HEAVY METALS AND THE LIVER HISTOPATHOLOGICAL CHANGES INDUCED BY THEM

II.2.3.1.1 BIOACCUMULATION OF HEAVY METALS IN THE LIVER

The normal physiological levels of copper and zinc in the liver of the control group individuals were very close, ranging from 24.12±9.97 mg/kg to 25.77±6.21 mg/kg for copper and 21.48±2.28 mg/kg and 24.64±5.38 mg/kg for zinc. For species of the Acipenseridae family, a normal range of concentrations of these two metals in the liver has not been determined so far (Brucka-Jastrzebska et al., 2009), so this study brings new information for completing the literature in the field.

II.2.3.1.1.1. Bioaccumulation of copper and zinc in individual experiments

As shown in Table II.2.3.1.1, after 7 and 14 days exposure to copper concentration of 0.054 mg/l, a significant increase ($p < 0.05$) of metal concentration in the liver was observed. In the case of zinc, after exposure to 3.42 mg/l for 7 days, its concentration increased by 8.24% ($p > 0.05$) compared to the control group, and after 14 days the concentration increased significantly ($p < 0.05$) with 49.63%.

At the highest concentration of zinc, 8.55 mg / l, a significant increase ($p < 0.05$) of the metal concentration in the tissue, with a percentage of 45.90%, can be observed, and after 14 days there was an insignificant decrease ($p > 0.05$) compared to the previous period, but the zinc concentration remained higher than the control group, the increase being only 19.56%.

Table II.2.3.1.1. Total heavy metals concentrations (mean ± standard deviation mg / kg wet weight) in the liver of *Acipenser stellatus* juveniles (significant differences between control and exposed individuals: * p <0.05, ** p <0.01, *** p <0.001) (n=5)

		Concentrations	**Copper**		**Zinc**	
			7 days	**14 days**	**7 days**	**14 days**
Liver		**Control**	24,12± 9,97	25,77± 6,21	21,48± 2,28	24,64± 5,38
	Cupru	10% LC50	40,22± 9,01*	54,55± 5,13***	-	-
		25% LC50	44,63 ± 12,83*	53,22± 4,71***	-	-
	Zinc	10% LC50	-	-	23,25± 4,83	36,87± 6,38 *
		25% LC50	-	-	31,34± 7,67*	29,46± 5,05

II.2.3.1.1.2. Bioaccumulation of copper and zinc in the binary mixture

With regard to the copper and zinc binary mixture (Table II.2.3.1.2), after 7 days of exposure, there was a significant increase at lower concentrations (0.054 mg /l Cu and 3.42 mg/l Zn) of both metals (p <0.05), with 66.95% for copper and 41.52% for zinc, respectively, compared to the control group individuals.

In the binary mixture with copper values of 0.136 mg/l and zinc 8.55 mg/l, after 7 days, a significant increase (p <0.05) of the metal concentration by 45.5% for copper, and 29% for zinc was observed compared to individuals in the control group.

If we look at the increase in the concentration of metals in the binary mixture at lower concentrations (0.054 mg/l copper and 3.42 mg/l zinc), compared to the individual experiment, after 7 days at the same concentrations, we can see that it was close to the concentration copper in the individual experiment, while the zinc concentration increased by 30.75% in the presence of low copper concentrations in the binary mixture.

However, in the binary mixture, at elevated concentrations (0.136 mg /l Cu and 8.55 mg / l Zn), the concentration of both metals was reduced, statistically insignificant (p> 0.05) compared to the metal concentration in the individual experiments with 21.36% for copper and 11.59% for zinc respectively) but also for the low metal binary mixture (with 12.84% for copper and 8.85% for zinc).

Table II.2.3.1.2 Total heavy metals concentrations (mean ± standard deviation mg/ kg wet weight) in the liver of *Acipenser stellatus* juvenile after 7 days of exposure (significant difference between control and exposed individuals: * p <0.05, ** p <0.01 , *** p <0.001) (n=5)

		Concentration	Copper	Zinc
Liver		Control	24,12± 9,97	21,48± 2,28
	Copper and zinc	10%+10% CL50	40,27± 6,63*	30,4± 8,46*
		25% +25% CL50	35,10± 9,97*	27,71± 9,34*

The trend of accumulation observed at higher concentrations in the binary mixture indicates that both metals were in a concentration sufficiently high to cause significant competitive interaction.

II.2.3.1.2. HISTOPATHOLOGICAL CHANGES INDUCED BY HEAVY METALS IN THE LIVER

II.2.3.1.2.1 Normal histological structure of the liver

The sturgeon liver is wrapped in a conjunctive capsule, generally made up of collagen fibers and fibroblasts (Muscalu et al., 2011), being an irregularly shaped gland.

In the present study, the liver of control juveniles showed a macroscopic appearance of fatty liver, a phenomenon often observed in sturgeon liver but also in other fish species due to high-fat food (Zaharia and Dumitrescu, 2010; Jalilpour et al. 2016).

The normal histology of the liver of *Acipenser stellatus* juveniles from the control group is shown in Figure II.2.3.1.11. Parenchyma of the liver is structured in morphological formations made up of hepatocytes. Hepatocytes showed a polygonal appearance with a diameter of approximately 11.13 ± 0.96 μm, disposed along the sinusoids, organized in rows converging towards the central vein (Figure II.2.3.1.11).

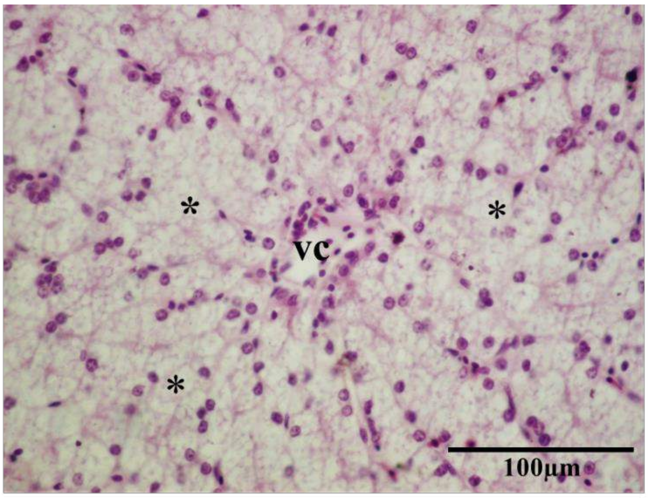

Fig.II.2.3.1.11 General aspect of hepatic parenchyma in *Acipenser stellatus* (Control), H&E staining: vacuolization (*),central hepatic vein (vc), x40 (original foto)

The morphological aspect of the hepatocytes is slightly heterogeneous. Depending on the degree of lipid loading of the cytoplasm and according to the classification made by Muscalu et al., 2011, predominantly hepatocytes with an exocentric spherical nucleus were observed. The nucleus presented one or more prominent nucleoli together with heterochromatin granules, the cytoplasm exhibiting numerous lipid vacuoles.

On the histological sections performed in the liver of the control juveniles, the presence of eosinophilic granulocytes (CGE), known as mast cells, was revealed. From the study of literature, it is the first time that the presence of these cells is histologically shown in the liver of the stellate sturgeon.

II.2.3.1.2.2 Histopathological changes induced by copper and zinc in individual experiments

After exposure to copper, stellate sturgeon liver showed marked histopathological changes compared to juveniles belonging to the control group. The observed alterations increased with dose and duration of exposure as follows:

After 7 and 14 days of exposure, in the copper exposed individuals, both concentrations determined morphological changes compared to the controls group. Thus histological changes included changes in hepatic parenchyma such as necrosis (changes in normal tissue architecture) and dilation of interhepatic space, increased melanomacrophage but also circulatory changes such as dilation of sinusoidal space and mild congestion of blood vessels.

Exposure to a concentration of 0.054 mg/l Cu^{2+} for 7 days also resulted in a progressive increase in mast cells in connective tissue and around blood vessels, demonstrating the presence of two mast cell populations in the *Acipenser stellatus* species: resident and circulating

In the liver, after 7 days after exposure to copper, it was observed the infiltration of inflammatory cells, respectively of lymphocytes, these being often observed in aggregates.

At the same concentration (0.054 mg / l Cu^{2+}), but after 14 days of exposure, the hepatic parenchyma necrosis and the dilation of the interhepatocyte space are observed. As the period of exposure increases, there is an aggravation of the circulatory changes, by dilatation of the sinusoidal spaces, the congestion of the blood vessels, and the increase of hemorrhagic areas.

Infiltration of inflammatory cells (lymphocytes) is more common. Massive aggregations of lymphocytes have often been observed with melanomacrophages.

The analysis of the sections performed for individuals exposed to the higher concentration of copper, 25% CL50 (0.136 mg / l), for 7 days, showed the same changes as in the case of the concentration of 0.054 mg / l, respectively changes of the hepatic parenchyma - necrosis , interhepatocyte corridors but also circulatory changes - dilation of sinusoids and appearance of bleeding areas. Mast cells were often observed along with other inflammatory cells such as lymphocytes but also melanoma macrophages.

At the highest concentration (0.136 mg / l Cu2 +), after 14 days, it is observed the induction of the same changes as necrosis, the expansion of the interhepatocyte space but also the expansion of the sinusoidal space and the congestion of the blood vessels.

Compared with the lower concentration (0.054 mg / l), but also with the previous period of experiment, there is also a marked increase in the number of lymphocytes, with the increase of the exposure period, both large and small lymphocytes. Commonly, lymphocytes have appeared mast cells.

In the liver of individuals exposed for 7 and 14 days, at a concentration of 3.42 mg / l zinc appeared interhepatocyte corridors and necrosis zones.

At the level of the liver, aggregates of melamomacrophages, mast cells, both large and small lymphocytes could also be observed. It can be observed that zinc ions may also have immunotoxic action, but these ions could not induce a marked increase in the number of large lymphocytes, as observed with copper exposure.

From the circulatory changes, dilations of the sinusoidal space, blood vessel congestion, but also hemorrhagic areas could be observed.

At the highest zinc concentration (8.55 mg / l), after 7 days of exposure, the presence of necrosis and interhepatocyte spaces can be observed, some individuals also presenting with vacuolization of the hepatic parenchyma.

There is a marked increase in melanoma macrophages and mast cells. There was no increase in the number of lymphocytes, as with copper exposure.

After 14 days of exposure to a concentration of 8.55 mg / l zinc, stellate sturgeon individuals presented necrosis, interhepatocyte corridors, vessel congestion. There has been an increase in the incidence of melanoma macrophages and rare lymphocytes.

Among the nuclear alterations, in the liver, for the specimens exposed to zinc ions, nuclear hypertrophy was observed, at all the metal concentrations and the exposure periods.

II.2.3.1.2.3 Histopathological changes induced by copper and zinc in the binary mixture

Exposure to the copper and zinc mixture (concentrations of 0.054 mg/l copper and 3.42 mg/l zinc) caused liver tissue necrosis and dilatation of interhepatocitar space, an increase in sinusoidal capillary dilation with haemorrhagic areas.

After 7 days of exposure, there was a marked increase in the number of melanomasacrophages and mast cells.

There was an increase in lymphocyte number compared to the control group, but fewer compared to those exposed only with copper ions (0.054 mg / l copper, 7 days), which may suggest that the copper still has a strong influence on the immune system.

Regarding the binary mixture of the two metals at the highest concentrations, it can be noticed that the histopathological changes are less pronounced, which can correlate with the much smaller amount of metals accumulated in this binary mixture compared to the individual experiments. Hepatocyte vacuolization has been frequently reported.

However, the mixture caused dilation of the sinusoidal capillaries and vascular congestion. It can be observed that the binary mixture did not lead to a marked increase in the number of melanomacrophages, with an increase in the number of small lymphocytes and mast cells. Large lymphocytes were rarely reported compared to exposure to a previous binary mixture.

II.2.3.1.2.4 Semi-quantitative assessments of histopathological changes

Applying the lesion index to the liver according to Bernet et al., 1999, associated with image analysis, allowed a more accurate assessment of both the individual and binary experiments to evaluate the histopathological effects induced by the two metals. The establishment of the index allowed a comparison of the level of lesions between the two metals, their concentrations and exposure periods.

The semi-quantitative analysis according to the method of Bernet et al., 1999, indicated that both metals in both individual experiments and binary experiments can cause significant histopathological changes in the liver after exposure for 7 and 14 days.

The histopathological index recorded for all metals, concentrations and periods of exposure is very high, which according to the classification made by Zimmerli et al., 2007, indicates severe alterations in the architecture and normal morphology of the liver.

II.2.3.1.3 PARTIAL CONCUSIONS

Both concentrations of copper and zinc used in this study determined a significant increase in liver concentration after 7 and 14 days of exposure. In the case of copper exposure, there was a saturation of metal uptake in the liver, reflecting the essential nature of the metal and the ability of fish to restore physiological control.

It can be seen that the zinc adjustment mechanism is different from that of copper. Thus, in the case of exposure to the highest concentration of zinc (8.55 mg / l), a two-phase accumulation occurs, respectively an increase in bioaccumulation during the first 7 days, followed by a decrease after 14 days of exposure.

The interaction way of metals at low concentrations in the mixture was significantly different from the interaction of metals at high concentrations but also during individual exposures.

The trend of accumulation observed at higher concentrations in the binary mixture indicates that both metals were in a concentration sufficiently high to cause significant competitive interaction.

The morphological appearance of the hepatocytes in the control specimens is slightly heterogeneous, with predominantly hepatocytes observed with an exocentric spherical nucleus, the cytoplasm exhibiting numerous lipid vacuoles. Due to vacuolization, the cells become hypertrophic, the nucleus is pushed to the periphery, while a hepatic degeneration (steatosis) is installed.

On the histological sections made at the liver of the control juveniles, was highlighted for the first time in *Acipenser stellatus* individuals, the presence of eosinophilic granulocyte cells (CGE), known as mast cells.

After exposure to copper and zinc, the stellate sturgeon liver showed marked histopathological alterations compared to individuals in the control group. The observed changes generally increased with the dose and duration of exposure.

II.2.3.2. BIOACCUMULATION OF HEAVY METALS AND THE GILL HISTOPATHOLOGICAL CHANGES INDUCED BY THEM

II.2.3.2.1. BIOACCUMULATION OF HEAVY METALS IN THE GILL

Normal levels of copper, at the gill level, in control individuals showed values between 1.766 ± 0.18 mg/kg and 1.919 ± 0.19 mg/kg. In the case of zinc, the concentrations were higher than those recorded for copper, respectively between 16,573 ± 3,37 mg/kg and 21,317 ± 3,75 mg/kg.

II.2.3.2.1.1 Bioaccumulation of copper and zinc in individual experiments

In the case of copper exposure, a markedly significant ($p < 0.01$) increase in copper concentration is observed throughout the experiment period and at all concentrations.

In the case of zinc, after 7 days of exposure at a concentration of 3.42 mg/l, its concentration increased significantly ($p<0.01$) by 41.82% compared to the control group, the zinc concentration at the gill level reaching after 14 days an increase of only 15.96% ($p> 0.05$).

At 8.55 mg/l, after 7 days of exposure, a very significant ($p < 0.001$) increase in metal concentration occurred with 65.64%, and after 14 days of exposure, the concentration increased by 32.25 % ($p> 0.05$).

Table II.2.3.2.1 Total heavy metals concentration (mean ± standard deviation mg/kg wet weight) in the gill of *Acipenser stellatus* juvenile (significant differences between control and exposed individuals: * p <0.05, ** p <0.01, *** p <0.001) (n=5)

		Concentration	Copper		Zinc	
			7 days	14 days	7 days	14 days
Gill		Control	1,766±0.18	1,919±0,19	16,573±3,37	21,317±3,75
	Copper	10% LC50	3,039±0.40**	3,118±0,44**	-	-
		25% LC50	3,161±0.57**	3,307±0,44***	-	-
	Zinc	10% LC50	-	-	23,505±2,77**	24,721±7,21
		25% LC50	-	-	27,453±2,31***	28,192±6,81

After completion of the experiment, it was found that there were no significant accumulations (p> 0.05) of zinc in the gill of stellate sturgeon individuals after 14 days exposure at both concentrations (3.42 mg/l and 8.55 mg/l). The lack of significant accumulations compared to the control group may be due to the elevated zinc background values seen in control samples after 14 days compared to 7 days.

For both zinc concentrations, it can be noticed that there are no significant differences (p> 0.05) between the zinc concentration after 7 days and 14 days of exposure.

Reducing zinc accumulation after 14 days may be due to a process of acclimatization, perhaps by restoring calcium influx, and a reduction in zinc influx (Olsson et al., 1998) due to decreased affinity of the Ca^{2+} / Zn^{2+} transport system.

II.2.3.2.1.2 Bioaccumulation of copper and zinc in the binary mixture

With regard to the binary metal experiments (Table II.2.3.2.2), after 7 days of exposure, at low concentrations of the two metals (0.054 mg/l Cu and 3.42 mg/ l Zn), the concentrations of both metals increased by 74.57 % for copper (p<0.01) and 22.94% respectively for zinc (p> 0.05) compared to the values recorded for the control. At higher concentrations in the binary mixture (0.136 mg / l Cu and 8.55 mg / l Zn), after 7 days, the increase was only 59.79% for copper (p <0.01) and 49.86% for zinc (p <0.01) compared to control group.

Table II.2.3.2.2 Total heavy metals concentrations (mean ± standard deviation mg / kg wet weight) in gill of *Acipenser stellatus* juveniles after 7 days of exposure (significant differences between control and exposed individuals: * p <0.05, ** p <0 , 01, *** p <0.001) (n=5)

		Concentration	Copper	Zinc
Gill		**Control**	1,766±0,18	16,573±3,37
	Cupru and zinc	**10%+10% CL50**	3,083±0,46**	20,376±1,91
		25% +25% CL50	2,822±0,26**	24,837±5,76**

If we compare the degree of metals bioaccumulation in the binary mixture at reduced concentrations (0.054 mg/l Cu and 3.42 mg /l Zn), compared to the individual experiment, at 7 days of exposure, it can be seen that the values are close for copper, while the zinc concentrations are lower but statistically insignificant (p> 0.05).

However, in the binary mixture at high concentrations (0.136 mg / l Cu and 8.55 mg / l Zn) the concentration of both metals was reduced compared to individual metal exposure, with only 10.73% for copper and 9 , 53% for zinc.

The decrease in bioaccumulation of both metals at the gills level at higher metal concentrations (0.136 mg /l Cu and 8.55 mg / l Zn) compared to individual exposures may indicate that the metals are at a concentration sufficiently high to determine a strong competitive interaction for the two metal binding sites.

Significant decreases in zinc concentration at the gill level at the lower concentration of the two metals can be attributed by removing zinc ions from copper binding sites, much more competitive in the mixture of the two metals.

II.2.3.2.2. HISTOPATHOLOGICAL CHANGES INDUCED BY HEAVY METALS IN GILL

II.2.3.2.2.1. Normal histological structure of the gill

The normal morphological appearance of the gill in *Acipenser stellatus* is similar to other fish species. The gills are situated on the branchial arches and formed by gill filaments and secondary lamellae.

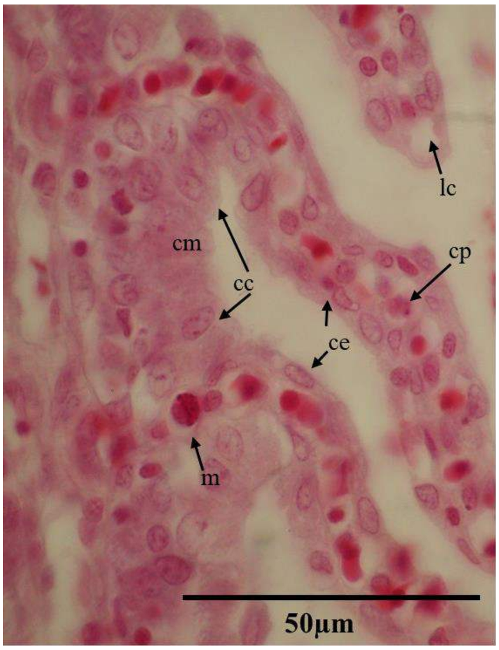

Fig.II.2.3.2.2.3 Normal structure of the gill *Acipenser stellatus* (control), H&E staining:

chloride cells (cc), mucous cells (cm), pillar cells, epithelial cells (ce), mast cells (m), marginal blood channel (lc), x100 (original foto)

Different types of cells can be identified on epithelial lamina: superficial pavement cells, mucous cells and chloride cells (Figure II.2.3.2.2.3). The lamella are lined with a simple squamous epithelium consisting of epithelial cells, covering the blood vessels separated by pillar cells.

In the present study, some gill changes were observed in individuals in the control group, such as mild hyperplasia and hypertrophy of chloride and mucous cells, edema and melanomacrophage aggregates. No irreversible gill injury was observed to affect the normal functioning of the respiratory epithelium.

II.2.3.2.2.2. Histopathological changes induced by copper and zinc in individual experiments

By comparing the histological appearance of the gills in the control group, with those of individuals exposed to copper and zinc, remarkable pathological changes are observed in the branchial architecture.

The changes included dilation of the intercellular spaces and tissue necrosis after exposure to copper. Hypertrophy and hyperplasia of the respiratory epithelium have been observed both in the branchial filamnent and apical areas, with no evidence of hypertrophy and hyperplasia of the mucous and chloride cells. Also at the level of the secondary lamellae could be evidenced the presence of edema. Other changes observed were the fusion of the secondary lamellae tips, the curvature and shortening of the secondary lamellae. From circulatory changes were seen dilation of blood vessels and blood congestion at the level of the gill filaments. Of the inflammatory processes, lymphocytes and melanomacrophage aggregates have been observed.

As the period of exposure increases, hypertrophy and hyperplasia of epithelial, mucous and chloride cells are observed. Also during this period the presence of edema is also observed. Dilatation of the intercellular spaces as well as the fusion of the secondary lamellae were observed. There is an increase in the number of melanomacrophage aggregates in the gill filaments. Of the circulatory changes, clavate lamellae appear.

In individuals exposed for 7 and 14 days at a zinc concentration of 3.42 mg / l, the same changes were observed as in the case of copper exposure, for exemple dilation of the intercellular spaces, secondary lamellae curvature, and very rarely the hyperplasia and hypertrophy of epithelial cells, hypertrophy and hyperplasia of mucous and chloride cells. The lamellar epithelium rupture was also observed.

At 7 days exposure to zinc concentration of 8.55 mg/l, a strong hyperplasia and hypertrophy of the lamellar epithelium and mucous and chloride cells was observed. The shortening but also the curvature of the secondary lamellae, the lamellar epithelium rupture were observed. At this concentration, nuclear hypertrophy was observed. Circulatory changes have seen telangiectasia and enlargement and congestion of blood vessels.

Paradoxically, with the increase of the exposure period, at the same concentration a mild hypertrophy of the chloride and mucous cells, and hyperplasia and hypertrophy of epithelial cells were seen. The presence of dilation of the intercellular spaces but also the rupture of the lamellar epithelium was observed. Circulatory changes were rarely observed, these being the same as at previous concentrations and periods of exposure.

II.2.3.2.2.3. Histopathological changes induced by copper and zinc in the binary mixture

In the case of exposure to a binary mixture of the two metals, 0.054 mg / l Cu and 3.42 mg / l zinc, dilation of the intercellular spaces and necrosis were observed. Edema and melanomacrophages have been reported. Only fusion of the secondary lobe tips was observed. Circulatory changes consisted of telangiectasia and the presence of clavate lamellae.

It should be mentioned that hyperplasia and hypertrophy of epithelial cells, mucous and chloride cells and complete lamellar fusion were not observed. With the increase in the concentration of metals in the mixture (0.136 mg / l copper and 8.55 mg / l zinc) at the end of the 7 days of exposure, dilatation of the intercellular spaces and necrosis, as well as the hypertrophy and hyperplasia of the epithelial, mucous and chlorogenic cells, were found. These processes of hyperplasia and hypertrophy have led to the fusion of the secondary lamellae but also the complete lamellar fusion. Melanomacrophages and lymphocyte infiltrations were a constant presence.

Circulatory changes are constant, namely the presence of clavate lamellae and the widening and congestion of blood vessels.

Histopathological examinations showed similar changes at the gill level due to copper and zinc exposure but also after exposure to a mixture of the two metals, but this was normal since histopathological changes induced by heavy metals but also by other toxic substances are not specific to a single pollutant, which is also confirmed by similar alterations under the influence of a wide range of contaminants (see Mallatt, 1985).

II.2.3.2.2.4. Semi-quantitative assessments of histopathological changes

Since the histopathological changes induced by the two metals in both individual experiments and binary experiments were the same, we performed a semi-quantitative assessment of these changes by which we could highlight the fact that there are differences in the exposed individuals depending on degree and extent lesion, as well as the lesion importance factor.

Two methods for establishing a histopathological index were applied, the first according to the method of Bernet et al., 1999, and the second according to the method of Poleksic and Tutundzic, 1994.

The values recorded for the histopathological index confirm once again that the binary mixture at high concentrations causes significant changes at the gill level, but at elevated concentrations of the two metals in the mixture, the two metals appear to be in sufficient concentration so that there is a competition between them.

According to the method of Poleksic and Mitrovic-Tutundzic (1994), a histopathological index of 8 was determined for the control group, indicating functional glands. It is evident as shown by the histopathological index value calculated for both metals, all concentrations and periods that gills are irreparably affected by exposure to heavy metals.

II.2.3.2.3 PARTIAL CONCLUSIONS

In the present study, a significant increase in copper concentration at the gill level was observed with the increase in concentration and exposure time. The zinc adjustment mechanism appears to be different from copper at the gill level, with a reduction in gill uptake of zinc as the time of exposure increases.

By comparing the bioaccumulation of copper and zinc, in individual experiments, a higher bioaccumulation of copper than zinc at the gill level was observed. This was to be expected, since copper has a covalent index much higher than zinc, which makes it more competitive for intracellular ligands, rich in nitrogen and sulfur.

With regard to experiments on metal binary mixtures, at low concentrations of the two metals (0.054 mg/l Cu and 3.42 mg/l Zn) only significant copper accumulations were observed, while in the binary mixture, high concentrations of the two metals determined a significant bioaccumulation of the two metals.

By comparing the degree of bioaccumulation of metals in the binary mixture at reduced concentrations (0.054 mg/l Cu and 3.42 mg/l Zn), compared to the individual experiment, at 7 days of exposure, it was observed that the values are approximately close for copper while the zinc concentration was lower but statistically insignificant.

However, in the binary mixture at high concentrations (0.136 mg / l Cu and 8.55 mg / l Zn) the concentration of both metals was reduced compared to individual metal exposure. The decrease in bioaccumulation of both metals at the gills at higher metal concentrations (0.136 mg/l Cu and 8.55 mg/l Zn) compared to individual exposures may indicate that the metals are at a concentration sufficiently high to determine a strong competition interaction for the two metal binding sites.

The present study shows that the high water hardness (high concentration of calcium and potassium ions) does not provide protection against the accumulation of heavy metals at the gill level.

Following histopathological analysis, some gill changes were seen in control group, such as mild hyperplasia and hypertrophy of chloride and mucous cells, edema and melanomacrophage aggregates. No irreversible gill injury was observed to affect the normal functioning of the respiratory epithelium.

By comparing the histological appearance of the gills of juveniles in the control group, with those of the individuals exposed to copper and zinc, marked pathological changes are noted in the branchial architecture.

Histopathological examinations revealed a number of gill tissue changes such as hypertrophy and epithelial cell hyperplasia, hypertrophy and hyperplasia of mucous and chloride cells, edema, lamellar fusion resulting in increased diffusion distance between the external environment and the blood. All of these changes have the consequence of affecting oxygen uptake, as well as affecting osmoregulation.

The results of histopathological analysis indicate that the identified lesions that affect gill morphology can disturb physiological processes and cause oxidative stress.

II.2.3.3 HISTOPATHOLOGICAL CHANGES INDUCED BY HEAVY METALS IN THE INTESTINE

II.2.3.3.1 Normal histological structure of the digestive tract and the adjacent glands

The digestive system of stellate sturgeon (*Acipenser stellatus*) is typical for a primitive carnivore, (Singer and Ballantyne 2005), both the morphology and the anatomy of the digestive tube being similar to other sturgeon species. The digestive tract consists of the oral cavity (a protractile mouth, preceded by a rostrum), pharynx, esophagus, stomach, intestine with pyloric appendix, spiral valve and anus.

Each segment of the digestive tube is made of four layers: mucosa, submucosa, muscularis, and serosa.

Pharynx is distinguished by the presence of pyramidal mucous papillae arranged in longitudinal series on its inner surface. In the transition region between pharynx and the esophagus, the high papillae are replaced by longitudinal folds of reduced dimensions (Dapra et al. 2009).

Esophagus, in stellate sturgeon is relatively long and narrow compared to other sturgeon species. (Vajhi et al. 2013). From a histological point of view, the esophagus has two distinct regions. The anterior portion has an epithelium consisting of cylindrical cells with microvilli and numerous mucosal cells responsible for the production of neutral and acidic mucosubstances. The epithelium of the posterior region of the esophagus has a pseudo-layered appearance, containing numerous ciliated cells and mucous cells. The mucosa is surrounded by connective tissue and a circular muscular layer (Martos et al. 2015).

In stellate sturgeon, during embryonic development, approximately 7 days after hatching, **the stomach** is differentiated in the anterior glandular stomach and the posterior pyloric stomach. The two regions are provided with anatomical and histological adaptations (Buddington and Doroshov 1986) indicating a high degree of specialization.

Fig.II.2.3.3.3 Transversal section of glandular stomach, *Acipenser stellatus,* H&E staining, x10 (original foto)

The anterior glandular stomach (fig II.2.3.3) is characterized by the presence of many branched tubular gastric glands occupying most of the mucous *lamina propia*. The gastric epithelium is simple, cylindrical, composed of ciliated and mucous cells. The mucous-secretory cells of the stomach have a cylindrical appearance, with a basal nucleus of irregular shape, their apical surface showing microvilli.

The gastric glands are composed of a single cell type - pyramidal or cuboidal oxyntic- peptic cells, free of cilia and microvilli.

The posterior stomach (pyloric) (fig. II.2.3.4) shows some mucous glands, the mucosa having a smooth aspect. The smooth muscle of the stomach is well developed, accounting for about 80% of the gastric wall thickness.

The epithelium in this region is predominantly composed of secretory mucous cells, including ciliary cells. Submucosa contains numerous nerve bundles consisting of myelinated and unmyelinated fibres, as well as neuronal bodies (Martos et al. 2015).

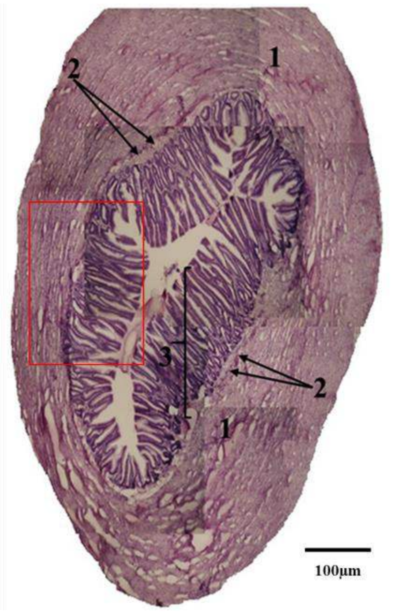

Fig.II.2.3.3.4 Transversal section of pyloric stomach, *Acipenser stellatus*, H&E staining : 1- muscularis, 2-submucoasa, 3-mucoasa, x10 (original foto)

The pyloric appendix (fig.II.2.3.5) consisting of several pyloric caeca, is located between the intestine and the stomach. The caeca are encapsulated in a prominent circular smooth muscle, traberculae (musculature), also separating pyloric caeca into lobules of different sizes. Each lobe consists of mucosal folds (villi) consisting of a columnar epithelium rich in mucosal cells.

On the analyzed sections, the following histological structure could be emphasized mucosa, submucosa, muscularis (smooth musculature). Each lobe consists of mucosal folds. The epithelium of villi consists of simple columnar cells with a large eccentric nucleus.

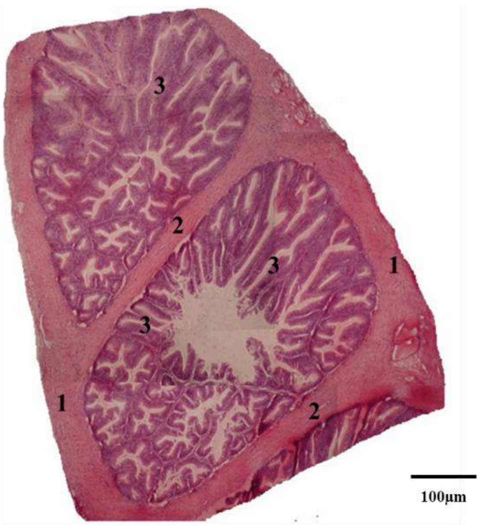

Fig. II.2.3.3.5 Transversal section of pyloric appendix, *Acipenser stellatus*, H&E staining: 1- muscularis, 2- traberculae, 3- villi, H&E staining, x10 (original foto)

The sturgeons **intestine** consists of three parts: the anterior intestine, the spiral valve, and the terminal portion of the intestine or rectum. Histological analysis has shown that the organization of the intestinal wall is similar to that of other vertebrates. The anterior intestine comprised a mucosa with an epithelium consisting of enterocytes and mucous cells, *lamina propria* of connective tissue and mucosa muscularis. There are also visible the submucosa, the external muscularis consisting of two layers of smooth muscle cells, and on the outside serosa

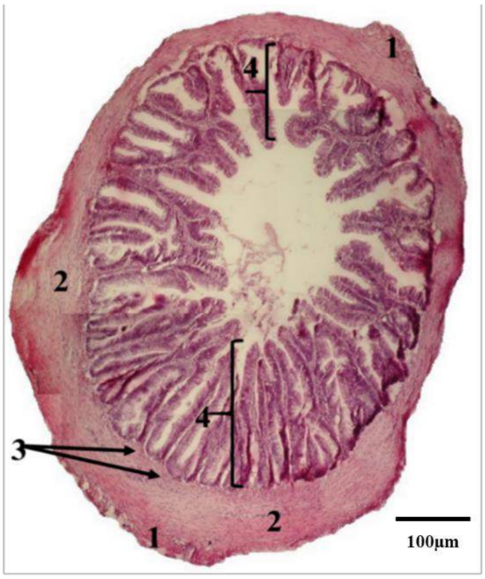

Fig. II.2.3.3.6 Transversal section of intestine *Acipenser stellatus,* H&E staining: 1-serosa, 2-muscularis, 3-submucosa, 4-mucosa, x10 (original foto)

The anterior part of the intestine is the site of digestive processes under the action of its own enzymes and those secreted by the accessory glands. Also in this region is the absorption of nutrients that are taken by blood and lymph.

In sturgeons, a special feature of the digestive tract (primitive trait) is the presence of **the spiral valve,** which occupies most of the intestinal lumen (fig.II.2.3.7).

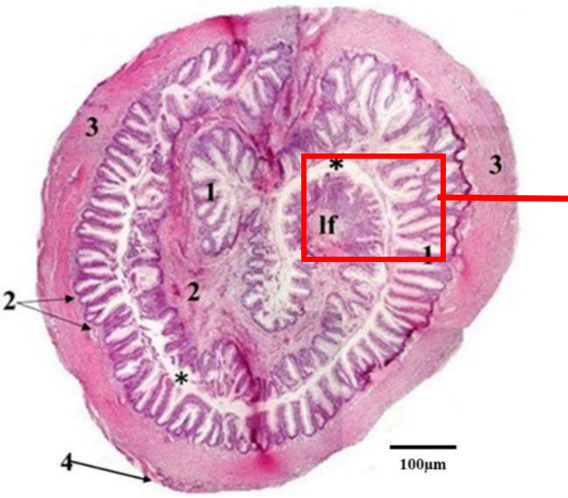

Fig.II.2.3.3.7 Transversal section of spiral valve, *Acipenser stellatus,* H&E staining: *intestinal lumen, 1-mucosa, 2-submucosa, 3-muscularis, 4-serosa, lf-lymphoid structure , x10 (original foto)

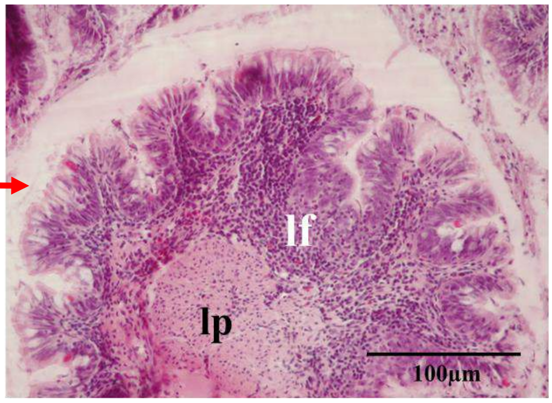

Fig.II.2.3.3.7a Aspect of lymphoid structure, *Acipenser stellatus*, H&E staining: lymphocytes (lf), *lamina propria* (lp), x40 (original foto)

Sturgeons compensate for the reduced bowel length by the presence of this spiral valve, respectively the growth of the intestinal surface by folding the mucosa and the submucosa, the number of folds depending on the species and the species. The spiral valve together with the anterior intestine constitutes up to 45-50% of the total length of the digestive tract (Martos et al. 2015).

At the level of the spiral valve, the present study revealed the presence of lymphoid tissue (fig.II.2.3.3.7a), considered by some authors to be involved in immune defense against parasites and bacteria (Martos et al. 2015).

The spiral valve (fig.II.2.3.7) has a thick muscular wall, but the mucosa is thinner. In this region, the *lamina propia* of the intestinal wall contains a large number of lymphocytes.

The mucosa consists of a simple columnar epithelium, with many mucosal cells, some of which are vacuolated.

In the intestine, but especially at the level of the spiral valve, eosinophilic granulocytes could be observed, both distributed to the mucosa and *lamina propria*, but also to the muscularis.

The presence of these cells in the digestive tract at acipenseriformes was first histologically revealed also for other sturgeon species (*Acipenser transmontanus, Polyodon spathula)* by Radaelli et al. (2000), Petrie-Hanson și Peterman (2005). Thus, the present study is the first to demonstrate the presence of these cells in the digestive tract of stellate sturgeon (*Acipenser stellatus*).

The rectum is short, showing a thin muscular tunic compared to the other components of the digestive tract, while the flattened mucosa has large folds.

The histological analysis was able to reveal the presence of the four tunics, while in the mucosa a stratified epithelium containing mucous glands and numerous mucous cells could be observed.

II.2.3.3.2 Histopathological changes induced by copper and zinc in individual experiments

Copper-induced histopathological changes in the intestine

The reduced concentration of copper (0.054 mg / l) after 7 days of exposure induced significant changes in the intestine of exposed individuals.

Individuals intoxicated with 0.054 mg / l copper for 7 days and 14 days have changes in intestinal epithelium - hypertrophy and the appearance of intercellular spaces, the lamina propria - retraction of connective tissue, capillary hyperemia, edema, and inflammatory cell infiltration into the submucosal and muscular levels.

In individuals intoxicated with 0.136 mg / l copper for 7 days, histopathological changes are evident in the intestine, including apical dilation and fusion of villi and pronounced hypertrophy of the epithelium. Inflammatory cells infiltrating into the epithelial layer and the underlying connective tissue may also be observed.

In both individuals exposed for 7 days and those exposed for 14 days, the high dose of copper led to the appearance of necrotic lesions in the intestinal villi.

Histopathological changes induced by zinc in the intestine

Individuals intoxicated 3.42 mg / l zinc for 7 days frequently present capillary hyperemia of the intestinal villi, associated with the infiltration of inflammatory cells.

Fish intoxicated with 8.55 mg / l zinc for 14 days show pronounced changes in the intestinal epithelium. The accumulation of eosinophilic granules in the cytoplasm of epithelial cells is commonly encountered. Greater vacuolization of the enterocyte cytoplasm is also observed and the glandular cells show large mucus vacuoles, as well as the appearance of intercellular spaces, hypertrophy of the epithelium and nuclei. At the level of the external muscle, the inflammatory cells infiltrate, which has an atypical appearance, with wide corridors between the muscle fibers.

II.2.3.3.3 Histopathological changes induced copper and zinc in the binary mixture

In individuals exposed to a mixture of 0.054 mg / l copper and 3.42 mg / l zinc (10% CL50), predominantly changes in the intestinal mucosa were observed: hypertrophy of the intestinal epithelium and mucosal cells, fusion of the villi, retraction of the connective tissue, emergence of intercellular spaces in the intestinal epithelium and increased stratification. Among the circulatory changes, hyperemia of the capillaries was observed. Some intestinal portions showed necrosis of intestinal tissue. Inflammatory processes such as increased lymphocyte infiltration into the epithelial layer or muscle were also observed.

Individuals exposed to a mixture of 0.136 mg / l copper and 8.55 mg / l zinc also showed changes in the intestinal mucosa, such as hypertrophy of the intestinal epithelium and mucosal cells, fusion of villi, retraction of connective tissue. Inflammatory cells were observed in the epithelial layer and adjacent connective tissue. Of the circulatory changes, hyperemia of the blood vessels appears.

II.2.3.3.4. PARTIAL CONCLUSIONS

The intestine of the fish, together with the gill, is one of the important organs in heavy metals uptake, either through water or food.

Histopathological analysis has highlighted the existence of a digestive system characteristic of a primitive carnivore. In the individuals of the stellate sturgeon, as with other sturgeon species, the digestive tract consists of pharynx, esophagus, stomach (which differentiates in two regions: the glandular stomach and non-glandular stomach), pyloric appendix, intestine, spiral valve, and rectum. All these regions of the digestive tract have a characteristic histological structure, being specialized in different degrees for digestion and absorption processes

Exposure to heavy metals has marked effects on the histological organization of the digestive system. The results of the present study on the intestine in *Acipenser stellatus* show that heavy metals exert their toxic effects on different intestinal layers.

Most lesions were observed in the intestinal mucosa, most of which constituted defense mechanisms against heavy metal toxicity.

Both metals, both in individual and mixed experiments, caused changes in the intestinal mucosa after just 7 days of exposure.

In the case of copper exposure, a correlation between the metal concentration and the severity of the lesions is observed. Unlike copper, exposure to zinc did not cause major histopathological changes in the intestinal mucosa.

In the case of metals mixture, numerous changes in the intestinal mucosa are observed at low concentrations (0.054 mg/l copper and 3.42 mg/l zinc (10% LC50). Changes due to the direct action of metals were observed, as intestinal epithelial rupture and necrosis. When exposed to high concentrations of the two metals, defense changes of the intestinal epithelium are observed, with no changes such as necrosis or epithelial rupture observed.

All these observations indicate that in the case of low metal concentration mixtures there is a synergism of the two metals, most likely the changes being induced predominantly by copper ions, as observed in the individual experiments.

Since most intestinal changes were recorded in the mucosa, this indicates that the bioaccumulation of metals occurs in the intestinal mucosa.

Since heavy metals in the intestine can also affect bacterial communities, along with induced changes in the intestinal epithelium, suggests that sturgeons may have problems with normal digestive processes for the absorption of nutrients.

II.2.4. GENERAL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

The present study is the first to highlight the sensitivity of juvenile stellate sturgeons (*Acipenser stellatus*) to heavy metal intoxication in both individual experiments and binary experiments.

The paper was structured on two major categories of objectives: the study of acute toxicity of heavy metals and the study of the chronic toxicity induced by the two metals in the stellate sturgeon (*Acipenser stellatus*) - namely the determination of the bioaccumulation of heavy metals and the histopathological changes induced by them in various organs.

Thus, in the first part of the PhD thesis, I aimed to highlight the novelty contribution of the PhD thesis, with particular emphasis on the study of heavy metals on sturgeons, especially on Danube sturgeon species. The character of novelty is the selection of the sturgeon species, *Acipenser stellatus*, a less studied species, compared to other sturgeon species in which concerns the bioaccumulation of heavy metals in different organs and their histopathological changes induced by them.

The study of literature in the field, presented in the first part of the doctoral thesis, led to the general conclusion that there are insufficient studies to assess the acute and chronic toxicity of heavy metals, on sturgeons, especially in the field of histopathology.

In the second part of the thesis are presented the laboratory experiments and the results (bioaccumulation, histology) that ultimately led to the determination of the effects of heavy metals and the sensitivity of stellate sturgeon juveniles. Thus, in the thesis through experimental research I researched both the acute and chronic toxicity of the two metals with the highest concentrations in Danube waters and sediments (copper and zinc), following their bioaccumulation and histopathological changes at the level of three important organs for the uptake and metabolism of metals (gills, intestines and liver).

The first objective of the doctoral thesis, was the achievement of an acute toxicity test for copper and zinc, namely the determination of lethal concentration LC50 96 h in sturgeons. The study of the literature on the toxicity of heavy metals to sturgeons has highlighted the lack of data on the toxicity of these metals for sturgeons in waters of different hardnesses.

The present study is first conducted to determine the acute toxicity of copper and zinc in the stellate sturgeon (*Acipenser stellatus*) in a water with a high hardness of 340 mg/l $CaCO_3$. The lethal copper concentration was estimated to be 0.547 mg / l and zinc to 34.225

mg / l. It was found that the mortality rate increases with increasing concentration of copper and zinc ions but also with the exposure period.

The lethal CL50 concentration of copper at 24 hours of exposure was estimated at 1184 µg / l, at 48 hours at 623.75 µg / l, and at 72 and 96 hours at 547.22 µg / l .

The lethal concentration of zinc CL50 at 24 hours of exposure was estimated at 34,821.6 µg / l, while the lethal concentration of CL50 at 48, 72 and 96 hours was the same, respectively 34225.04 µg / l. Thus, in the case of both metals, a reduction in the lethal CL50 concentration can be observed with the increase of the exposure period.

The calculation of the toxicity factor of copper and zinc, based on the lethal concentration CL50 96h, showed a toxicity ratio (TF) of 62.56: 1, indicating that copper ions are 62.5 times more toxic compared to zinc.ions.

Although calcium provides clear protection against the toxicity of copper and zinc, *Acipenser stellatus* remains a species sensitive to the toxicity of the two metals, with low values of lethal CL50 concentration 96 h, compared to other fish species exposed to heavy metals at near water hardness. of the present study.

The second objective of the doctoral thesis was to perform a chronic toxicity test for copper and zinc. Within these tests, the bioaccumulation of metals was pursued at the level of the two main organs, involved in the acquisition of metals (gills), but also their metabolism (liver).

Determining the concentration of copper and zinc in the liver of the control individuals indicated that the physiologically normal values are very close. Thus, the values of the concentration of heavy metals in the liver, ranged between 24.12 ± 9.97 mg / kg and 25.77 ± 6.21 mg / kg for copper and 21.48 ± 2.28 mg / kg and 24, 64 ± 5.38 mg / kg for zinc. Normal levels of copper, at the level of the gill, in the control individuals showed values between 1.766 ± 0.18 mg / kg and 1.919 ± 0.19 mg / kg. In the case of zinc, it had higher concentrations than those recorded for copper, respectively between 16,573 ± 3.37 mg / kg and 21,317 ± 3.75 mg / kg.

For the species of the family Acipenseridae, a normal range of concentrations of these two metals in the liver and gills has not been determined so far, so this study brings new information to supplement the literature in the field.

Although the general pattern of accumulation for each metal has been studied, for different fish species, this study is the first to directly compare the pattern of chronic accumulation of copper and zinc in stellate sturgeon, both in an individual experiment and in the binary mixing of those. two metals, under similar exposure conditions.

In the present study, the accumulation of metals in the species Acipenser stellatus was performed according to the concentration and the exposure period, as well as the interactions between the binary mixture.

Both concentrations of copper and zinc, used in the present study, determined after 7 and 14 days of exposure, a significant increase in the concentration of metals in the liver. In the case of exposure to copper, at a concentration of 0.136 mg / l, there was a saturation of the metal uptake in the liver, after 14 days of exposure, which reflects the essential nature of the metal and the ability of the fish to restore physiological control.

It has been found that the mechanism of regulation of zinc is different from that of copper. Thus, in the case of exposure to the highest concentration of zinc (8.55 mg / l), a two-phase accumulation of zinc takes place, respectively an increase of bioaccumulation in the first 7 days, followed by a decrease after 14 days of exposure.

Within the present study, a significant increase of the copper concentration at the branchial level was observed with the increase of the concentration and the exposure period. As in the liver, the mechanism of regulation of zinc at the branchial level seems to be different from that of copper, with a reduction in the branchial takeover of zinc as the exposure time increases (the values of the metal concentration at the branchial level, after 14 days of exposure, they were close to the values recorded after only 7 days of exposure).

Compared with the control group, after 14 days of exposure to zinc, no significant accumulations at the branchial level were observed. The lack of significant accumulation compared to the control group, may be due to the increased background values of zinc, observed in the control specimens, after 14 days, compared to 7 days.

Comparing the bioaccumulation of copper and zinc, in the individual experiments, we could observe a greater bioaccumulation of copper, than of zinc both in the liver and in the bran. This was to be expected, since copper has a much higher covalent index than zinc, which makes it much more competitive for intracellular ligands rich in nitrogen and sulfur.

Although many studies on metal accumulation had the main conclusion that the metals in the mixture accumulate more intensely than each individual metal (synergistic effect), in the present study we showed that in the mixtures of copper and zinc, the synergistic effect varies with the time of exposure and concentration of metals in the mixture.

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Although many studies on metal accumulation had the main conclusion that the metals in the mixture accumulate more intensely than each individual metal (synergistic effect), in the present study we showed that in the mixtures of copper and zinc, the synergistic effect varies with the time of exposure and concentration of metals in the mixture.

The mode of interaction of metals at low concentrations in the binary mixture was significantly different from the mode of interaction of metals at high concentrations but also to that during the individual exposures.

If, in the case of exposure to low concentrations of metals in the binary mixture, there was a significant increase in the concentration of metals in the liver, in the case of exposure to high concentrations of the two metals, a decrease in the bioaccumulation of metals was found, in this organ, comparatively with previous concentrations.

Regarding the experiments on the binary mixing of metals at the branchial level, at low concentrations of the two metals, significant accumulations of copper were observed only. The binary mixing at high concentrations of the two metals resulted in a significant bioaccumulation of the two metals, compared to the control group, but lower compared to the bioaccumulation values recorded in the individual experiments.

This accumulation tendency observed at higher concentrations in the binary mixture at the hepatic and branchial levels, indicates that both metals were in a concentration sufficiently high to cause significant competitive interaction.

The present study shows that high water hardness (high concentration of calcium and potassium ions) does not provide protection against accumulation of heavy metals at the branchial level.

Exposure to hard water provides an abundance of the necessary cations (Ca2 +, Mg2 +) to compete with the copper and zinc ions for the binding sites of the gills. In the present study, the physico-chemical parameters of the water, respectively a pH of 8.4 together with a high water hardness of 340 mg / l CaCO3, suggest that a large fraction of metals was present as inorganic complexes (mainly CuCO3). and ZnCO3). Because it was observed at the branchial and hepatic level, an increase in bioaccumulation during exposure to the two metals, the results of the present study show that the complexes of the two metals are also bioavailable for bioaccumulation by juvenile stellate sturgeon

It is obvious that metals behave differently in the mixture than during individual exposures for different species, and these cannot be explained solely on the basis of single data on bioaccumulation in binary mixture, so further studies are needed.

The present study is a first attempt to determine the histopathological effects of heavy metals, simultaneously in an individual and binary experiment, on a species of sturgeon. Thus, we aimed to highlight the histopathological changes induced by the intoxication with the two metals, in the liver, the gills and the intestine, which revealed the toxic potential of these metals.

Histopathological examinations showed that the alterations of the main target organs (liver, gland, and intestine) generally increase in severity as the concentration of metals and the duration of exposure increases.

The observation of the normal histological structure of stellate sturgeon liver, revealed the vacuolization of hepatocytes most often a reversible process, which can have long-term consequences, through severe disruptions of cell function.

On the histological sections made in the liver of the control specimens, for the first time in individuals of *Acipenser stellatus* the presence of eosinophilic granular cells (CGE), known as mast cells, an essential component of the immune system, was revealed.

After exposure to copper and zinc, the liver of stellate sturgeon showed marked histopathological alterations compared to individuals in the control group. Alterations generally observed increased with dose and duration of exposure.

In general, the range of histopathological changes was similar. Thus, in the case of exposure to heavy metals, both in the individual experiments and in the binary experiments, both changes of the hepatic parenchyma, such as necrosis, dilation of the interhepatocyte space, but also circulatory changes such as dilation of the sinusoidal space, appearance of hemorrhagic areas and congestion of blood vessels were observed..

Inflammatory changes were also observed, such as the numerical growth of mast cells and lymphocyte infiltration, a change reported predominantly in individuals intoxicated with copper ions, and at high concentrations of this metal.

Regarding the weight of structural changes, due to exposure to heavy metals, it can be seen that this is almost the same, both in the case of individual and binary experiments. The only major difference is in the case of exposure to copper, is the the increase of inflammatory processes (increasing lymphocyte presence with increasing metal concentration).

These observations highlight the strong immunotoxic action of copper ions as compared to zinc ions. All of these observations point out once again that copper ions are much more toxic to stellate sturgeon (*Acipenser stellatus*) as compared to zinc ions.

Following histopathological analyzes, several branchial changes were observed in individuals in the control group, such as slight hyperplasia and hypertrophy of chlorogenic and mucosal cells, and aggregates of melanoma macrophages. No irreversible glandular lesions were observed that affect the normal functioning of the respiratory epithelium.

Comparing the histological aspect of the gills in control group, with those of the individuals exposed to heavy metal poisoning, both in individual and binary experiments, marked pathological changes are observed in the branchial architecture.

In general, the range of histopathological changes was similar. Histopathological examinations revealed a series of changes in defense of the branchial tissue against the toxic action of heavy metals such as hypertrophy and hyperplasia of epithelial cells, hypertrophy and hyperplasia of the mucous and chlorogenic cells, edema, lamellar fusion, which have as a result the diffuse fusion, which have as a consequence between the external environment and the blood, the decrease of the oxygen uptake capacity, but also the osmoregulation.

The second type of lesions encountered consists of modifications such as necrosis, rupture of the branchial epithelium, which reflects the direct effects of metals on the branchial cells.

As the histopathological changes induced by the two metals in both individual and binary experiments were similar, we performed a semi-quantitative evaluation of these changes both at the hepatic and branchial level, by which we could show that there are differences between the groups. intoxicated, depending on the degree and extent of the lesion, as well as on the factor of importance of the lesion.

Applying the method of Bernet et al., 1999, showed that the histopathological index recorded for all metals, concentrations and exposure periods was very high, indicating severe alterations of the normal architecture and morphology of the liver.

At the branchial level, the same index showed that the gills are irreparably affected by the exposure to heavy metals, by inducing irreversible histological changes, such as necrosis, which lead to functional disturbances of this organ.

At the hepatic and branchial level, significant increases of the histopathological index were observed, with the increase of the concentration of metals both in the individual and binary experiments, and implicitly with the bioaccumulation of the metal in the tissue. In the case of the binary mixture, it is confirmed once again that the binary mixture at high concentrations, causes significant changes at the branchial level, but compared to the individual exposures, they appear to be in a sufficient concentration, so that between the two metals there is a competition.

In the case of binary mixing, a slight decrease in the value of the histopathological index is observed in individuals exposed to high concentrations of metals (25% CL50), compared to the histopathological index recorded for individuals exposed to low concentrations (10% CL50). This correlates with the decrease in the bioaccumulation of metals in the liver, as the concentration of metals in the mixture increases, and underlines once again.

This correlates with the decrease in the bioaccumulation of metals in the liver, as the concentration of metals in the mixture increases, and again emphasizes that in the binary mixture the metals were in a concentration sufficiently high to determine a competitive interaction.

In the present experiment, we followed the normal histological structure of the digestive tract in stellate sturgeon (*Acipenser stellatus*) but also the effects that heavy metals can induce on the normal histological structure of the intestine.

Histopathological analysis revealed the existence of a digestive system characteristic of a primitive carnivore. In trstellate sturgeon as with other sturgeon species, the digestive tract is made up of the pharynx, esophagus, stomach (which differentiates into two regions: the glandular stomach and the non-glandular stomach), pyloric appendix, intestine, spiral valve, and rectum. All these regions of the digestive tract have a characteristic histological structure, being specialized in different degrees for the processes of digestion and absorption.

In the digestive tract of the species Acipenser stellatus, we were able to histologically highlight, for the first time the presence of eosinophilic granular cells, essential elements of the immune system in sturgeons.

Exposure to heavy metals has significant effects on the histological organization of the digestive system. The results of the present study on the gut in Acipenser stellatus show that heavy metals exert their toxic effects on different intestinal layers.

Most lesions were observed in the intestinal mucosa, most of them constituting defense mechanisms against the toxicity of heavy metals.

In case of exposure to high concentrations of the two metals, changes in the defense of the intestinal epithelium are observed, without changes such as necrosis or rupture of the epithelium.

All these observations indicate that in the case of mixing metals at low concentrations, there is a synergism of the two metals, most likely the changes being predominantly induced by copper ions, as observed in the individual experiments.

Given that heavy metals in the intestine can affect bacterial communities, along with induced changes in the intestinal epithelium, it suggests that sturgeons may have problems with normal digestive processes of nutrient uptake. At the same time, given the role of the intestine in the osmoregulation process, heavy metals can influence this process through the observed histopathological changes.

As a general conclusion, it can be said that the species *Acipenser stellatus*, remains a sensitive species, in the face of the toxicity of heavy metals, even in water with a very high hardness.

The presence of histopathological changes in the liver level of the breeding specimens raises the question of the efficiency of the breeding programs. Due to the reduction of the sturgeon stock in the Danube, the practice of repopulating the natural basins with individuals obtained in artificial environment and raised in aquaculture conditions has spread lately.

The histopathological study at the branchial level indicated that both heavy metals can induce a series of modifications could lead to osmoregulation disturbances - which implicitly affects the adaptability and survival of the specimens.

A better understanding of the toxicity of heavy metals to sturgeons is necessary for their better conservation. The results obtained in the present study are critical for the efforts to repopulate the Danube with sturgeons, many more studies being needed on the acute but also chronic toxicity of heavy metals in sturgeons, due to the effects of water quality on the survival of juveniles.

Future research directions

After analyzing the results obtained and the difficulties encountered in finding studies / researches on the histopathological effects of heavy metals on sturgeon populations in the Danube, new research directions on the subject presented in the chapters of this thesis are anticipated.

I emphasize the need for further in-depth studies to determine the susceptibility of sturgeon species to heavy metals exposure. Integrative studies quantifying species response to a wide range of heavy metals and their concentrations in individual experiments and mixture experiments are required.

Given the limited number of studies on the bioaccumulation of heavy metals in the intestine, additional studies are needed to determine the degree of bioaccumulation of heavy metals and the preference for a particular region of the intestine (pyloric appendix, intestine, spiral valve).

The literature study indicated the lack of semi-quantitative quantification of histopathological changes at the intestinal level. Since intestinal changes induced by exposure to metals have been observed in the present study, it is considered necessary to establish semi-quantitative quantification indices similar to those established at the liver and branhial levels.

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