ISSN: 1220-3173; E-ISSN: 2457-7618

EVALUATING THE EFFECTS OF LEAD ON STERLET LARVAE (*ACIPENSER RUTHENUS*) – A CYTOHISTOLOGIC STUDY

EVALUAREA EFECTELOR PLUMBULUI PE LARVE DE CEGĂ (*ACIPENSER RUTHENUS*) – STUDIU CITOHISTOLOGIC

M. BAROGA[1),2)], Diana BREZOVAN[1)], Jelena SAVICI[1)], Eugenia DUMITRESCU[1)], F. MUSELIN[1)], S.A. ORĂȘAN-ALIC[1)], R.F. MORUZI[1)], R.T. CRISTINA[1),*)]

ABSTRACT | REZUMAT

Methods for identifying toxicity to fish larvae can detect the types of acute and/or lethal toxicity of different chemical matrices. The study aimed the cytohistological confirmation of our anterior published study and to identify, upon exposure to lead acetate solutions, the main changes in the architecture of the organs and tissues (vitellus and intestine, liver, kidney, skin, muscles, and heart) as a comparison with the main obtained indicators. The study used two-day-old hatched-free embryos and the samples were divided into groups A and B. After acclimatization period, free larvae were inserted and exposed to decreasing soluble lead solutions as follows: 50 individuals/aquarium, total: 100 larvae/concentration: E1-A/E1-B=400 μgL^{-1};E2-A/E2-B=200 μgL^{-1}; E3-A/E3-B=100 μgL^{-1}; E4-A/E4-B=50 μgL^{-1}. The control group consisted of 100 vigorous larvae. Acipenser ruthenus larvae were monitored by direct observation at baseline, then at 12, 24, 48, 72, and 96 hours. The first dead larvae were detected after 12 hours of lead exposure, with the tip at an interval of 72-96 hours. Statistics revealed a significant association between the influence of lead on the parameters of length ($^{*}p<0.05$) and weight ($^{**}p=0.01$; $^{***}p<0.01$) and the number of dead larvae at the same concentration, which statistically demonstrates the detrimental activity of lead in water. Cytohistological architecture confirmed the morphological changes observed on the larvae exposed to lead toxicity, with the signaling of significant cytoarchitectural changes especially for the high concentrations used, (E1A/1B = 400 ppm and E2A/2B = 200 ppm).

Keywords: juveniles, *Acipenser ruthenus*, lead, cytohistology

Metodele de identificare a toxicității pentru larvele de pește pot detecta tipurile de toxicitate acută și / sau letală a diferitelor matrice chimice. Studiul a vizat confirmarea citohistologică a studiului publicat anterior și identificarea, la expunerea la soluții de acetat de plumb, a principalelor modificări ale arhitecturii organelor și țesuturilor (vitelus și intestin, ficat, rinichi, piele, mușchi și inimă), ca un comparabil cu principalii indicatori obținuți anterior. Studiul a folosit embrioni liberi eclozați în vârstă de două zile, iar probele au fost împărțite în grupele A și B. După perioada de aclimatizare, larvele libere au fost inserate și expuse la soluții de plumb solubile după cum urmează: 50 larve / acvariu, total: 100 larve/concentrație: E1-A/E1-B = 400 pgL^{-1}; E2-A/E2-B = 200 pgL^{-1}; E3-A/E3-B = 100 pgL^{-1}; E4-A/E4-B = 50 pgL^{-1}. Grupul martor, a fost compus din 100 de larve sănătoase. Larvele de *Acipenser ruthenus* au fost monitorizate prin observare directă la momentul inițial, apoi la 12, 24, 48, 72 și 96 ore. Primele larve moarte au fost detectate după 12 ore de expunere la plumb, cu vârful la un interval de 72-96 ore. Statistica a relevat o asociere semnificativă între influența plumbului asupra parametrilor de lungime ($^{*}p$ <0,05) și greutate ($^{**}p$ = 0,01; $^{***}p$ <0,01) și numărul de larve moarte la aceeași concentrație, care statistic demonstrează activitatea dăunătoare a plumbului în apă. Histologia a confirmat modificările morfologice observate la larvele expuse la toxicitatea plumbului, cu semnalizarea unor modificări citoarhitecturale semnificative în special pentru concentrațiile mari utilizate, (E1A / 1B = 400 ppm și E2A / 2B = 200 ppm).

Cuvinte cheie: juvenili, *Acipenser ruthenus*, plumb, citohistologie

Pollutants from freshwater sources, through their potential risk, can affect human and animal health, with numerous risk tests, guidelines, and standards currently being validated / validated and implemented (2, 26). It is known that the groups of vertebrates that respond without delay if the environment is polluted as a result of anthropogenic activities are fish. In the ecotoxicological and bio-medical field, adult fish and their early stages are used as a means to test various substances, from industrial pollutants to medicinal active substances (1, 3, 5, 7, 9, 11, 18, 20, 31, 32).

For this reason, the benefit of using acute toxicity tests on fish is the applicability for a wide range of substances, this methodology being increasingly used to

1) Banat's University of Agricultural Science and Veterinary Medicine "King Michael I of Romania" Faculty of Veterinary Medicine, Timișoara, Romania
2) Sanitary Veterinary and Food Safety Directorate Dolj County, Romania
*) *Corresponding author: romeocristina@usab-tm.ro*

compare the sensitivity of other aquatic species (3, 4, 12, 35).

The advantages of using acute toxicity tests on fish were: applicability for a wide range of substances and chemical combinations. The fact that fish repro-duce easily, are small in size so do not take up too much space, is cheap biological material compared to other animal species used in experiments, are accep-ted by the scientific community having applicability in many branches of human and veterinary medicine, uses to compare sensitivity to other species and orga-nisms. Initial studies have shown that fish are sensi-tive enough to detect pollutants in water, both in acute and long-term studies. More and more researchers are using toxicity tests on embryos and larvae as viable alternatives to acute or chronic toxicity tests for adult fish, as they have proven to be perfectly valid tools for assessing the potential for toxicity (1, 6, 9, 11, 17, 19, 22, 31). Methods for identifying toxicity to larvae / fish detect the types of acute and / or lethal toxicity of different chemical matrices on fish. The method requires the knowledge of the physicochemical properties of the investigated substances such as hydrophilicity, hydrostatic pressure, physicochemical stability, biodegradability, etc. for choosing the right method (9, 14, 16, 27, 31, 36-38).

The development of toxicity tests can bring new data on the toxicological effects of different matrices present in water on different fish species and their evolutionary stages. Of these, acute embryo tests are very useful because they provide speed, accessibility at low cost, but also accurate results (3, 7, 10, 14, 17, 21, 31, 34). Also, the early stages of fish development can allow a large number of repetitions to be performed in a short period, which will increase the availability and reproducibility of the results (1, 2, 5, 7, 17, 19). To this end, zebrafish (*Danio rerio*) was initially the first to be tested due to the many benefits offered compared to other species of laboratory animals currently used in experiments (1, 7, 11, 12, 18, 32).

Our previous initial observations revealed similarities between zebrafish and another native species, the pikeperch (*Sander lucioperca*) arguing that also other fish species could be successfully used in such experiments (10, 14-16, 21, 27, 29, 36). In this regard, we began further studies on sterlet (*Acipenser ruthenus;* class: *Actinopterygii*; order: *Acipenseriformes*; family: *Acipenseridae*), a valuable species from the sturgeon family from the Danube area (6, 13, 30, 35, 36).

In this sense, the aim was to evaluate the toxic effects of lead find as an essential water polluter, through acute tests on larvae of *A. ruthenus* and monitoring the behavioral disorders depending on the concentration of the substance used as other valuable studies in the field (4, 6, 14-16).

This original test was conceived as a useful procedure that comes to the aid of known OECD methodologies in the field of eco-toxicity testing (23-25). Our reason was to provide new and reliable information on the activity of lead in juvenile forms of sturgeons exposed to various decreasing concentrations of soluble lead acetate in a static 96-hour acute toxicity test, intenting to identifying the main clinical symptoms, the anatomical and morphological changes, the minimum / maximum lethal concentrations, and mortality (all these being considered critical control points), as well as to accomplish correlations with the specific parameters, like temperature, pH, dissolved oxygen (all these data being presented in an anterior paper) (6).

The present study aimed the cytohistological confirmation of our anterior published study and to identify, upon exposure to lead acetate solutions, the main changes in the cytoarchitecture of the main organs and tissues: vitellus and intestine, liver, kidney, skin, muscles, and heart as a parallel with main obtained indicators.

MATERIALS AND METHODS

The larvae and them examination

Experimental aquariums and juvenile *A. ruthenus* forms were provided by the Department of Fisheries at the Faculty of Bioengineering of Animal Resources Timisoara, adult fish being raised in aquaculture systems with recycling (RAS). Their reproduction was artificial, the incubation of eggs and the hatching of free embryos were carried out in accordance with the accepted methodologies for the sturgeons (13, 30).

The embryos obtained were healthy and without visible somatic defects. The study used two-day-old hatched-free embryos (natural mortality on the first day of life can reach 50%). Feeding was not necessary for the entire study period, knowing that free embryos of *Acipenser ruthenus* begin to feed from day 6-7 of life, after vitellus absorption (13, 30).

The samples were divided into groups A and B and the mirror-aquariums were filled with water (10 litres each). After acclimatization period, free larvae were inserted and exposed to decreasing soluble lead solutions (28) as follows: 50 individuals / aquarium, total: 100 larvae/concentration: E1-A, E1-B = 400 μgL^{-1}; E2-A, E2-B = 200 μgL^{-1}; E3-A, E3-B = 100 μgL^{-1}; E4-A, E4-B = 50 μgL^{-1}. The control group = C, consisted of 100 vigorous larvae The main critical points of toxicity were observed according to the OECD instructions (24-26), presented in Table 1.

Sterlet larvae were monitored by direct observation at baseline, then at 12, 24, 48, 72, and 96 hours (one hour / reading). From the beginning of each examination, every change in behavior or in the somatic body was observed, the measurements performed including: individual weighing and total length, pre-anal

Table 1

Critical toxicity points after OECD guidelines [24, 26]

Somatic / behavioral changes of Sterlet larvae
1. Not detaching the tail
2. Cardiac arrhythmia
3. Swelling and / or bleeding into the chest cavity
4. Enlargement of the thoracic cavity / accumulation of fluids
5. Body pigmentation
6. Tail in the V; L or S shape or unfinished tail growth
7. Impaired /severe /slow /rapid mobility - alternative mobility
8. Swimming vertically or like a propeller
9. A larva was dead if no gill movements were observed and if the touch of the caudal peduncle does not registered reaction

length and total height of the larva. At the beginning of the study, the second day after hatching, the initial measured average length of a sturgeon embryo was 9.7 mm (all larvae being normal, with small eyes, nose openings, well-developed folds of the wings, and yolk sac) and with an individual body weight ranging from 0.191 to 0.195 g.

Used instrumentation and fish euthanasia

To examine with the minimum aggression,vital and somatic alterations, short-term immobilization and after that euthanasia of larvae was achieved with tricaine mesylate (Metacaine, Tricaine or MS-222; Finquel-Merck, Germany) an accepted substance, used in fish in the EU and the US (33). The powder was diluted to 100 mg × L^{-1} (as an anesthetic) and respectively 500 mg × L^{-1} (as euthanasiant) for 2 to 10 minutes (33). Somatic changes were observed under a Kruss optronic GmbH (Germany) microscope using the ×10 lens (i.e., WF10 × /20), and the weight was assessed using an electronic Kern ABJ 220-4M Sohn GmbH (Germany) scale with the error of max. 0.01 mg.

Statistical analyzis

The statistical values were expressed graphically by GraphPad Prism 6.0. Windows (GraphPad, San Diego USA). To evaluate the differences between the groups, it was performed by two-way ANOVA, with *t*-test and, respectively, Bonferroni's multiple corrections, the statistical difference being set at the value of $^{*}p < 0.05$ or less.

Citohistologic architechture

The aim was to identify and confirm the main critical changes observed in individuals subjected to different concentrations of lead. The staining methodology was the one known in the literature. The larval samples were processed by the Hematoxylin Eosin (H.E.) staining method, a simple method which reveals the cytoarchitecture of changes in tissues and organs (e.g., respiratory system, digestive tract, liver, kidney, muscle, etc) (6, 29).

H.E. staining went through the steps: staining of nuclei with Harris hematoxylin for 3 minutes; washing and differentiation in tap water; staining the cytoplasm with Eosin 2% for 3-5 minutes; dehydration in increasing solutions of ethyl alcohol: 70°, 80°, 96°, absolute alcohol, 10 minutes for each bath; clarification in two benzene baths lasting 15 minutes each; mounting in Canadian conditioner. The examination and reporting will be finalized by microscopy where the stained samples will be compared with the control samples. The cytoarchitectural changes will be identified/analyzed/described, and photographed, the images being captured using the Olympus CX41 microscope software (Olympus, Germany).

Table 2

The variation of mortality, temperature, pH, and dissolved oxygen during the experiment

Dilution	**Dead larvae**					**pH**					**Water temperature** (°C)					**O_2 Concentration** (%)				
Interval (h)	12	24	48	72	96	12	24	48	72	96	12	24	48	72	96	12	24	48	72	96
Control 1	-	-	-	-	-	7.10	7.15	7.20	7.07	7.04	18.3	18.3	18.3	18.5	21.2	77.2	75.0	73.0	70.1	70,0
Control 2	-	-	-	-	-	7.10	7.11	7.06	7.05	7.07	18.0	18.0	18.2	18.3	18.9	73.5	74.8	72.3	70.3	70.1
E1A	**1**	**3**	**5**	**5**	**7**	7.03	7.07	7.37	7.70	7.08	18.1	18.5	18.7	19.0	19.2	76.0	75.0	72.1	70.6	70.8
E1B	-	**1**	**4**	**6**	**6**	7.01	7.02	7.06	7.02	7.06	18.4	18.5	18.5	18.5	19.0	77.2	73.7	70.6	70.2	70.1
E2A	-	**1**	**2**	**3**	**3**	7.04	7.03	7.07	7.01	7.06	17.7	18.0	18.4	18.5	18.7	73.7	74.5	70.7	70.1	70.7
E2B	-	**1**	**2**	**2**	**1**	7.05	7.06	7.04	7.08	7.09	18.6	18.5	18.4	18.7	18.8	72.4	71.4	70.1	70.9	70.9
E3A	-	**-**	**-o-**	**1**	**2**	7.03	7.09	7.03	7.02	7.06	18.1	18.1	18.5	18.4	19.0	74.5	72.7	70.9	70.9	70.0
E3B	-	-	**-**	**1**	**1**	7.08	7.0	7.02	7.02	7.10	18.2	18.2	18.5	19.0	18.9	75.0	72.5	70.4	70.2	70.3
E4A	-	**-**	**-**	**-**	**-**	7.10	7.05	7.08	7.03	7.08	18.0	18.2	18.6	18.6	18.8	73.6	74.2	70.8	70.4	70.1
E4B	**-**	**-**	**-**	**-**	**-**	7.06	7.07	7.07	7.08	7.10	18.1	18.2	18.6	18.7	19.0	74.6	74.0	70.1	70.1	70.8

RESULTS AND DISCUSSIONS

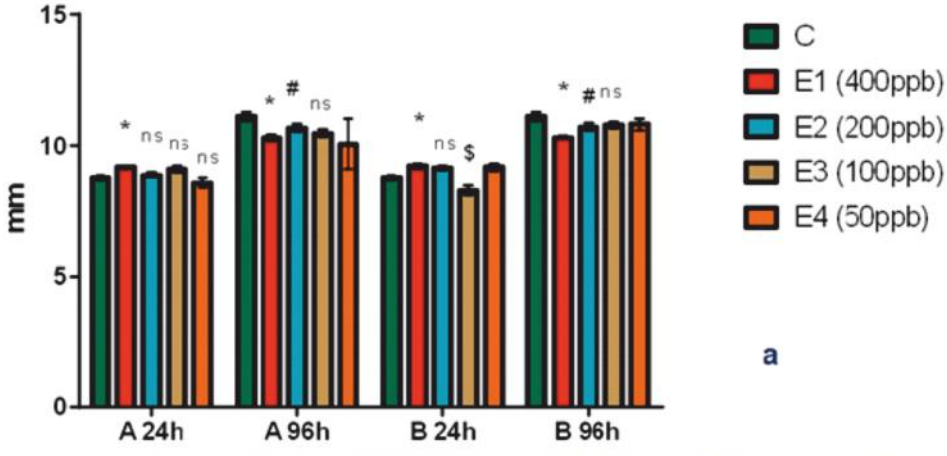
Behaviour and clinical observation

Two strong critical points were acknowledged during testing: **a**. attempt of larvae to escape on introduction into tanks at a concentration of 400 µg × L^{-1};

b. the negative response of the larvae to swimming and movement after descending to the aquarium's bottom, followed by the installation of a variety of behavioral disorders.

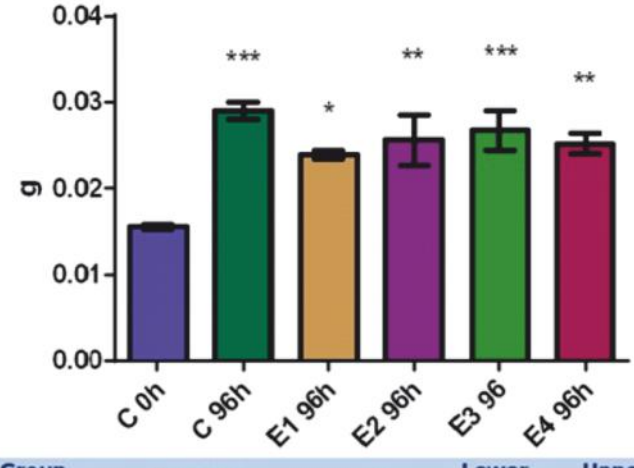
The main changes considered as critical points identified by us, and then studied cytohistologically and which are considered evidence of the harmful activity of lead, included: delayed resorption of the yolk sac;deformed/modified tail;undeveloped eyes; hemorrhages (cardiac / pericardial / peritoneal / generalized); altered pigmentation and finally exitus.

Sturgeon's larvae mortality was observed at lead's concentrations of 400 and 200 µgL^{-1}.



Group / replica (n=10)	Control	E1 (400ppb)	E2 (200ppb)	E3 (100ppb)	E4 (50ppb)
	X±SEM	X±SEM	X±SEM	X±SEM	X±SEM
A 24h	8.78±0.10	9.17±0.05*	8.88±0.08^{ns}	9.09±0.12^{ns}	8.59±0.20^{ns}
A 96h	11.12±0.16	10.29±0.11*	10.67±0.15$^{\#}$	10.47±0.15^{ns}	10.06±0.96
B 24h	8.78±0.10	9.22±0.11*	9.13±0.08^{ns}	8.30±0.19$^{\$}$	9.17±0.14
B 96h	11.12±0.16	10.30±0.07*	10.71±0.14$^{\#}$	10.79±0.14^{ns}	10.82±0.23

Fig. 1. The evolution of the length parameters at various lead concentrations at 24 and 96 hours of exposure
Where: *p < 0.05; **p = 0.01; ***p < 0.01; #comparative to Control p< 0.05; *ns* = not significant



Group (n = 10)	X ±SEM	SD	Lower 95% CI	Upper 95% CI
C 0h	0.0155±0.000279	0.000882	0.01487	0.01613
C 96h	0.02901±0.000987***	0.003122	0.02678	0.03124
E1 96h	0.0239±0.0005*	0.00158	0.02277	0.02503
E2 96h	0.02558±0.002969**	0.009388	0.01886	0.0323
E3 96h	0.02673±0.002337***	0.007389	0.02144	0.03202
E4 96h	0.02519±0.001191**	0.003766	0.0225	0.02788

Fig. 2. The statistical significance of the weight parameters at various lead concentrations at day 0 and 96 h.
Where: *p < 0.05; **p = 0.01; ***p < 0.01; #comparative to Control p< 0.05; *ns* = not significant

The first dead larvae were detected after 12 hours of lead exposure, with the tip at an interval of 72-96 hours. In Table 2 the variation of mortality, pH, temperature, and O_2 load at the moments of the experiment is shown.

Figures 1 and 2 presents the statistics of the evolution of growth parameers (average length).

In addition to the changes in movement and flotation that amplified during the experiment, were also observed: cardiac hemorrhage, relaxed abdomen, and V-shaped, L or S-shaped tails. Compared to the control group, the results revealed statistically significant values, recorded for the average length parameters, measured at 24 and 96 hours, and for the main weight parameters at the time 0 and 96 hours for all experimental concentrations.

Statistical evaluation revealed a significant association between the influence of lead on the parameters of length ($^{*}p$ <0.05) and weight ($^{**}p$ = 0.01; $^{***}p$ <0.01) and the number of dead larvae at the same concentration, which statistically demonstrates the detrimental activity of lead diluted in water.

Cytohistological architecture

Histological samples confirmed the cytomorphological changes observed on the larvae exposed to lead toxicity, with the signaling of significant cytoarchitectural changes especially for the high concentrations used, (E1A/1B = 400 ppm and E2A/2B = 200 ppm).

Vitellus and intestine samples

In the Control group, microscopic examination of the intestine revealed its normal structural appearance, with the columnar monolayered epithelium of the intact intestinal mucosa and the total resorption of the yolk sac. For groups E1, E2, E3, and E4, the 96-hour microscopic examination gradually showed the appearance of edema, installed between the mucosa and submucosa of the intestine, delayed resorption of the yolk sac, phenomena more obvious in E1 and E2 and less pronounced in group E4. Also, in group E1, the presence of cellular aggregates, containing necrotic enterocytes, detached from the epithelium, was highlighted in the intestinal lumen.

In areas with normal appearance, non-resorption / delayed resorption of the calf was found, considered an essential critical point that demonstrated the detrimental activity of lead. These irritative-alterative phenomena slow down the absorption function at the intestinal level, therefore, consequently, they decrease the viability of *A. ruthenus* larvae.

Figure 3 is presenting the main changes in the intestinal mucosa and stages of intestinal resorption 96 hours after the start of the study. The images were taken at the smallest microscope objective (ob.×4/1000 µm), respectively the largest, except for the immersion objective (ob. × 40/100 µm).

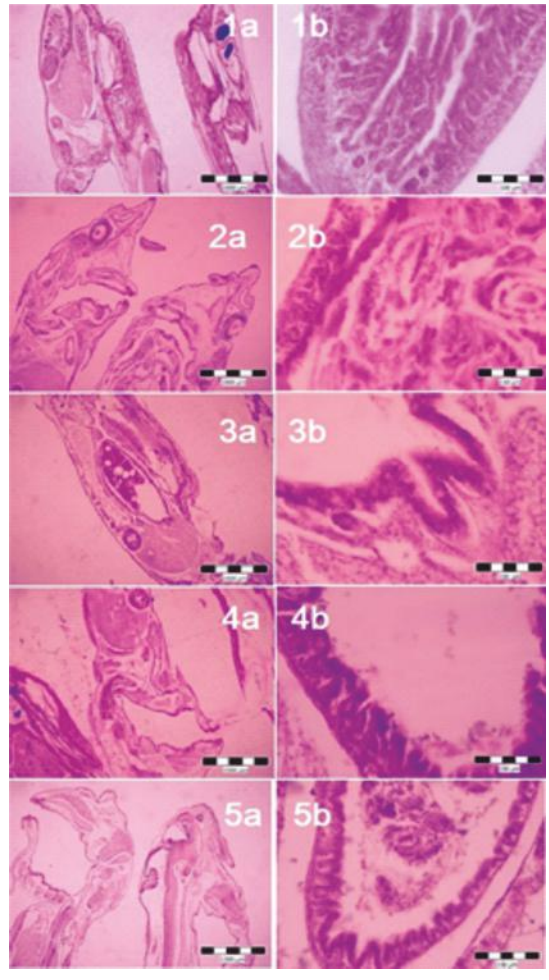


Fig. 3. The main changes in the intestinal mucosa and stages of intestinal resorption 96 hours after the start of the study: **1a and 1b** = **Control group**: Normal histological appearance of the intestine with complete resorption of the yolk sac (1a = ob. ×4 / 1b = ob. ×40); **2a and 2b** =**Group E1/400 ppm**: Necrotic cells in the intestinal lumen with the massive presence of unabsorbed calf edema between the mucous and submucosal tunica (2a = ob. ×4 / 2b= ob. × 40); **3a and 3b** = **Group E2/200 ppm**: The presence of unabsorbed vitellus and edema between the mucous and submucosal tunica (3a = ob. × 4 /3b = ob. × 40 (3b). **4a and 4b = Lot E3/100 ppm**. Absence of the calf and areas of much reduced edema between the mucous and submucosal tunica (4a = ob. × 4 left / 4b = ob. × 40 right). **5a and 5b** = **Lot E4/50 ppm**. Absence of edema, active intestinal mucosa with dividing enterocytes, vitellus resorbed almost completely (5a = ob. × 4 left / 5b = ob. × 40 right).

Liver samples

Figure 4 is presenting the main changes in liver tissue 96 h, after the start of the study. The images were taken with the objective of the microscope of × 20/200 µm, respectively × 40/100 µm.

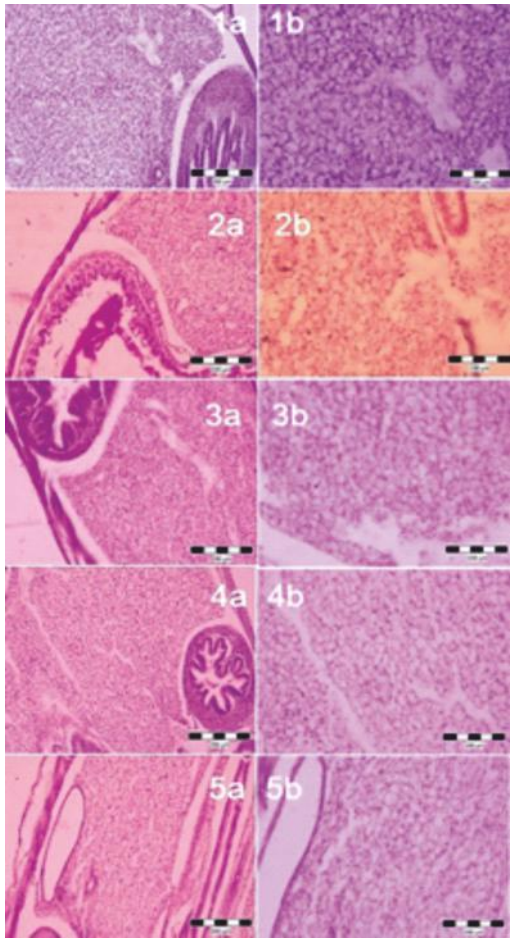


Fig. 4. The main changes in liver tissue 96 hours after the start of the study. **1a and 1b = Control group**. Normal image of the liver with numerous adipocytes and normal intestinal section (1a = ob. × 20 left / 1b = ob. × 40 right). **2a and 2b = Group E1/ 400 ppm**. Liver vasodilatation and massive marginal subcapsular hemorrhages, with the presence of erythrocytes in the lumen of the centro-lobular veins (2a = ob. × 20 left / 2b = ob. × 40 right). **3a and 3b = Group E2/200 ppm**. Vasodilation in the liver with marginal hemorrhages and the presence of erythrocytes in the lumen of the lobular centro-lobular veins (3a = ob. × 20 left / 3b = ob. × 40 right). **4a and 4b = Group E3/100 ppm**. Vasodilation in the liver with the highlighting of the centro-lobular veins (3a = ob. × 20 left / 3b = ob. × 40 right). **5a and 5b = Lot E4/50 ppm**. Apparently normal appearance with slight centro-lobular vasodilation (5a = ob. × 20 left / 5b = ob. × 40 right).

Kidney samples

Figure 5, reveal the main changes in renal tissue 96 hours after the start of the study. The images were taken only with the microscope objective of × 40/100 µm, at other lower resolutions, the cytohistological aspects being little observable.

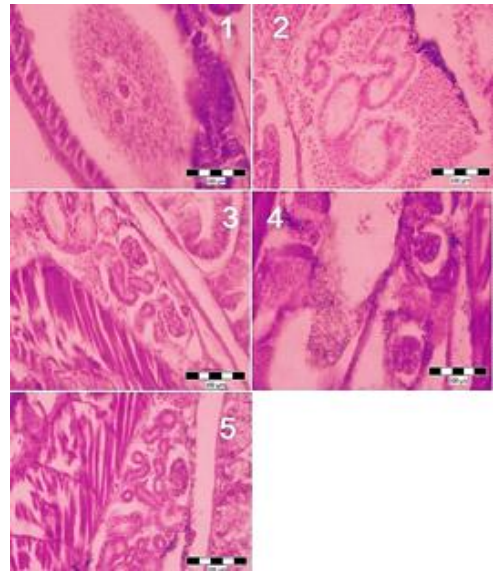


Fig. 5. The main changes in renal tissue 96 hours after the start of the study. **1 = Control group**. The normal image of the kidney per section (ob. × 40 H.E.) **2 = Group E1/400 ppm**. Hemorrhagic dilatation, the presence of erythrocytes in the capillaries of the renal tubules (ob. × 40 H.E.) **3 = Group E2 / 200 ppm**. Dilation of the Bowman's capsule with enlargement of the urinary space, non-hemorrhagic (ob. × 40 HE). **4 = Group E3 / 100 ppm**. Bowman's capsule dilation with moderately enlarged, nonhemorrhagic urinary space (ob. × 40 HE) **5 = Lot E4 / 50 ppm**. Bowman capsule, normal apparent appearance (ob. × 40 HE).

Skin samples

Figure 6 presents the main changes in the skin 96 hours after the start of the study. The images were taken only with the microscope objective of × 40/100 µm, at other lower resolutions, the cytohistological aspects being little observable.

Muscle and heart samples

Figure 7 are presenting the main changes in striated muscle and heart tissue 96 hours after the start of the study. The images were taken only with the microscope objective of × 40/100 µm, at other lower resolutions, the cytohistological aspects being little observable.

The methods for determining the effects of acute toxicity are intended to highlight changes in the anatomical and ethological development of the developmental stages, and in particular fish larvae (2, 6, 14, 17, 27, 29, 34, 38). Tests performed on fish larvae are chosen due to the easy availability of biological material, the advan-tages of the relatively low cost of materials needed to carry out the tests and especially the short period of performance, thus being able to perform a large number of repetitions of tests, increasing

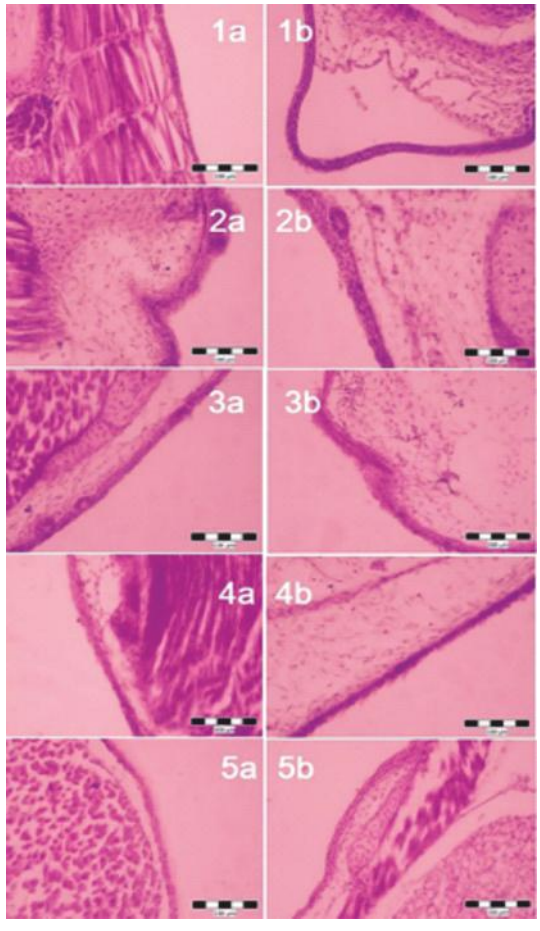


Fig. 6. The main changes in the skin 96 hours after the start of the study. **1a and 1b = Control group**. The normal cytohistological appearance of the sterlet's skin (ob. × 40 H.E.). **2a and 2b = Group E1/400 ppm**. Thickening of the epidermis with moderate to high epidermal stratification phenomena (ob.×40 H.E.). **3a and 3b = Lot E2/200 ppm.** Skin with localized phenomena of moderate epidermal thickening (ob. × 40 H.E.). **4a and 4b = Lot E3/100 ppm**. Normal appearance with slight epithelial desquamation of the superficial layer (ob. × 40 H.E.). **5a and 5b = Lot E4/50 ppm**. Normal appearance with very slight epithelial desquamation of the superficial layer (ob. × 40 H.E.)

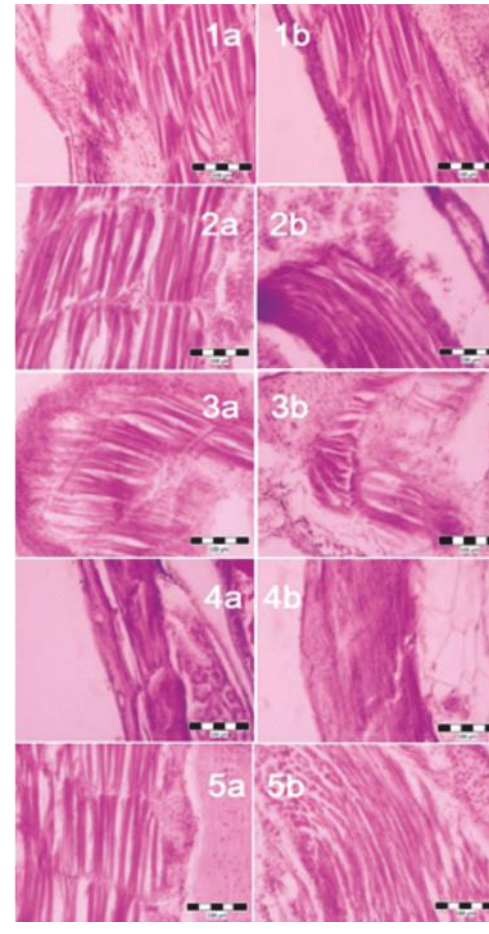


Fig. **7.** The main changes in striated muscle and heart tissue 96 hours after the start of the study. **1a and 1b=Control Group**. The normal cytohistological appearance of the striated muscle in sterlet larvae (ob. × 40 H.E.). **2a and 2b = Lot E1/400 ppm**. Striated muscle with thickened conjunctival tunic and capillary ectasia due to micro-hemorrhages (left) and cardiac muscle with massive pericardial hemorrhage (right) (ob. × 40 H.E.), **3a and 3b =Lot E2/200 ppm**. Muscle with conjunctival tunic illustrating capillary micro-ectasia due to micro-hemorrhages (left) (ob. × 40 H.E.) **4a and 4b = Lot E3/100 ppm**. Muscle with normal conjunctival tunic presenting erythrocytes and rare micro-ectasias (ob.×40 H.E.). **5a and 5b = Lot E4 / 50 ppm**. Ribbed muscle with an apparently normal connective tunic (ob. × 40 H.E.)

the reliability of statistical results, and interpretations (1, 9, 12, 15, 20, 27, 38). The extrapolation of methodologies to sturgeons is extremely important in the context of the dramatic decrease of these special resources in the idea of detecting sources of natural pollution (including the presence of lead in water) along the lower Danube, the habitat of this species (6, 13-16, 29, 30, 34, 35).As a research alternative, we agree with the authors that they evaluated that fish could be complementary to rodents or other laboratory mammals (especially in toxicological and drug research) and we appreciate that with the development of this in vitro model, researchers will be able to replace and provide significant results on water toxicity, which will be va-luable and applicable to vertebrate research (2, 3, 9, 10, 17, 19, 26, 31, 36). Based on this observation, we also noticed that sturgeons had characteristics suitable for temperate climatic conditions, making it an ideal candidate, as an alternative to zebrafish embryos and larvae, in some specific experiments (ecotox, embryo, pollution) from this climate zone (6). As a limitation, we point out that many of the pu-

blished aquatic toxicity experiments have the same shortcomings as the present study, chemical exposures are reported as limit concentrations. They do not have much more accurate and individual measurement responses, but even if the exposures studied in lead are not fully known (as individual fineness), this paper demonstrates that blind sturgeon can be credibly included in the list of suitable organisms for toxicity tests on juvenile forms (1, 3, 11, 12, 17, 19, 31, 32).

This study could also be considered as having strengths. For example, the presence of the mentioned behavioral and cytohistological changes (considered critical control points, practically identical to those found in zebrafish), identified for the early stages of sturgeons, support the results obtained by us and consider the sturgeons as a possible new species, fish that could be added to fish species already recognized and accepted internationally for acute environmental toxicology tests (2, 3, 29, 31). The use of juvenile forms of *Acipenser ruthenus* is beneficial because fish embryos and larvae are exempt from animal protection laws (8). According to European welfare regulations, this test is classified as an *in vitro* test and is therefore not subject to animal protection legislation which, in our view, can be easily extrapolated to any fish larva and can further develop studies on different species (8). The aspect of originality of this research is the use for the first time in Romania of *Acipenser ruthenus* larvae in an ecotoxicity test as an alternative extrapolation of the test method with zebrafish (*Danio rerio*) and the validation of primary results, in the light of OECD methodology.

CONCLUSIONS

Lead pollution through its potential ecological risk alters human and animal health. The detrimental effects of lead toxicity diluted in water were highlighted in the anatomical, ethological and cytoarchitecture changes identified in larvae. Although *Acipenser ruthenus* is not yet considered as standard in toxicology or pollution tests, we believe that this method could be developed as an alternative analysis. This species of fish is suitable as an assessment tool for ecological water pollution surveys. Larvae have the advantage of being used in an increased number of individuals, being preferred because studies on organisms in the embryonic / larval stage are not subject to current animal protection legislation. Significantly high statistical values were also found for the length and weight parameters of juvenile forms. Graphical assessment of larval lethality revealed a clear link between the number of dead embryos at the same concentration. The data obtained could be useful to determine the critical concentration of one or more contaminants that can cause considerable consequences and to update the information. Cytohistological changes as a result of exposure to lead acetate fully confirmed the morphopathological changes observed in living individuals exposed especially to high concentrations in the liver, intestines, and kidneys.

REFERENCES

1. *Ali S., Champagne D.L., Spaink H.P., Richardson M.K.,* (2011), Zebrafish embryos and larvae: a new generation of disease models and drug screens, *Birth Defects Res C Embryo Today*, 93(2):115-133
2. *ASTM (American Society for Testing and Materials),* (1988), ASTM E1241-05. Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. Available at: https://www.astm.org/Standards/E1241.htm (Accessed: 12.12.2020)
3. *Braunbeck T., Böettcher M., Hollert H., Kosmehl T., Lammer E., Leist E., Rudolf M., Seitz N.,* (2005), Towards an alternative for the acute fish LC_{50} test in chemical assessment: the fish embryo toxicity test goes multi-species - an update. *ALTEX,* 22:87-103
4. *Calfee R.D., Little E.E., Puglis H.J., Scott E., Brumbaugh W.G., Mebane C.A.,* (2014), Acute sensitivity of white sturgeon and rainbow trout (*Oncorhynchus mykiss*) to copper, cadmium, or zinc in water-only laboratory exposures. Environ Toxicol Chem*,* 33:2259-2272
5. *Chakraborty C., Hsu C.H., Wen Z.H., Lin C.S., Agoramoorthy G.,* (2009), Zebrafish: a complete animal model for *in vivo* drug discovery and development. Curr Drug Metab*,* 10:116-124
6. *Cristina R.T., Baroga M., Grozea A.,Mihailov S.A., Sirb N. M., Dumitrescu E., Muselin F.,* (2019), An early embryo model of sterlet (*Acipenser ruthenus*) for the assessment of lead effects. Appl Ecol Environ Res, 17(1):917-929
7. *De Oliveira R*., (2009), Zebrafish early life-stages and adults as a tool for ecotoxicology assessment. Disertation Biology Department, University of Aveiro, Portugal. Available at: https://ria.ua.pt/bitstream/10773/8838/1/6237.pdf (Accessed: 12.12.2020)
8. *Directive 2010/63/EU* of the European Parliament and of Council of 22.09.2010 on the Protection of Animals Used for Scientific Purposes. Available at:http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=oJ:L:2010:276:0033:0079:en:PDF (Accessed: 12.12.2020)
9. *Embry R.M., Belanger S.E., Braunbeck T.A., Galay-Burgos M., Halder M., Hinton D.E., Léonard M.A., Lillicrap A., Norberg-King T., Whale G*., (2010), The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aqua Toxicol*,* 97:79-87
10. *Hariharan G., Purvaja R., Rame R.,* (2016), Environmental safety level of lead (Pb) pertaining to toxic effects on grey mullet (*Mugil cephalus*) and Tiger perch (*Terapon jarbua*). Environ Toxicol*,* 31:24-43
11. *Hill A.J., Teraoka H., Heideman W., Peterson R.E.,* (2005), Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci*,* 86:6-19
12. *Lammer E, Carr G.J., Wendler K., Rawlings J.M., Belanger S.E., Braunbeck T.,* (2009), Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a po-

tential alternative for the fish acute toxicity test? Comp Biochem Physiol Part- C:Toxicol Pharmacol, 149:196-209
13. *Lazu D., Patriche N., Talpeș M., Cristea V., Metaxa I.,* (2008), Sturgeons from Romania, (Ed.) Excelsior Art, Timișoara, Romania (in Romanian)
14. *Linhartova P., Gazo I., Shaliutina-Kolesova A., Hulak M., Kaspar V.,* (2015), Effects of tetrabrombisphenol A on DNA integrity, oxidative stress, and sterlet spermatozoa quality variables. Environ Toxicol, 30:735
15. *Little E.E., Calfee R.D., Linder G.,* (2012), Toxicity of copper to early-life stage Kootenai river White sturgeon, Columbia river White sturgeon and Rainbow trout. Arch Environ Contam Toxicol, 63:400-408
16. *Little E.E., Calfee R.D., Linder, G.,* (2014), Toxicity of smelter slag-contaminated sediments from Upper Lake and associated metals to early life stage White sturgeon (*Acipenser transmontanus*). J Appl Ichtyol, 30:1497-1507
17. *Luckenbach T., Kilian M., Triebskorn R., Oberemm A.,* (2001), Fish early life stage tests as a tool to assess embryotoxic potentials in small streams. J aquat ecosyst stress recovery, 8:355-370
18. *McGrath P., Li C.Q.,* (2008), Zebrafish: a predictive model for assessing drug-induced toxicity. Drug Discov Today, 13:394-401
19. *McKim J.M.,* (1977), Evaluation of tests with early life stages of fish for predicting long-term toxicity. *J Fish Res Board Can,* 34:1148-1154
20. *Moșneang C.L., Dumitrescu E., Muselin F., Ciulan V., Grozea A., Cristina R.T.*, (2015), Use of zebra fish eggs as early indicators of aquatic environmental pollution. *Pol J Environ Stud,* 24:2079-2085
21. *Moșneang C.L., Grozea A., Dumitrescu E., Muselin F., Cristina R.T.* (2015). A correlation between two different species of fish embryos used in a freshwater qualitative pollution test. Rom Biotechnol Lett, 20:10352-10357
22. *Noel L., Chekri R., Millour S., Merlo M., Leblanc J.C., Guerin T.,* (2013), Distribution and relationships of As, Cd, Pb and Hg in freshwater fish from five French fishing areas. Chemosphere, 90:1900-1910
23. *OECD*, (2009), Fish, Acute Toxicity Test. Guidelines for the Testing of Chemicals, Section 2, Effects on Biotic Systems, Test No. 203. Adopted: 17 July 1992, Updated: 10 Dec. 2009. Available at: http://dx.doi.org/10.1787/9789264069961-en (Accessed: 20.05.2020)
24. *OECD*, (2013), Guidelines for the Testing of Chemicals Fish, Early-life Stage Toxicity - Fish, Early-life Stage Toxicity Test, Test No. 210, Adopted: 26 July 2013. Available at: http://dx.doi.org/10.1787/9789264203785-en (Accessed: 20.05.2020)
25. *OECD*, (2013), Fish Embryo Acute Toxicity (FET) Test, *Test No. 236*. Adopted: 26 July 2013. Available at: http://dx.doi.org/10.1787/9789264203709-en (Accessed: 20.05.2020).
26. *OMEWMR*, (2005), Methodology for assessing the risk of Schedule I and II hazardous substances and priority / priority hazardous substances in the aquatic environment by mathematical modeling and Methodology for assessing the impact of Schedule I and II hazardous substances and priority / priority hazardous substances on the aquatic environment by ecotoxicological tests - green algae, daphnia, fish. Order no *245,* 26/03/2005. Available at: http://lege5.ro/en/Gratuit/gy4tkmzz/metodologia-din-26032005-de-evaluare-a-impactului-substantelor-periculoase-din-listele-i-si-ii-si-al-substantelor-prioritare-prioritar-periculoase-asupra-mediului-acvatic-prin-teste-ecotoxicologice-al/3 (Accessed: 20.05.2020)
27. *Osman A.G., Wuertz S., Mekkawy I.A., Exner H.J., Kirschbaum F.,* (2007), Lead induced malformations in embryos of the African catfish *Clarias gariepinus* (Burchell, 1822). Environ Toxicol, 22:375-389
28. *PubChem, (2018), Open Chemistry Database*. SID 24864770: Lead (IV) acetate. Available at: https://pubchem.ncbi.nlm.nih.gov/substance/24864770#section=Top (Accessed:20.05.2020).
29. *Raskovic B., Poleksic V., Visnjic-Jeftic Z., Skoric S., Gacic Z., Djikanovic V., Jaric I., Lenhardt M.,* (2015), Use of histopathology and elemental accumulation in different organs of two benthophagous fish species as indicators of river pollution. Environ Toxicol, 30:1153-1161
30. *Reinartz R.,* (2017), Sturgeons in the Danube River Biology, Status, Conservation. Available at:http://www.dstf.eu/assets/Uploads/documents/Sturgeons-in-the-Danube-RiverReinartz2002.pdf (Accessed:20.05.2020)
31. *Schirmer K., Tanneberger K., Kramer N.I., Volker D., Hafner C., Lee L.E., Bols N.C., Hermens J.L.,* (2008), Developing a list of reference chemicals for testing alternatives to whole fish toxicity tests. Aquat Toxic, 90:128-137
32. *Scholz S., Fischer S., Gündel U., Küster E., Luckenbach T., Voelker D.,* (2008), The zebrafish embryo model in environmental risk assessment-applications beyond acute toxicity testing. Environ Sci Poll Res Int, 15:394-404
33. *Science Direct Topics*, (2015), Tricaine mesylate - an overview, Tricaine mesylate (MS-222). Available at: http://www.sciencedirect.com/topics/medicine-and-dentistry/tricaine-mesylate (Accessed: 20.05.2020).
34. *Subotic S., Spasic S., Visnjic-Jeftic Z., Hegedis A., Krpo-Cetkovic J., Mickovic B., Skorić S., Lenhardt M.,* (2013), Heavy metal and trace element bioaccumulation in target tissues of four edible fish species from the Danube River (Serbia). Ecotoxicol Environ Saf, 98:196-202
35. *Vardy D.W., Santore R.C., Ryan A.C., Giesy J.P., Hecker M.,* (2014), Acute toxicity of copper, lead, cadmium, and zinc to early life stages of white sturgeon (*Acipenser transmontanus*) in laboratory and Columbia River water. Environ Sci Pollut Res, 21:8176-8187
36. *Wang N., Ingersoll C.G., Dorman R.A., Brumbaugh W.G, Mebane C.A., Kunz J.L., Hardesty D.K.* (2014). Chronic sensitivity of White sturgeon (*Acipenser transmontanus*) and rainbow trout (*Oncorhynchus mykiss*) to cadmium, copper, lead, or zinc in laboratory water-only exposures. Environ Toxicol Chem, 33:2246-2258
37. *Waszak I., Dabrowska H.,* (2009), Persistent organic pollutants in two fish species of *Percidae* and sediment from the Sulejowski reservoir in central Poland. Chemosphere, 75:1135-1143
38. *Weber D.N.,* (1996), Lead-induced metabolic imbalances and feeding alterations in juvenile fathead minnows (*Pimephales promelas*). Environ Toxicol, 11:45-51.