EFFECTS OF GLYPHOSATE-BASED HERBICIDES AND THEIR COMPONENTS ON THE EMBRIONAL DEVELOPMENT OF ZEBRAFISH (*DANIO RERIO*): ASSESSMENT OF THE ROLE OF RETINOIDS

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Abstract
Intensification of agricultural technologies led to a worldwide increase in the use of agrochemicals. Herbicides may exert toxicity to non-target organisms water ecosystems. In this study individual and combined teratogen effects of herbicide active ingredient glyphosate and surfactants applied in formulated herbicides were investigated on embryonal development of zebrafish. Retinoic acid-retinol (RA/ROH) and 13-cis-RA/ROH ratios are useful parameters in teratogenicity studies to assess xenobiotic effects, thus analytical determination of three retinoids were determined by HPLC method.

Introduction
Pesticides applied at cultivation areas may be absorbed, washed-off or leached to other environmental compartments. These chemicals can trigger harmful effects at very low concentrations on living organisms in soil or surface water, and cause teratogenic, carcinogenic or endocrine disrupting abnormalities. Upon the ban of atrazine in the EU [1], glyphosate has become the most widely used herbicide: its use has risen globally almost 15-fold since the introduction of genetically modified glyphosate-tolerant crops [2,3]. Due to the continuously expanding application of glyphosate, this herbicides active ingredient is a common pollutant in surface waters worldwide. Surfactants are used as common additives in agrochemical formulations to enhance it pesticide efficacy [4]. Surfactants are declared as inert ingredients regarding the main pesticide effect, but recent studies found that these compounds may exert toxicity, moreover, synergistic toxic effects were also indicated [5,6].

Retinoic acid (RA) is of major importance during vertebrate embryonic development and its levels need to be under strict endocrine regulation, otherwise congenital malformations will develop [7]. RA, as an active metabolite of vitamin A, is vital for vertebrate embryonic development, namely in somitogenesis, neurogenesis and organogenesis. It acts as a ligand for nuclear RA receptors, converting them from transcriptional repressors to activators. The distribution and levels of RA in embryonic tissues are tightly controlled by regulated synthesis through the action of specific retinol (ROH) and retinaldehyde dehydrogenases and by degradation via specific cytochrome P450s [8]. Possible functions of RA during embryo-genesis were first inferred by studying its teratogenic effects, i.e. how the administration of excess doses of RA, either globally or by local implantation using RA-impregnated beads, interferes with normal developmental processes. Studies have been performed in a wide range of species including amphibians, zebrafish, chick and rodents [9,10]. Teratogenic effects of glyphosate-based herbicides in embryonal development of *Xenopus laevis* via disturbances in RA cell signals was reported by Paganelli et al. [11,12]. Embryotoxicity and teratogeneicity by glyphosate on zebrafish embryos were also published, where cardiac and pericardiac

alterations, digest-vitelline and musculo-eskeletic malformations were detected [13]. In our study, we first report individual and combined teratogenic effects of components of formulated herbicides on zebrafish (*Danio rerio*) embryos.

Experimental

Teratogen effects of glyphosate-based herbicides and their components

Teratogenic effects of the herbicide active ingredient glyphosate, four formulated glyphosate-based herbicides and four surfactants frequently applied in herbicides were investigated based on the OECD 236 guideline [14]. Xenobiotics investigated were obtained by Lamberti S.p.A, Abizzate, Italy (Table 1).

Table 1. Chemical characteristics of xenobiotics investigated [15,16].

	Trade Name	**AI**	**AI (%)**	**Present in**
Surfactants	Emulson AG GPE 3SS	POE tallow amine	100	Roundup Classic, Glyfos
	Rolfen Bio	POE alkyl phosphate ether	70	
	Eucarol Age SS	sodium-APG sulfosuccinate	45	
	Eucarol Age EC	sodium-APG citrate	30	
		Glyphosate salt		**Surfactant (%)**
Formulated herbicides	Roundup Classic	IPA (486 g L^{-1})*		POEA (15.5%)
	Total	IPA (486 g L^{-1})*		un. (58.5%)
	Glyfos	IPA (486 g L^{-1})*		POEA (9%)
	Medallon Premium	DA (433 g L^{-1})*		APG (10-20%)
AI	glyphosate IPA	IPA (486 g L^{-1})*		

AI – active ingredient, POE – polyethoxylated, IPA – isopropylammonium salt, DA – diammonium salt, APG – alkyl polyglucoside, un. – unknown. *Concentrations are equivalent to 360 g L^{-1} of the free acid form of glyphosate

Authorisation of animal housing and experiments according to the corresponding legal regulation in the EU [17] have been completed prior to the study. Maintenance of the zebrafish colonies was performed in 80 litre aquaria at 26±1 °C and under 16:8 hrs light:dark photoperiods. Fish were fed daily with living and frozen food (*Artemia salina, Culex pipiens*) and plate fish food. Health conditions of the fish and maintenance conditions were supervised regularly by a veterinarian. In the 96-hr ecotoxicity assay, newly fertilised embryos were treated with glyphosate active ingredient, herbicides or surfactants at five concentrations. Tests were carried out in triplicates in 24-well plates. One day before the test males and females at a ratio of 2:1 per were placed in spawning tanks a few hours before the onset of darkness. Twenty embryos per concentration at 8-16-cell developmental stages were exposed to chemicals. FET control water was used as negative control and as internal plate control. A positive control 3,4-dichloroaniline at concentration of 4 mg L^{-1} was performed. Development and mortality was recorded upon 24, 48, 72 and 96 hrs of exposure by an Olympus IX73 inverse microscope (Unicam Kft., Budapest, Hungary), LC_{50} values were determined at 96 hrs and calculated by statistical software ToxRat® (ToxRat Solutions GmbH, Alsdorf, Germany).

Analytical determination of retinoids in zebrafish

For analytical determination of retinoids in full body homogenates, fish were collected at different developmental stages and sizes of 2-4 hr eggs, 10-day juveniles (DR1), 10-14 mm (DR3) and 25-30 mm fish (DR6). Eggs and fish were placed into 5 ml stabilizing buffer (0.5% ascorbic acid, 0.5% EDTA, 0.3% sodium sulphate in phosphate buffered saline, pH 7.3), then homogenised (Heidolph Silent Crusher M) for 2x1 min at 15000 rpm for eggs and DR1 group and 4x4 minutes at 26000 rpm for DR3 and DR6 groups. Chemical analyses of the samples were performed on Younglin YL9100 high pressure liquid chromatography (HPLC) equipped with a YL9150 autosampler, using a Kinetex EVO (Phenomenex) C18 core-shell column (150 mm × 4.6 mm i.d., 5µm) at 30ºC and UV detector signals recorded at λ=330 nm and λ=300 nm for each compound. The HPLC method was optimised with standard solutions of 13-cis-retinoic acid (13-cis-RA), retinoic acid (RA) and retinol (ROH). External calibration was used in the range between 20 ng mL^{-1} and 15 µg mL^{-1}. The eluent flow rate was 1.2 mL min^{-1} with isocratic elution for 5 minutes (20:80 = A:B eluents, A = 2% acetic acid in water, B = ACN). Retention times were 3.30, 3.50 and 3.77 minutes for 13-cis-RA, RA acid and ROH, respectively. Two sample preparation methods were assessed (Figure 1). Finally, the method using ethyl acetate:hexane = 1:1 mixture for the extraction was applied and recoveries were determined with standard solutions from spiked water samples at two levels (2 and 20 µg mL^{-1}) and from fish samples (1-, 2-, 3- and 4-day old eggs) as well.

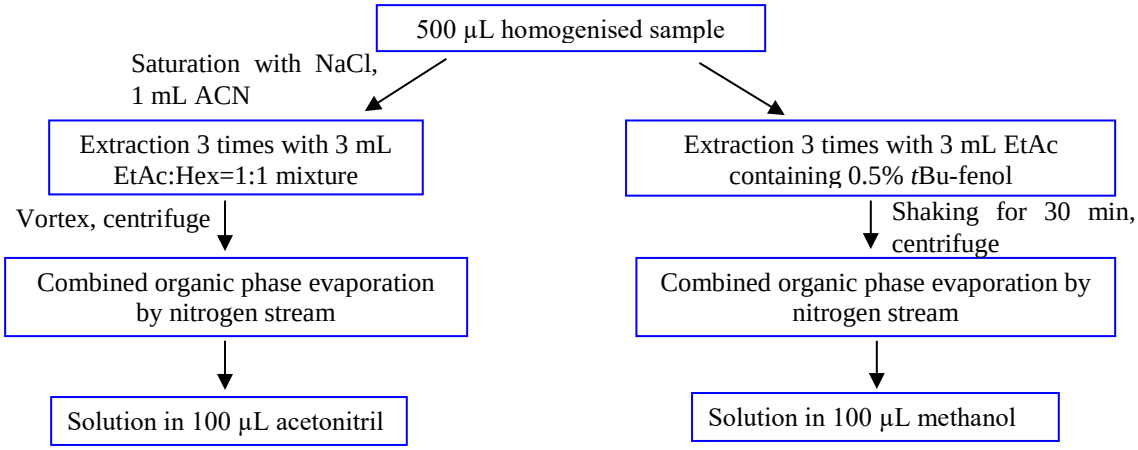

Figure 1. Extraction methods tested for preparation of *D. rerio* samples for HPLC measurement

Results and discussion

Teratogen effects of glyphosate-based herbicides and their components

Coagulation of the embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat were recorded as indicators of lethality. Other defromites (e.g., inhibition of heartbeat and circulation, lack of eyes or pigmentation, scoliosis, lordosis, lack of pigmentation, oedema) were also recorded. 96-hr LC_{50} values showed the acute toxic effects of chemicals investigated. Herbicide active ingredient glyphosate was found the least toxic on embryos. Its LC_{50} was higher than 9.8 g L^{-1} (glyphosate concentration in 2% Roundup Classic applied in agricultural practices). Formulated herbicides were 44-336 times more toxic than glyphosate, that were considered to be due to the chemical characteristics and quantity of the surfactants, since the nominal concentration of the active ingredient did not differ in these products. The least toxic one among formulated herbicides was Medallon Premium that

contains an alkyl polyglucoside surfactant; while the most toxic were Roundup Classic and Glyfos that contain a non-ionic surfactant polyethoxylated tallow amine. The pure surfactants were identified as the most toxic components: their LC_{50} values were 232-2438 times lower than that to glyphosate. Below or near the LC_{50} values, defromites, oedema (pericardial), inhibition of heartbeat and circulation were the most frequently detected non-lethal malformations in every treatments (Figure 2).

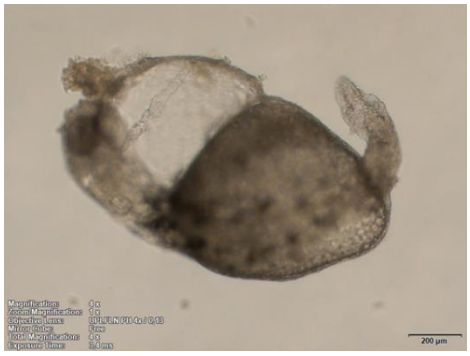

Figure 2. Pericardial oedema after 48 hrs exposition of Glyfos at concentration of 20 mg L^{-1}.

Analytical determination of retinoids and assessment of their roles in teratogenic effects

The biochemical scheme for the conversion of these compounds is depicted in Figure 3. As the method using ethyl acetate:hexane=1:1 for the extraction was more efficient and resulted in higher recoveries (Table 2) and cleaner extracts, than extraction with ethyl acetate, this solvent was applied for fish samples. Limits of detections, determined with standard solutions were 50, 25 and 10 ng mL^{-1} for 13-cis-RA, RA and ROH, respectively. Results of analytical determinations from embryos and small fish are summarised in Table 3.

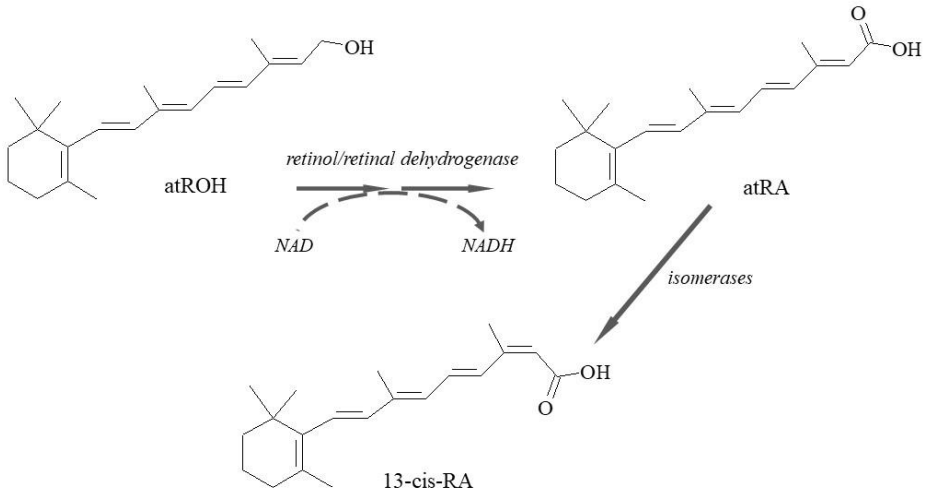

Figure 3. Enzymatic conversion of vitamin A (atROH) into all-trans-retinoic acid (atRA) and 13-cis-retinoic acid (13-cis-RA) during embryonic development.

Table 2. Recoveries (%) from spiked water samples concentrations and from fish eggs

	Spiked water samples		**Fish egg samples (days after fertilisation)**			
	2 μg mL^{-1}	20 μg mL^{-1}	1 day	2 days	3 days	4 days
13-cis-retinoic acid	82.3±6.7	86.5±8.7	36.9±2.4	69.3±5.1	68.2±4.5	56.6±4.9
retinoic acid	82.1±9.4	95.2±6.2	43.7±3.7	78.7±6.8	72.9±5.2	56.4±4.2
retinol	37.5±8.9	64±10.4	51.4±4.9	48.5±5.1	59.2±5.3	59.6±5.4

Table 3. Concentration of retinoids in the final extract (μg mL^{-1}) of different aged and sized zebrafish. DR1 - 10-day juveniles, DR3 – 10-14 mm length, DR6 – 25-30 mm length.

	DR1	**DR3**	**DR6**
13-cis-retinoic acid	<LOD	<LOD	<LOD
retinoic acid	<LOD	<LOD	<LOD
retinol	<LOD	0.035±0.003	0.289±0.039

DR1 - 10-day juveniles, DR3 – 10-14 mm length, DR6 – 25-30 mm length.

Retinoids regulate differentiation, development and embryogenesis of vertebrates [18,19], and induce changes in their endogenous levels eventually lead to teratogenic effects. Vitamin A or all-trans-retinol (atROH) is converted enzymatically in a sequential process to all-trans-retinoid acid (atRA), and is further isomerised into its isoform 13-cis-RA. The progression of this enzymatic process triggers numerous cellular effects, the retinoic acid signalling pathway (RA pathway) [20], and is an indicator of its activation. The RA pathway has been implicated in various developmental processes, e.g. during early embryonic development, retinoids act as important morphogens, and participate in regulating apoptosis, differentiation and cell fate specification. Surfactants used in glyphosate-based herbicides and the active ingredient glyphosate itself have been indicated to interfere with the formation of retinoids and the RA pathway [11,12]. Thus, we have determined levels of atROH, atRA and 13-cis-RA in *D. rerio* embryos and young fish emerging. Retinoic acid-retinol (RA/ROH) and 13-cis-RA/ROH ratios are useful parameters in teratogenicity studies to assess xenobiotic effects. In our hand, as RA and 13-cis-RA were found to be below LODs, such ratios could not be calculated, and therefore, assessment has to rely on ROH levels detected. Nonetheless, this preliminary survey indicated that ROH (and possibly RA and 13-cis-RA, if detected by more sensitive analytical methods) are suitable biomarkers of pesticide exposure in exposed *D. rerio*.

Acknowledgements

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