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RESEARCH ARTICLE

Effects of glyphosate on early life stages: *comparison* between *Cyprinus carpio* and *Danio rerio*

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Abstract

Glyphosate (*N*-(phosphonomethyl)glycine) is an active substance of many herbicides. According to literature studies, glyphosate residues and their metabolites have been commonly detected in surface waters and toxicological reports confirmed negative effects on living organisms. In this study, the acute embryo toxicity of glyphosate into two different fish species—common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*)—was investigated. Lethal endpoints, development disorder, and, in addition, other sublethal endpoints such as hatching rate, formation of somites, and development of eyes, spontaneous movement, heartbeat/blood circulation, pigmentation, and edema were recorded to indicate the mode of action of the toxic compound. Hatching retardation ($p < 0.05$) was observed in experimental groups of common carp exposed to glyphosate with significant statistical difference especially at the highest concentration after 72, 96, and 120 hpf. The significantly highest cumulative mortality at concentration of 50 mg/l was observed. In contrast, hatching stimulation was observed in embryos of zebrafish exposed to the highest concentration of glyphosate. The significantly highest cumulative mortality for zebrafish was observed only at concentration of 50 mg/l. Based on our results, early life stages of common carp are more sensitive in comparison to zebrafish to the toxic action of glyphosate.

Keywords Fish · Embryo toxicity tests · Mortality · Hatching · Malformation · Herbicide · Glyphosate

Introduction

Land mistreatment over the last few decades, and especially lakes, rivers, and shores, is putting the aquatic habitats under hard test. The responsible reasons for the poor quality of surface waters are the lack or inadequate treatment of municipal wastewater which discharges into river organic materials (Faggio et al. 2016; Pagano et al. 2016) and wastewater from industrial activities that reverse also their chemical products (Aliko et al. 2015; Burgos-Aceves and Faggio 2017; Pagano et al. 2017; Savorelli et al. 2016; Torre et al. 2013). However, the major impact on poor water quality is represented by fertilizers and agricultural pesticides, which during rainfall events reaches the shores, from the fields to the lakes and rivers (Botta et al. 2009). The widespread use and public debate about the use of these herbicides have aroused social concern and a scientific controversy about their environmental toxicity (Tarazona et al. 2017).

Glyphosate (*N*-(phosphonomethyl)glycine) is the active substance of the widely used non-selective, broad-spectrum, systematic herbicide (Bai and Ogbourne 2016; Nešković et al. 1996). Glyphosate-based formulations are applied directly on the leaves and then translocated through the plants. The herbicidal activity of glyphosate constrains plant growth through inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme in the biosynthesis of aromatic amino acids such us phenylalanine, tyrosine, and tryptophan biosynthesis (Piešova 2005). The annual global production has been estimated to be about one million t annually, and there are few indications of reduced use (Cuhra et al. 2016). This herbicide

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is one of the main water contaminants, and its use has rapidly increased following the development of cultivations genetically resistant to the substance. Glyphosate is used on woody and herbaceous crops, but also on areas not used for agriculture, such as industrial, civil, in embankments, and roadsides (Paris et al. 2016). The most widespread glyphosate-based formulations on the market are composed of its isopropylamine salt, a surfactant (commonly polyethoxylated tallow amine) and water (Saunders and Pezeshki 2015). The glyphosate binds strongly to the ground where it undergoes microbial degradation with production of its main metabolite, aminomethylphosphonic acid (AMPA), which has a biological activity of potency comparable to the parental compound. Therefore, despite the degradation of glyphosate, the toxic effects on target organisms extend over time (Paris et al. 2016).

According to literature, glyphosate residues have been commonly detected in surface and ground water (Jofré et al. 2014; Samanta et al. 2014). The maximum contaminant limit (MCL) for glyphosate in the European Union for drinking water is 0.1 μg/l and an environmental quality standards (EQS) of 28 μg/l. However, in many cases, the detected concentrations of glyphosate exceed this value in concentrations up to 86 μg/l (Friends of the Earth Europe 2013). Paris et al. (2016) during the ISPRA biomonitoring of pesticides in the water indicated concentrations for glyphosate and AMPA of 5.2 and 26.8 μg/l, respectively. They also reported that among the herbicides is the largest number of cases of exceeding the EQS limits with percentages of 25.2, 52.2, and for MCL of 1.1 and 2.3%. The main sources of water contamination by glyphosate and AMPA are represented by runoff or accidental spills (Bai and Ogbourne 2016).

Many scientific studies confirmed that fish are a good model to evaluate the toxicity in aquatic system due to their ability to metabolize xenobiotics, their sensitivity to pollutants (Bartoskova et al. 2013; Chromcova et al. 2015; Fazio et al. 2014a; Lauriano et al. 2016), and the position into the aquatic food chain (Fazio et al. 2014b). The use of fish embryos is considered a viable alternative that allows to evaluate the toxicity, the way we act, and teratogenic effects in the aquatic environment (Sehonova et al. 2016), because these development stages are not legislatively protected from the Directive 2010/63/EU. The advantage in working with embryos is that they allow simultaneous screening of many pollutants, because they can be manipulated and exposed in multiwall plates, thereby reducing costs and time as well (Glaberman et al. 2017).

Moreover, ecotoxicological testing with zebrafish embryos has been used to clarify the sublethal effects (developmental or behavioral abnormalities) of xenobiotics in fish (Sehonova et al., 2017a, b). Several morphological abnormalities such as edema, body curvature, and absence of swim bladder inflation are common sublethal effects of xenobiotics that are induced at lower concentrations than those that cause lethal effects, and these morphological abnormalities can lead to delayed development or mortality (Horie et al. 2017). Furthermore, common carp embryos were already used for toxicology studies to understand the effects of other pesticides on the developmental stages of aquatic animals. Many indices were monitored during the tests such us hatching rate, several morphological abnormalities, yolk sac absorption, growth rate, early ontogeny, and mortality (Köprücü and Aydın 2004; Velisek et al. 2015). The increase in agricultural areas, with glyphosate-resistant crops, makes necessary to assess its impacts on non-target organisms (Zanuncio et al. 2018).

Therefore, it is important to understand not only the lethal but also the sublethal effects of xenobiotics in aquatic organisms. The aim of this study was to assess the acute embryo toxicity of glyphosate and to compare the way of action and the effects on the development of this compound into two different fish species—common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*).

Materials and methods

Experimental design

The embryonal toxicity test was performed through the modified method of Fish Embryo Acute Toxicity (FET) Test inspired by the OECD guideline 236 (OECD 2013). Two different types of fish species were used as model organisms—common carp (*C. carpio*) and zebrafish (*D. rerio*). Certified eggs were obtained from the Mendel University in Brno, Czech Republic.

The eggs were visually selected using binocular microscope, and only fertilized eggs that showed no obvious irregularities during cleavage were selected for the test. For both fish species, fertilized eggs, at the latest at the 16-cell stage, were exposed to glyphosate (Sigma-Aldrich, Milan, Italy; chemical purity ≥ 99%) in concentrations of 0.005 (environmental concentration); 0.05; 5; 10 and 50 mg/l for 120 h. A pesticide stock solution was prepared in dilution water. Fertilized eggs were distributed on microwell plates with 48 wells on each plate. Twenty-four eggs were used for each experimental concentration and control as well. The control group was exposed only to dilution water. The test was performed in duplicate. Dilution water was prepared according to ISO 7346 (ISO 1996). The pesticide solutions and dilution water in the control group were renewed daily by gently draining each chamber and adding new solution slowly to avoid disturbing embryos, and the temperature was maintained at 26 ± 1 °C. The daily photoperiod consisted on 12 h of light and 12 h of darkness.

Embryos were daily observed for mortality, hatching rate, and occurrence of lethal and sublethal endpoints. The

guidelines for the determination of lethality were recorded in accordance with the OECD 236 (OECD 2013). This observation included coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat. In addition, other sublethal endpoints such as hatching rate, formation of somites, and development of eyes, spontaneous movement, heartbeat/blood circulation, pigmentation, and edema were recorded to indicate the mode of action of the compound. Screening for developmental disorders indicating teratogenic effects of glyphosate was performed according to Nagel (2002) and included modified structure of the chorda, malformations of the head, otoliths, tail and heart, scoliosis, and deformity of yolk.

Statistical analysis

Statistical analysis was performed using the Unistat 5.6 for Excel statistical software (Czech Republic). Data on mortality, malformations, and hatching rate were tested using 2 × 2 contingency tables (χ^2 test). Differences between the control and the tested groups of the same fish species were considered significant at $p < 0.05$.

Results

Fish embryo acute toxicity test on *Cyprinus carpio*

The mortality of carp embryos was recorded at 48, 72, 96, and 120 h post fertilization (hpf), and the results of cumulative mortality are shown in Fig. 1. Statistical analysis was calculated between the control and the experimental groups at the same time. Cumulative mortality did not exceed 10% in the control group. In common carp exposed to glyphosate, we observed an increase in cumulative mortality that raises with the concentration and the time of exposure, but significant difference was found after 48 hpf only at 10 mg/l; after 96 hpf at 5, 10, and 50 mg/l; after 120 hpf at 0.05, 5, 10, and 50 mg/l compared to the control. At the end of the test, the significantly highest cumulative mortality was observed at concentrations of 50 mg/l and it reached up to 54.1%.

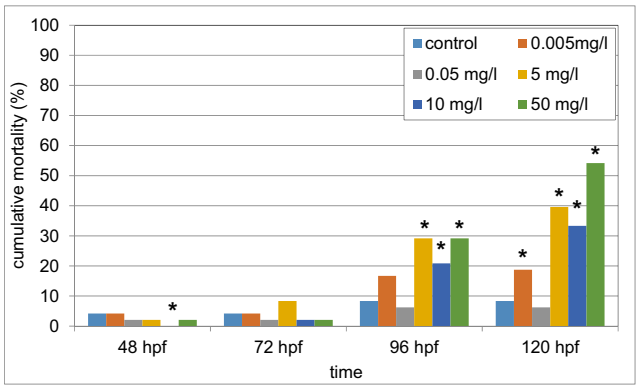

Fig. 1 Cumulative mortality of *C. carpio* embryos exposed to glyphosate (hpf hours post fertilization). Asterisk indicates significant difference ($p < 0.05$) between control and tested groups of the same time of exposure

Table 1 Hatching rate of *C. carpio* embryos exposed to glyphosate (hours post fertilization (hpf)). Asterisk indicates significant difference ($p < 0.01$) between the control and the tested groups

Group	Hatching rate (in %)		
	72 hpf	96 hpf	120 hpf
Control	47.9	72.9	89.6
0.005 mg/l	47.9	81.3	83.3
0.05 mg/l	77.1*	85.4*	100.0*
5 mg/l	35.4	62.5	62.5*
10 mg/l	2.1*	72.9	75.0*
50 mg/l	10.4*	56.3*	60.4*

Results of hatching rate are shown in Table 1. Hatching began after 72 hpf in the control and experimental groups as well. In common carp exposed to glyphosate, we observed a hatching retardation ($p < 0.05$); especially, the results were significant in the highest concentrations of glyphosate at 72 hpf (10 and 50 mg/l), 96 hpf (only 50 mg/l), and 120 hpf

Table 2 Occurrence of malformations in the control and tested groups of *C. carpio* embryos exposed to glyphosate (hours post fertilization (hpf)). Only surviving embryos were used for the calculation of the malformation rate

Time (hpf)	Malformation type	Group	Malformation rate (in %)
48	Yolk sac edema	Control	8.3
48	Yolk sac edema	0.005 mg/l	18.8
48	Pericardial edema	0.005 mg/l	6.3
96	Hematoma	0.005 mg/l	12.5
120	Late development	0.005 mg/l	58.3
48	Yolk sac edema	0.05 mg/l	6.3
48	Pericardial edema	0.05 mg/l	18.8
96	Hematoma	0.05 mg/l	22.9
120	Late development	0.05 mg/l	72.7
48	Yolk sac edema	5 mg/l	43.8
48	Pericardial edema	5 mg/l	14.6
96	Hematoma	5 mg/l	8.3
120	Late development	5 mg/l	100
48	Yolk sac edema	10 mg/l	33.3
48	Pericardial edema	10 mg/l	18.8
96	Hematoma	10 mg/l	20.8
120	Late development	10 mg/l	81.3
48	Yolk sac edema	50 mg/l	37.5
48	Pericardial edema	50 mg/l	31.3
96	Hematoma	50 mg/l	12.5
120	Late development	50 mg/l	95.5

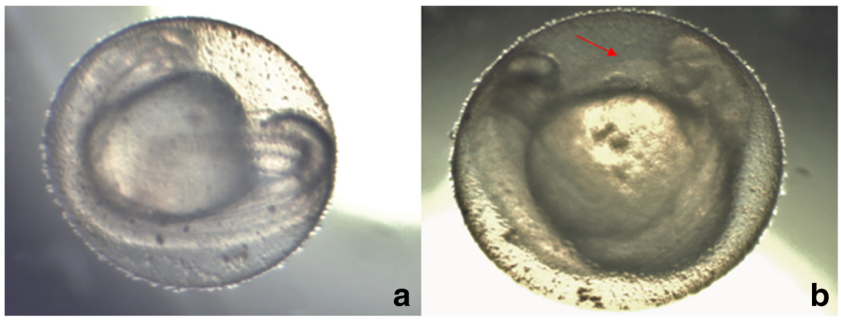

Fig. 2 Edema occurrence in *C. carpio* embryos after glyphosate exposure at 48 hpf. **a** Control. **b** 10 mg/l. The red arrow indicates yolk sac edema

(5; 10 and 50 mg/l). By contrast, the significantly higher hatching rate was found at a concentration of 0.05 mg/l of glyphosate during the whole hatching period.

Results of malformation occurrence are presented in Table 2. Only surviving embryos were used for the calculation of the malformation rate. Screening for development disorders showed only rare occurrence of yolk sac edema in the control group at 48 hpf. In contrast, higher malformation disorders such as yolk sac and pericardial edema, hematoma, and late development in most of the tested groups were observed (Fig. 2). Surprisingly, numerous malformations and late development were also observed in the lowest tested concentration. No malformations indicating teratogenic effects of glyphosate were found in embryos of common carp.

Fish embryo acute toxicity test on *Danio rerio*

The mortality of zebrafish embryos was recorded at 48, 72, 96, and 120 hpf, and results of cumulative mortality are shown in Fig. 3. Statistical analysis was calculated between the control and the tested groups at the same time. Cumulative mortality did not exceed 3% in the control group. Significant differences were found in all concentrations tested after 48, 72, and 96 hpf but only at 5 and 50 mg/l after 120 hpf. At the end of the test, the significantly highest cumulative mortality was observed at concentrations 50 mg/l and it reached 17.5%.

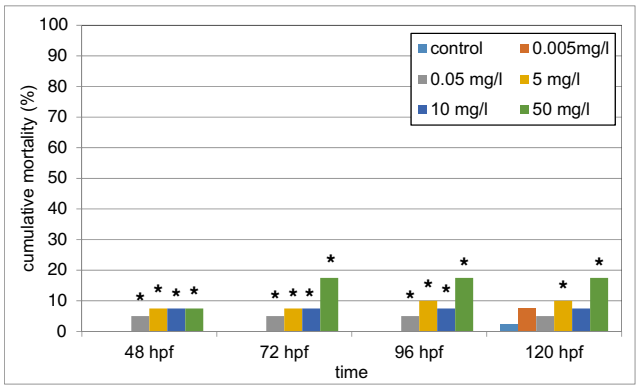

Fig. 3 Cumulative mortality of *D. rerio* embryos exposed to glyphosate (hpf hours post fertilization). Asterisk indicates significant difference ($p < 0.05$) between control and tested groups of the same time of exposure

Results of the hatching rate are shown in Table 3. Hatching began at 96 hpf in both the control and in the experimental groups exposed to the lowest concentrations of glyphosate (0.005 and 0.05 mg/l). In contrast, embryos exposed to glyphosate at concentrations of 5, 10, and 50 mg/l started to hatch already at 72 hpf. A significantly higher hatching rate was also found in all experimental groups at 96 hpf. No significant differences in hatching rate between the control and all experimental groups at 120 hpf were observed.

Results of malformation occurrence are presented in Table 4. Only surviving embryos were used for the calculation of the malformation rate. No malformations were observed in the control group, and only few morphological anomalies including pericardial and yolk sac edema, hematoma, and late development were found in experimental groups exposed to glyphosate (Fig. 4). No malformations indicating teratogenic effects of glyphosate in embryos of zebrafish were found.

Discussion

The extensive application of pesticides may result in their accidental introduction in fresh and marine surface waters. These contaminants pose a high ecological risk for aquatic organism particularly for early life stages (Banaee et al. 2011; Haarstad et al. 2011; Hostovsky et al. 2014; Velisek et al. 2015). An extensive number of studies have confirmed that aquatic organisms including zooplankton, fish, and

Table 3 Hatching rate in *D. rerio* embryos exposed to glyphosate (hours post fertilization (hpf)). Asterisk indicates significant difference ($p < 0.01$) between the control and the tested groups

Group	Hatching rate (in %)		
	72 hpf	96 hpf	120 hpf
Control	0.0	50.0	95.0
0.005 mg/l	0.0	72.5*	90.0
0.05 mg/l	0.0	82.5*	97.5
5 mg/l	37.5*	90.0*	95.0
10 mg/l	20.0*	92.5*	92.5
50 mg/l	37.5*	85.0*	85.0

Table 4 Occurrence of malformations tested groups of *Danio rerio* embryos exposed to glyphosate (hours post fertilization (hpf)). Only surviving embryos were used for the calculation of the malformation rate

Time (hpf)	Malformation type	Group	Malformation rate (in %)
48	Pericardial edema	0.05 mg/l	2.5
72	Yolk sac edema	0.05 mg/l	2.5
72	Hematoma	0.05 mg/l	2.5
120	Yolk sac edema	0.05 mg/l	2.7
120	Late development	0.05 mg/l	2.7
48	Pericardial edema	5 mg/l	2.5
120	Late development	5 mg/l	5.4
48	Yolk sac edema	10 mg/l	5.0
120	Late development	10 mg/l	10.8
48	Pericardial edema	50 mg/l	2.5
120	Late development	50 mg/l	5.9

amphibians are known to be highly sensitive to glyphosate-based herbicide formulations (Sarıgül and Bekcan 2009; Wagner et al. 2013). Glyphosate exposure is very stressful for them; thus, their use should be subject to strict limitation and monitoring as well (Rzymski et al. 2013). Various authors also documented that pure glyphosate may be relatively less toxic for aquatic organisms (Bridi et al. 2017; Solomon and Thompson 2003); however, its formulations are often more toxic for aquatic organisms due to the addition of the surfactant that is used to improve its penetration into plants (Sánchez et al. 2017; Stehr et al. 2009).

Many authors evaluated potential effects and ecotoxicological risks of glyphosate exposure on early life stages of different types of fish species (Li et al. 2017; Lopes et al. 2017; Sobjak et al. 2017; Stehr et al. 2009; Sulukan et al. 2017) or other aquatic organisms (Amid et al. 2017; Mottier et al. 2013; Schaumburg et al. 2016; Wagner et al. 2015) To the best of our knowledge, there are no ecotoxicological studies providing us with assessment of glyphosate exposure on early life stages of common carp. In our study, we compare embryo toxicity of glyphosate for two different fish species—*D. rerio* and *C. carpio*—using toxic effect endpoints such as mortality, hatching rate, and malformations.

The increase in mortality of early life stages of fish after exposure to glyphosate and glyphosate-based herbicide has been reported by many authors. For instance, Zhang et al. (2017) reported 100% of the mortality in *D. rerio* embryos treated with 600 mg/l of glyphosate at 6 hpf. Yusof et al. (2014) observed that only 50% of the embryos of Java medaka (*Oryzias javanicus*) exposed to 100 mg/l of glyphosate survived after 16 days exposure. In addition, Webster et al. (2014) found an increased mortality in glyphosate-treated embryos of *D. rerio* that originated from a glyphosate-exposed parental population. Mortality predominantly occurred in the earlier stages of development especially before 3.5 hpf. Authors assumed that increase in mortalities in so early life stages could be attributed to potential damage of the gametes occurring during gametogenesis and fertilization, rather than because of a direct embryo exposure. Moreover, they observed an evidence of developmental delay, numerous abnormalities, and premature hatching. In our study, a higher mortality was observed in embryos of *C. carpio* compared to embryos of *D. rerio*. At 50 mg/l, 54.1 and only 17.5% of the embryos of carp and zebrafish were dead at 120 hpf, respectively. Accelerated hatching was documented in many studies, which dealt with evaluation of fish embryotoxicity of different pollutants. For instance, Zhang et al. (2017) studied the adverse effects of glyphosate on embryos of zebrafish within a broad concentration range (0.1; 1, 10, 100, 200, and 400 mg/l). The first hatching activity was recorded at 48 hpf, but it was only in the experimental group exposed to 400 mg/l. At 96 hpf, hatching was recorded in all groups, but glyphosate exposure increased hatching rate in all treated groups compared to the control. Statistically significant difference only in the highest concentration was observed. Furthermore, they also documented other changes such as delay occurred in the epiboly process, reduction of body length, eye, or head area. In addition, embryonic exposure to glyphosate significantly elevated locomotor activities attributed to motoneuronal damage. Thus, the damaged primary motoneurons might cause an increase in spontaneous movement, which further increased hatching rate. Similarly, a significant increase in hatching rate was observed in our study performed in the toxicity test on *D. rerio* at most of the tested groups (5; 10 and 50 mg/l) at

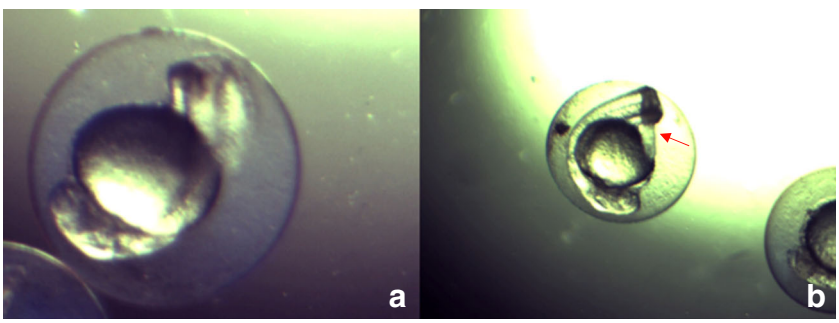

Fig. 4 Yolk sac edema (indicates with red arrow) in *D. rerio* embryos after glyphosate exposure at 48 hpf. **a** Control. **b** 5 mg/l

72 hpf and in all of the tested groups at 96 hpf. However, no significant differences were found in hatching rate compared to the control at the end of the test at 120 hpf, the increased in hatching rates might be caused by the increased spontaneous movement at early life stage. Those changes might influence the response to danger and decrease the survival of organism under risky circumstance. Further, Zivna et al. (2016) described a stimulatory effect of ciprofloxacin on hatching as a result of influence on the motor and respiratory intensity of embryos and the need to remove their fish egg covers. By contrast, hatching activities of *C. carpio* decreased significantly with increasing glyphosate concentration and this trend was evident at concentration 5 mg/l and higher at 120 hpf. In addition, 48% of the carp embryos in the control group were hatched at 72 hpf compared to zebrafish embryos, where first hatching activity in the control group was noticed at 96 hpf. An earlier hatching of carp was considerably influenced by temperature, because they need lower temperature for hatching in comparison to zebrafish (Peňáz et al. 1983).

Embryotoxicity of glyphosate-based herbicide was studied by Yusof et al. (2014) who observed significant decrease in survival and hatching percentage in early life stages of Java medaka (*Oryzias javanicus*) exposed to glyphosate in concentration range from 100 to 500 mg/l. They also found many malformations such as absence of pectoral fins and cornea, permanently bent tail, abdominal enlargement or cell disruption. In addition, they reported that glyphosate exposure initially increased the heartbeat compared to normal condition, later on fluctuated ones and finally slowed down or halted. Occurrence of numerous malformations and delay in development after glyphosate exposure were also noticed in our study especially in test on *C. carpio*. At the end of the test, delayed development was observed in all tested groups of *C. carpio* and it ranged from 58 to 100%. Further, malformations such as pericardial edema, hematoma, or yolk sac edema were often found in all tested groups of *C. carpio* with the highest frequency in the experimental groups exposed to the highest concentrations. In contrast, a delay in development only in range from 2.7 to 10.8% was found in tested groups of *D. rerio*. A similar trend was also observed in rate of malformations in this fish species, which did not exceed 2.5, 2.5, and 5% for hematoma, pericardial, and yolk sac edema, respectively, during the experiment. In contrast to our results, Sulukan et al. (2017) documented a higher percentage of malformations (pericardial edema, yolk sac edema, spinal curvature, and body malformations) in zebrafish embryos after exposure to similar concentrations. For instance, they observed some malformations mentioned in more than 15 and 30% of the embryos at 10 and 100 mg/l, respectively. For years, glyphosate has been considered harmless; however, the problem should not be underestimated due to the effects found in environmentally relevant concentration.

Overall, our results showed that lower concentration of glyphosate (0.005 mg/l) that is possible to find in environment can cause significant changes in common carp and zebrafish as well. This was mainly reflected by significant changes in mortality and occurrence of some malformations, and this can reduce biodiversity. It is also evident that early life stages of *C. carpio* are more sensitive and have lower tolerance to acute exposure to glyphosate compared to *D. rerio*. Higher sensitivity of *C. carpio* is obvious especially from occurrence of the numerous malformations and delay in development. This difference might have also resulted from natural characteristics of these two types of species.

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