

## CONTRIBUTIONS TO THE STUDY ON THE EFFECTS OF STOMP 330 EC HERBICIDE UPON THE *MARSH FROG*

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### Abstract

Pesticides are the best examples of risky compounds because they spread all over the environment. The experiments were performed to determine the effect of the Stomp 330EC herbicide at concentrations of  $0.5 \times 10^3$ ,  $1 \times 10^3$ ,  $2 \times 10^3$  and respectively  $4 \times 10^3$  ml of herbicide/l of water on *Pelophylax ridibundus* tadpoles and adults. The herbicide produces delay and reduction of hatching (at the concentration of 0.004 ml/l), slowing of growth in volume and length as well as decreased oxygen consumption and survival of the tadpoles. The frequency of buco-pharyngeal movements in adults of marsh frog is influenced by the Stomp® 330 EC herbicide at a concentration of 0.002 and 0.004 ml/l of water. After 14 days of exposure to the herbicide, the average number of erythrocytes increases, the average number of leukocytes decreases and the level of glucose changes.

Keywords: erythrocytes, glycaemia, length, volume, marsh frog, tadpoles, oxygen consumption, Stomp 330 EC.

### 1. INTRODUCTION

Amphibians are good bioindicators of environmental quality, being affected both by changes in the aquatic ecosystem but also by those occurring at terrestrial ecosystems (Chovanec and Grillitsch, 1994). According to a study conducted by IUCN, 2010, amphibians represent the most endangered vertebrate group, with a 41% risk of extinction: 43% of amphibian species are declining, 32.5% are endangered globally, 34 species disappeared, and another 88 species are in danger of extinction.

The marsh frog - *Pelophylax ridibundus* (Pallas, 1771) is the largest species of frogs in Europe, being the most widespread of the anuras in Romania, representing 92% of the potentially edible; according to the Habitats Directive (92/43 / EEC), three species of frogs (*Rana esculenta*, *R. ridibunda* and *R. temporaria*) can be exploited economically but only on the basis of impact studies (Cogălniceanu, 2002).

The contamination of the aquatic environment with different chemicals is associated, among other things, with the decline of numerous populations of non-target organisms (Davidson et al., 2002, IUCN, 2004, Stuart et al., 2004, Davidson, 2004). Pesticides have attracted the attention of researchers as factors that have led to declining amphibians, acting alone or combined with other stress factors (Relyea and Mills, 2001; Relyea, 2004; Relyea, 2005; Relyea et al., 2005; Brühl et al., 2013; Wagner et al., 2014 ). The relatively low number of specialty studies on herbicide toxicity to amphibians may be due, at least in part, to the fact that the main components of these products

are not considered to be typically toxic to these animals, toxicity being due in many cases to adjuvants (Perkins et al., 2000; Williams and Semlitsch, 2009; Moore et al., 2012; Mullin et al., 2016).

Stomp herbicide® 330 EC (pendimethalin active substance) is highly toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment (EPA, 1987). Pendimethalin is a dinitroaniline herbicide used for the selective control of most annual grasses and many annual broad-leaved weeds in several crops (Tomlin, 1994; Engebretson et al., 2001). The pendimethalin compound is highly toxic to fish and aquatic invertebrates; the reported 96-hour LC50 for pendimethalin in bluegill sunfish is 199 ug/L, in rainbow trout is 138 ug/L, and in channel catfish is 420 ug/L (EPA, 1987; Weed Science Society of America, 1994).

The purpose of this paper is to complete the studies on the negative impact of the pendimethalin herbicide on the species *Pelophylax ridibundus*, both on mature specimens as well as on the hatching process and the tadpoles. In addition to aspects of hatching and growth of tadpoles, survival, a range of physiological parameters (oxygen consumption with tadpoles, the frequency of buco-pharyngeal movements in adults), hematological (number of erythrocytes and leukocytes) and biochemical (blood glucose and cholesterol).

## 2. MATERIALS AND METHODS

The experiments were conducted with a permission for animal testing from the of the local Committee of Bioethics according to the Romanian law 205/2004 art.7, 18, 22 and regulation number 143/400/2002 for care and use of animals for research purposes. Marsh frog adults and spawn were taken from Lacul Gării, Schitu Golești place, Argeș County. Sampling took place at the end of March 2016 in glass containers that were transported in isothermal packaging to avoid thermal and mechanical shocks. Adults were captured in April-May 2016; after capture, the animals were kept in clean aquaterrariums with water changed daily, to avoid the accumulation of toxins. Laboratory accommodation for adult frogs lasted 10 days.

The concentrations used were determined after investigations of the relevant literature works and taking into account the herbicide concentrations that can be accidentally produced in water. Three experimental variants were installed.

Variant I comprises 5 experimental lots (one control lot and 4 lots exposed to the herbicide at concentrations of 0.0005, 0.001, 0.002 and 0.004 ml/l respectively), corresponding to pendimethalin concentrations of 0.165, 0.33, 0.66 and 1.32 mg/ l respectively) made on spawn of *P. ridibundus* (30 eggs/ lot); the eggs were placed in aquariums with dechlorinated water, aerated with air pumps, at 18-20°C to hatching. For each of the five lots, the number of hatched tadpoles was recorded daily (these were subsequently removed and kept in dechlorinated tap water). The duration of the experiment was 9 days until the last tadpole appeared. Hatched larvae (tadpoles) were fed "*ad libitum*" during their evolution with boiled and minced salad.

Variant II comprises the experiments performed on tadpoles, three weeks from hatching (under laboratory conditions, without herbicide exposure) consisting of 5 experimental lots of 20 tadpoles each (every lot has 2 subplot of 10 tadpoles in jars with the capacity of 800 ml); lots II.1-II.4 being exposed to the same herbicide concentrations as in the previous variant and II.5 lot being free of herbicide added. Within this experimental variant, the following determinations were made:

- ✓ the total volume and length of the body of the tadpoles; of the measurements currently used in experiments on tadpoles (Relyea, 2001) we determined the total length of the body (for a traumatization as small as possible);
- ✓ oxygen consumption every 24, 48, 72, 96, 144 and 336 hours (according to the Winkler method, adapted after Picoş and Năstăsescu, 1988).

Variant III includes the experiments performed on adults by *Pelophylax ridibundus*, with an average weight of  $32 \pm 1.75$  g. It consists of five lots of 7 frogs each - the first four lots being exposed to the Stomp® 330 EC herbicide and a control lot with frogs kept in tap water. Within this variant, the frequency of buco-pharyngeal movements was determined in dynamics (at 24, 24, 48, 72, 96, 144 and 366 hours). At the end of the experiment (after two weeks), the frogs were sacrificed (after a previous anesthesia with ether) to make the blood intakes (from the heart, according to the method described by Picoş and Năstăsescu, 1988) necessary so as to determine the number of erythrocytes and leukocytes (at the optical microscope with Thoma counting chambers), blood glucose and cholesterol levels (with the device Accutrend® GCT).

All tests were semi-static, by "refreshing" the solution at 24 hours. For all experimental lots, survival was monitored daily, the dead specimens being periodically removed. Values are given as arithmetic means  $\pm$  standard error of the mean (SEM). The data was statistically analyzed using multiple comparison tests (LSD - SPSS / PC program version 10.0 for Windows).

### 3. RESULTS AND DISCUSSIONS

The results obtained in the first experimental variant, in which we followed the percentage and time required to hatch the *Pelophylax ridibundus* in the case of exposure to different concentrations of Stomp® 330 EC are given in table 1.

*Table 1. The influence of Stomp herbicide® 330 EC in different concentrations on the percentage of hatching with Pelophylax ridibundus*

Herbicide concentration (ml/l water)	Percentage of hatching (days)									
	1	2	3	4	5	6	7	8	9	
W (lot de control)	0	0	6.66	33.33	59.94	86.58	86.58	86.58	86.58	86.58
0.0005	0	0	3.33	36.63	59.94	83.25	83.25	86.58	86.58	86.58
0.001	0	0	0	49.95	63.27	79.92	83.25	83.25	83.25	83.25
0.002	0	0	6.66	33.33	49.95	63.27	73.26	73.26	73.26	83.25
0.004	0	0	0	6.66	26.64	33.33	56.61	63.27	63.27	66.6

A significant decrease in the percentage of hatching eggs occurred only at the concentration of 0.004 ml herbicide/l water, concentration at which a hatching delay was observed. A decreased

hatching success was also observed for eggs of a European frog species (*Rana arvalis*) that were exposed to the insecticide  $\alpha$ -cypermethrin (Greulich and Pflugmacher, 2003).

Survival of tadpoles and adults *Pelophylax ridibundus* in the case of exposure to the Stomp herbicide<sup>®</sup> 330 EC is presented in table 2.

**Table 2 Survival of *Pelophylax ridibundus* tadpoles and adults intoxicated with Stomp<sup>®</sup> 330 EC**

Experimental lots		Percentage of survival						
		Start point	24h	48h	72h	96h	168h	336h
<i>P. ridibundus</i> tadpoles	Control	100	100	100	100	100	100	95
	0.0005 ml/l	100	100	100	100	100	100	95
	0.001 ml/l	100	100	100	100	90	90	90
	0.002 ml/l	100	100	100	100	90	75	70
	0.004 ml/l	100	100	90	80	75	60	40
<i>P. ridibundus</i> adults	Control	100	100	100	100	100	100	100
	0.0005 ml/l	100	100	100	100	100	100	100
	0.001 ml/l	100	100	100	100	100	100	100
	0.002 ml/l	100	100	100	100	85.72	85.72	85.72
	0.004 ml/l	100	100	100	85.72	85.72	71.44	71.44

Comparing the survival curves of the tadpoles and adults shows a more pronounced toxicity of the herbicide stomp<sup>®</sup> 330 EC to tadpoles of *Pelophylax ridibundus*. In a study conducted on tadpoles of *Lithobates clamitans* regarding the toxic effects of several pesticides, including Prowl 400 EC (which has pendimethalin as active substance), Weir and Salice (2012) reported LC 50 96 hours of 2.47 mg pendimethalin / l water; at a concentration of 2 mg pendimethalin / l water the authors recorded a mortality of 5% after 96 hours of exposure. In our experiences, a 30% mortality was observed after 96 hours of exposure to an equivalent of Stomp concentration in pendimethalin of 1.32 mg/l. This difference suggests either a higher sensitivity of the mumps *Pelophylax ridibundus* to pendimethalin, or a different formulation of the two herbicides.

Stomp herbicide<sup>®</sup> 330 EC in concentrations of 0.0005 and 0.001 ml/ l water, did not have significant effects on the growth in volume of *Pelophylax ridibundus* tadpoles during the two weeks of exposure (figure 1). However, there is a significant reduction in volume growth at concentrations of 0.002 and 0.004 ml/l of water. The effect is less visible at the end of the acute test when the reduction of this index is 7.32% compared to the control group at both the 0.002 herbicide concentration and at 0.004 ml/l water. After 7 days of contact with the pesticide, differences in the volume increase of the tadpoles between the last two tested concentrations are more obvious - the average volume on the tadpoles at the concentration of 0.002 ml herbicide/l water is 74.19% of the value determined in the group control, while double this concentration produced a decrease in volume increase to 61.29% compared to the control group. The results determined after 14 days of contact with the herbicide did not show significant changes compared to those determined at 7 days. After 7 days of herbicide intoxication at concentrations of 0.002 and 0.004 ml/l, there was a 16% growth in length compared to the control group but without any significant differences between these concentrations (figure 2).

Inhibition of growth in length of tadpoles due to exposure to Stomp 330<sup>®</sup> EC is maintained over the next 7 days of treatment, in which case the results are significantly different between both

concentrations and those observed at 7 days post exposure (total mean body length of the tadpoles being 25% lower compared to the control at concentration of 0.002 ml herbicide/l water and 32.21% less than the control at the concentration of 0.004 ml herbicide/l water).

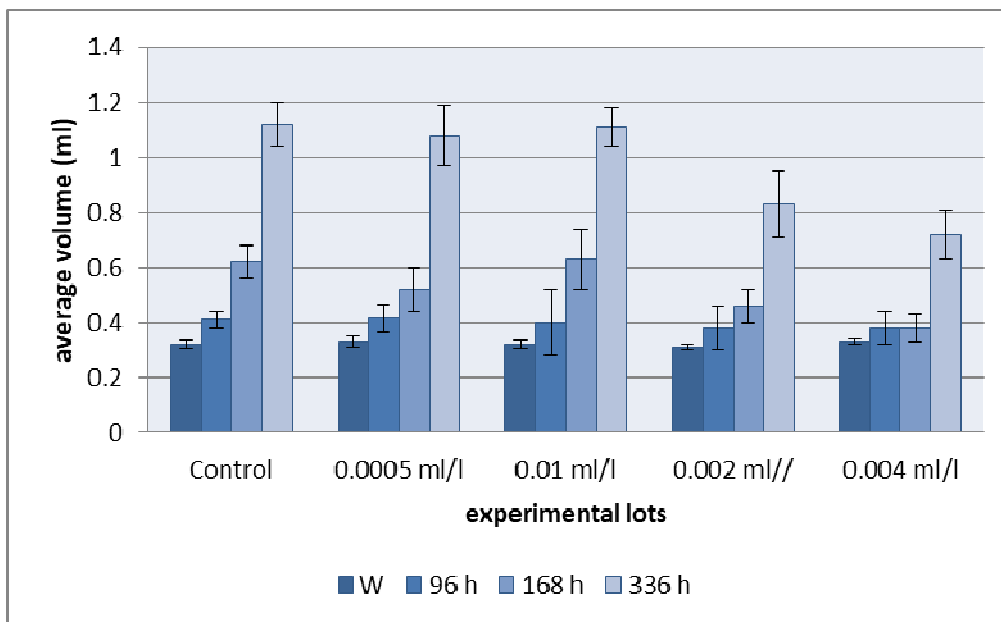


Figure 1. The variations in the volume of the tadpoles exposed to Stomp 330 EC in different concentrations and standard deviations

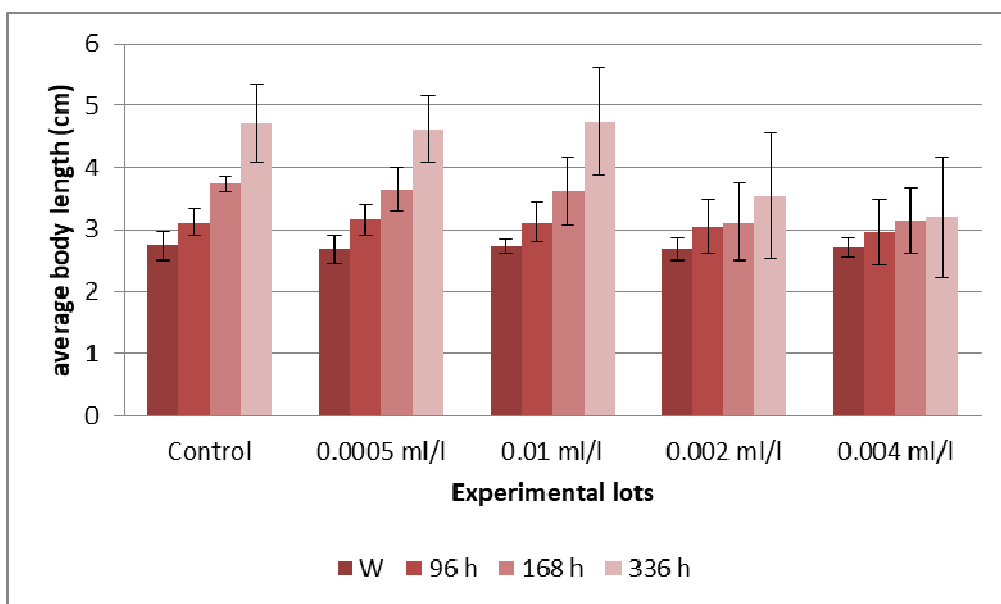
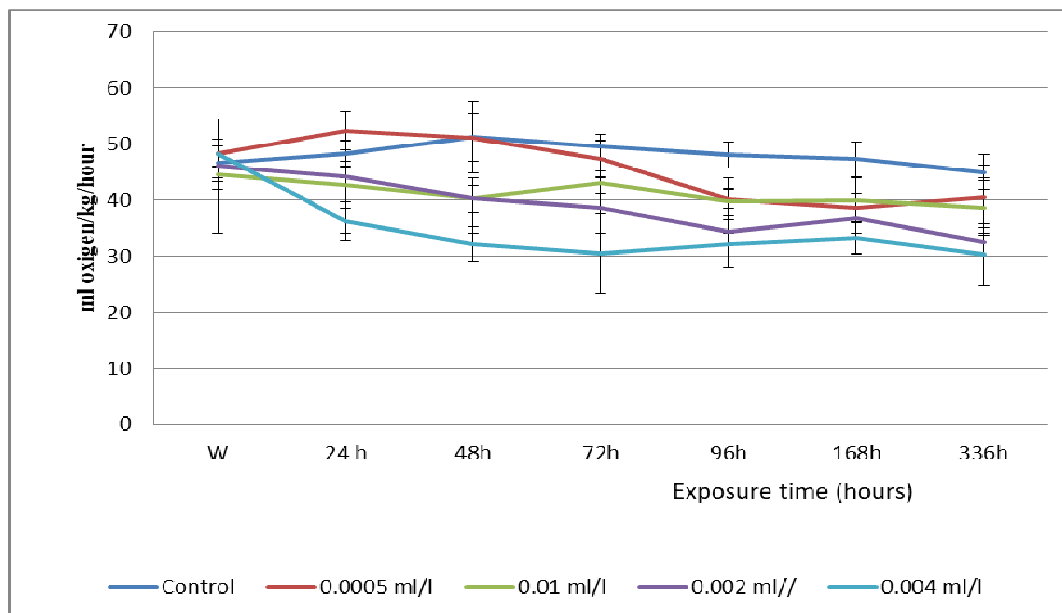


Figure 2. Average body length of tadpoles and standard deviation after exposure on Stomp® 330 EC herbicide

The results obtained, both in terms of volume and long growth in length, are in line with the data in specialty literature indicating a decrease in the body weight of animals exposed to sublethal doses

of pendimethalin (USEPA, 1997; El-Sharkawy et al., 2011). Lanctot et al. (2014) found that the length of the tadpoles decreased when exposed to glyphosate-based herbicide formulations.

Dynamic variations in average oxygen consumption in tadpoles intoxicated with Stomp® 330 EC in different concentrations are shown in figure 3.



**Figure 3. Variations in oxygen consumption and standard deviation in *Pelophylax ridibundus* tadpoles intoxicated with Stomp® 330 EC**

The evolution of oxygen consumption in tadpoles follows broadly the same pattern as the above-mentioned parameters except that the first two herbicide concentrations, 96 hours after exposure, significantly inhibited the oxygen consumption of the tadpoles (the values determined at the end of the experiment being 15.94 and 13.45% respectively lower than those recorded before the introduction of the tadpoles into toxic solutions).

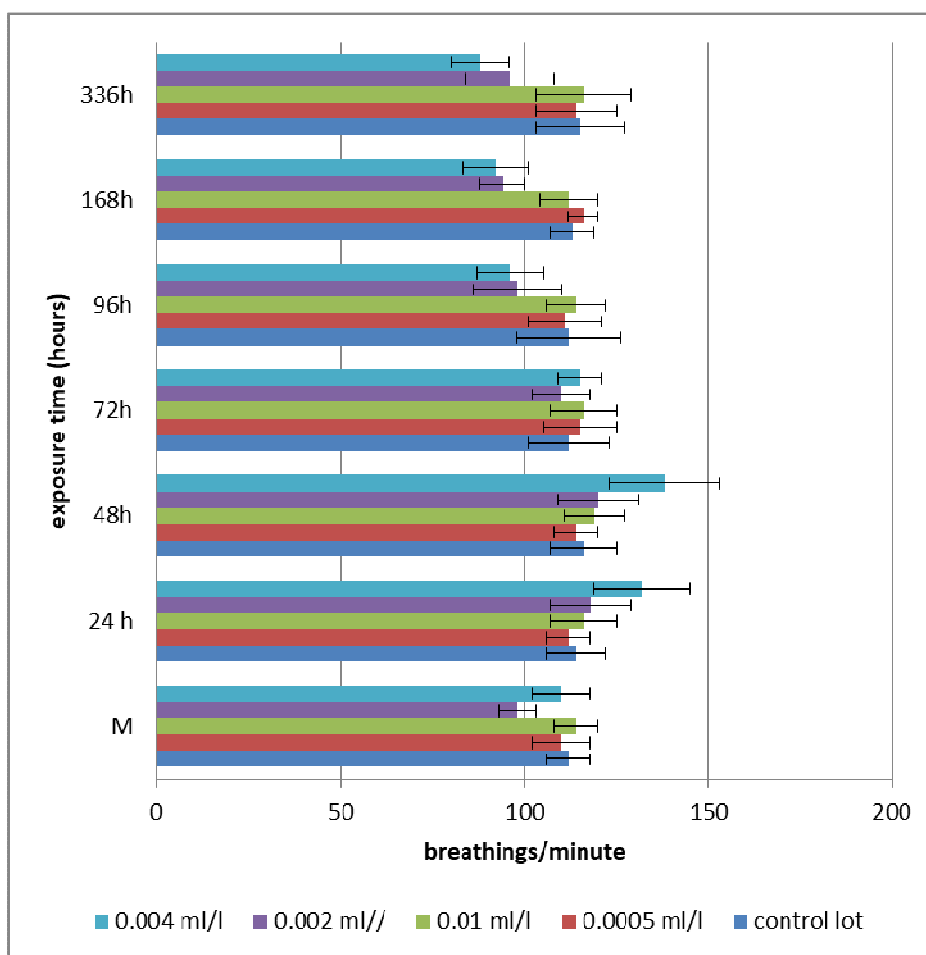
Reducing oxygen consumption as a result of exposure to the pendimethalin herbicide (commercial product Pendigan 330 EC) was also found by Păunescu et al. (2016) in the case of *Leuciscus cephalus* exposed to a concentration of 0.004 ml/l Pendigan for four weeks. USEPA, 1997 also reported as the effect of chronic and sub-chronic poisoning with pendimethalin in rats a decrease in tissue oxygen consumption.

No significant changes in the frequency of buco-pharyngeal movements occurred at the first two concentrations of Stomp® 330 EC studied (0.005 and 0.001 ml/l water), although slight stimulation thereof occurs at the concentration of 0.0005 ml/l even in the first hours after exposure (figure 4). The first significant changes in respiratory rate occur even from the first 24 hours of exposure at 0.002 and 0.004 herbicide/l water respectively (about 20% more than the mean values determined prior to exposure to toxic), increase which is maintained for 2-3 days (the highest increase was 25.45% compared to the control, 48 hours after the introduction of frogs in the herbicide at the concentration of 0.004 ml/l water).

Reduction of the respiratory rate as a result of exposure to the pendimethalin herbicide was also reported by Paunescu et al. (2016) at species *Leuciscus cephalus* exposed to a concentration of

0.004 ml/l Pendigan for four weeks, the authors also not having found a stimulating effect in a first phase.

Table 3 shows the mean values of hematological and biochemical parameters investigated in *Pelophylax ridibundus* adults exposed for 14 days at different concentrations of Stomp® 330 EC. The mean values of erythrocytes and leukocytes determined within the control group fall within the variation range specified for *Pelophylax ridibundus* by Schemer, 1967, quoted from Ciudin, 2004. The average number of erythrocytes after 14 days of intoxication with the herbicide Stomp® 330 EC shows significant changes only at concentrations of 0.002 and 0.004 ml/l water. The increase was 28.57% and 21.42%, respectively, compared to the control group at the two mentioned concentrations. Regarding the average number of leukocytes, the effect of the herbicide was the opposite, starting with the concentration of 0.001 ml of herbicide/l of water. The decreases in this index after 14 days of exposure of the frogs to herbicide were 7.31, 14.43 and 17.7% respectively compared to the control group at concentrations of 0.001, 0.002 and 0.004 ml/l of water.



**Figure 6. Average respiratory rhythm and standard deviation in *Pelophylax ridibundus* adults poisoned with Stomp® 330 EC**



**Table 3 Hematological and biochemical parameters in *Pelophylax ridibundus* adults after two weeks of exposure to the Stomp® 330 EC herbicide**

Experimental lots		Haematological parameters		Biochemical parameters (mg/dl blood)	
		erythrocytes/ml blood	leukocytes/ml blood	glucose	cholesterol
<i>Control lot</i>		420.000	5.200	42	112
<i>Lots treated with Stomp® 330 EC</i>	0.0005 ml/l	428000	5.250	44	102
	0.001 ml/l	435000	4.820*	48	108
	0.002 ml/l	540.000*	4.450*	68*	110
	0.004 ml/l	510.000*	4.280*	84*	122

\* significant for the variation threshold  $p < 0.05$  compared to the control group

The increase in the number of erythrocytes and the leucocyte decrease were also noted by Paunescu et al., 2016 at species *Leuciscus cephalus* exposed to Pendigan, a herbicide based on pendimethalin. Of the two biochemical parameters determined (glycaemia and cholesterol), only glycaemia showed significant changes as a result of the exposure of frogs to herbicide at concentrations of 0.002 and 0.004 ml/l of water.

Significant increase of glycaemia in *Leuciscus cephalus* exposed to Pendigan 330 EC in a concentration of 0.0004 ml / l water was reported by Păunescu et al., 2016. The 1997 USEPA report on the toxic effects of pendimethalin also contains a series of studies, especially in mammals, where the herbicide also resulted in an increase of glycaemia. Changes in the mean number of erythrocytes and leukocytes, glycaemia and cholesterol levels have been reported by Păunescu et al. (2011) at *Pelophylax ridibundus* in poisoning with the Roundup herbicide®.

El-Sharkawy et al. (2011) reported a significant decrease in body weight, an increase in blood glucose and cholesterol at *Oreochromis niloticus* exposed to Stomp® at a concentration of 0.355 mg / l and 0.177 mg / l - about 10% and 5% of the LC50 - 96 hours for this species.

#### 4. CONCLUSIONS

The Stomp® 330 EC herbicide produces a delay in *Pelophylax ridibundus* spawn hatching, and at the concentration of 0.004 ml/l water, it also produces a significant reduction in hatching. Lethal effects as a result of exposure to the Stomp® 330 EC herbicide were noted at the concentration of 0.004 ml herbicide/l water, the effect being much more pronounced in tadpoles. Growth in volume and length of the *Pelophylax ridibundus* tadpoles is not significantly influenced by the investigated herbicide at concentrations of 0.0005 and 0.001 ml/l water during the experiment. The



concentrations of 0.002 and 0.004 herbicide/l of water produce a slowing in volume growth starting on the 4<sup>th</sup> day after exposure and a slowing of growth in length on the 7<sup>th</sup> day of exposure.

The Stomp® 330 EC herbicide produces a decrease in the oxygen consumption of *Pelophylax ridibundus* tadpoles in all concentrations investigated, the effect of the concentration of 0.004 ml/l of water being stronger and occurring after a shorter exposure time. Frequency of bucco-pharyngeal movements in *Pelophylax ridibundus* adults is influenced by the Stomp® 330 EC herbicide at a concentration of 0.002 and 0.004 ml/l of water, with an increase in this physiological index from the first hours after frog immersion in the toxic solutions to the end of the acute test (96 hours) followed by the decrease of this index below the values determined at the control for the concentration of 0.004 ml/l of water.

The hematological chart indicates an increase in mean erythrocyte count at concentrations of 0.002 and 0.004 ml/l water and a decrease in mean leucocyte count at 0.001, 0.002 and 0.004 ml/l in the poisoned frogs for 14 days with Stomp® 330 EC/l water. The response of the adult frog body to Stomp® 330 EC herbicide aggression is also highlighted by changes in plasma glucose levels at concentrations of 0.002 and 0.004 ml/l water. The mean cholesterol values did not significantly change after the two weeks exposure to the herbicide in all four investigated concentrations.

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