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A glyphosate micro-emulsion formulation displays teratogenicity in *Xenopus laevis*

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ABSTRACT

Glyphosate is the active ingredient in broad-spectrum herbicide formulations used in agriculture, domestic area and aquatic weed control worldwide. Its market is growing steadily concurrently with the cultivation of glyphosate-tolerant transgenic crops and emergence of weeds less sensitive to glyphosate. Ephemeral and lentic waters near to agricultural lands, representing favorite habitats for amphibian reproduction and early life-stage development, may thus be contaminated by glyphosate based herbicides (GBHs) residues. Previous studies on larval anuran species highlighted increased mortality and growth effects after exposure to different GBHs in comparison to glyphosate itself, mainly because of the surfactants such as polyethoxylated tallow amine present in the formulations.

Nevertheless, these conclusions are not completely fulfilled when the early development, characterized by primary organogenesis events, is considered.

In this study, we compare the embryotoxicity of Roundup® Power 2.0, a new GBH formulation currently authorized in Italy, with that of technical grade glyphosate using the Frog Embryo Teratogenesis Assay–*Xenopus* (FETAX). Our results evidenced that glyphosate was not embryolethal and only at the highest concentration (50 mg a.e./L) caused edemas. Conversely, Roundup® Power 2.0 exhibited a 96 h LC50 of 24.78 mg a.e./L and a 96 h EC50 of 7.8 mg a.e./L. A Teratogenic Index of 3.4 was derived, pointing out the high teratogenic potential of the Roundup® Power 2.0.

Specific concentration-dependent abnormal phenotypes, such as craniofacial alterations, microphthalmia, narrow eyes and forebrain regionalization defects were evidenced by gross malformation screening and histopathological analysis. These phenotypes are coherent with those evidenced in *Xenopus laevis* embryos injected with glyphosate, allowing us to hypothesize that the teratogenicity observed for Roundup® Power 2.0 may be related to the improved efficacy in delivering glyphosate to cells, guaranteed by the specific surfactant formulation. In conclusion, the differences in GBH formulations should be carefully considered by the authorities, since sub-lethal and/or long-term effects (e.g. teratogenicity) can be significantly modulated by the active ingredient salt type and concentration of the adjuvants. Finally, the mechanistic toxicity of glyphosate and GBHs are worthy of further research.

1. Introduction

Pesticide contamination of surface and groundwater is well documented worldwide and constitutes a major issue that gives rise to concerns from local to global scale. Due to their direct application on the soil, pesticides or some of their residues may reach the aquatic environment through direct run-off and leaching.

According to the 2016 Italian National report by ISPRA (ISPRA, 2016) 21.3% of the surface waters monitoring points have pesticide concentrations beyond the Environmental Standard Quality limits. Herbicides including glyphosate and its metabolite AMPA exceed these limits in the 25.2% and 52.2% of the monitored sites respectively. European Glyphosate Environmental Information Sources (EGEIS, 1993–2009) have documented a similar glyphosate contamination across the whole European Union. Such a widespread contamination derives from the fact that glyphosate is the active ingredient of a high number of different broad spectrum herbicides. The applications of these Glyphosate Based Herbicides (GBHs) are measured in gross tonnage mainly during cultivation of genetically modified plants (i.e. soybean, canola, sugarbeet and cotton), engineered to tolerate

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glyphosate (Saunders and Pezeshki, 2015; Duke and Powles, 2008; Cerdeira and Duke, 2006). Although glyphosate has a short half-life (2–14 days) and high affinity for soil particles, background concentrations between 10 and 40.8 μg acid equivalents (a.e.)/L are nonetheless detectable in surface waters (Battaglin et al., 2009; Byer et al., 2008; Struger et al., 2008). Peruzzo et al. (2008) detected glyphosate concentrations ranging from 0.1 to 0.7 mg/L in Pampean rivers near to transgenic soybean cultivation areas and Edwards et al. (1980) found a concentration of 5.2 mg/L in watershed samples in a runoff study. This level of contamination could be reached temporarily in ephemeral and lentic waters, where contaminants may accumulate without substantial dilution. The presence of high concentrations of herbicides in these habitats, that are favorite by amphibians for their breeding and early life-stage development, has been pointed to be one of the causes of the global amphibian decline (Collins, 2010).

The many GBH formulations differ each other for the content of active ingredient, the form of G salt, the identity and concentration of the surfactant. Glyphosate targets 5-enolpyruvylshikimate-3-phosphate synthase enzyme, which is involved in the metabolism of aromatic amino acids in plants and some microorganisms (Rubin et al., 1982). Since this biochemical pathway does not exist in Vertebrates, glyphosate is generally considered low toxic to non-target organisms (Williams et al., 2012). Indeed, the extensive literature on glyphosate and GBH comparative toxicity points out detrimental effects only of GBHs on freshwater organisms such as microbial communities and planktonic algae (Bonnet et al., 2007; Pérez et al., 2011) as well as fish (Modesto and Martinez, 2010; Glusczak et al., 2011; Hued et al., 2012; Menezes et al., 2011) and most notably amphibians (Relyea, 2005; Govindarajulu, 2008; Mann et al., 2009). In particular, different GBHs formulations (i.e. Roundup® Original, WeatherMax, Ultramax, Transorb, Biactive) previously studied on various amphibian species highlighted mortality or growth effects during larval development in comparison to the sole active ingredient (Mann and Bidwell, 1999; Edginton et al., 2004; Howe et al., 2004; Relyea and Jones, 2009; Williams and Semlitsch, 2010; Fuentes et al., 2011). This discrepancy has been attributed mainly to the presence of surfactants in GBH formulations, which are supposed to be key factors in toxicity also in other aquatic organisms (Folmar et al., 1979; Giesy et al., 2000; Tsui and Chu, 2003). Glyphosate formulations containing polyethoxylated tallow amine (POEA) are generally more toxics to amphibian larvae than other formulations making use of other surfactants from the alkoxylated alkyl amine family (Howe et al., 2004; Relyea, 2005; Govindarajulu, 2008; Fuentes et al., 2011; Lajmanovich et al., 2011).

Nevertheless, these conclusions are not completely fulfilled when the early development is considered. Perkins et al. (2000) using the Frog Embryo Teratogenesis Assay–*Xenopus* (FETAX) test evaluated the toxicity of two glyphosate formulations (Roundup® and Rodeo®), both containing glyphosate isopropylamine (IPA) salt and the surfactant POEA only in Roundup®. They concluded that formulation with POEA was embryolethal with a 96 h LC50 of 9.3 mg a.e./L and that this toxicity was attributable to the surfactant, since Rodeo® was toxic only at very high concentrations (96 h LC50 7.29 gr a.e./L). Surprisingly no increment of malformations was observed in embryos exposed to sublethal concentrations of GBHs and POEA itself. A subsequent investigation performed in *X. laevis* embryos by Paganelli et al. (2010), evidenced that sublethal doses of Roundup® Classic (corresponding to 72 mg a.e/L) caused malformations at tadpole stages, such as shortening of the trunk, cephalic reduction, microphthalmy, cyclopia, and craniofacial malformations. Paganelli observed the same phenotypes also in embryos injected with glyphosate alone, suggesting that glyphosate itself was able to induce specific malformations, some of these referable to the alteration of the retinoic acid signaling pathway. These results undermine the idea of a safe glyphosate, although its toxicity was only evinced in the presence of the surfactant. In a more recent study, also Wagner et al. (2016) describe effects of another GBH formulation, Roundup® UltraMax, on *Xenopus* viscerocranial skeleton development with a 96 h EC50 of 37.35 mg active ingredient/L (corresponding to 27.64 mg a.e./L: conversion factor for glyphosate IPA salt 0.74, Giesy et al., 2000). This glyphosate formulation contains glyphosate IPA salt and an ether amine ethoxylate as surfactant.

Since a large number of studies indicates that differences in toxicity between different formulations of Roundup® may be largely attributable to the type of surfactant, there is a need to extend the investigations to new generation GBHs containing novel surfactants, especially during embryonic development.

In this study, we compare the embryotoxicity of Roundup® Power 2.0 (RU-PW) (Monsanto Italia S.p.A.), a relatively new GBH formulation, with that of technical grade glyphosate. RU-PW is a mixture of glyphosate in form of potassium salt, smaller than IPA salt and then characterized by a rapid assimilation, and an ethoxylated ether alkyl ammine described as an effective surfactant with high affinity for the cuticle waxes. The embryotoxicity was assessed using FETAX (ASTM, 1998), a standardized approach to screen teratogenic potential of environmental contaminants during early developmental stages and organogenesis of *Xenopus laevis* (Bantle et al., 1999; Bonfanti et al., 2004; Bonfanti et al., 2015; Williams et al., 2015; Colombo et al., 2017).

In addition to the median lethal concentration (LC50), we wanted to extrapolate the median concentration inducing malformations (EC50) and the teratogenicity index (TI), these latter poorly investigated previously, but imperative for assessing the risk in non-target species. Moreover, we performed a histological analysis on embryos at the end of the test, comparing the possible pathological fields with those from controls.

2. Materials and methods

2.1. Chemicals

All analytical-grade reagents, human chorionic gonadotropin (HCG), 3-amino-benzoic acid ethyl ester (MS222), salts for FETAX solution, technical grade *N*-(Phosphonomethyl)glycine (Glyphosate CAS Number: 1071-83-6), were purchased from Sigma-Aldrich S.r.l., Italy. The commercial formulation of glyphosate considered in this study was Roundup® Power 2.0 (Monsanto Italia S.P.A., commercially purchased), indicated in this work as RU-PW. RU-PW has been approved for 5 years in Italy (3/20/2013 to 12/31/2017) and was formulated with a guarantee of 360 g glyphosate acid equivalent (a.e.) per liter present as the potassium salt (CAS RN 70901-12-1). Six percent by volume of the RU-PW formulation consisted of ethoxylated ether alkyl ammine (CAS RN 68478-96-6) and 58.5% water and other ingredients not specified by the producer.

2.2. Roundup® Power 2.0 solutions

To evaluate the emulsion properties of RU-PW, the hydrodynamic diameter of the commercial formulation (without dilution) was measured by a Zetasizer Nano ZS (Malvern Instrument, UK) with prior shaking. DLS analysis revealed that RU-PW is characterized by microemulsion made of micelles of about 4 μm in diameter (for results see Supplementary materials S1).

Primary stock solution of RU-PW was prepared at nominal concentration of 100 mg/L, calculated as nominal concentrations of a.e. glyphosate, using FETAX solution and subsequently stirred for 15 min. The control FETAX solution composition in mg/L was NaCl 625, $NaHCO_3$ 96, KCl 30, $CaCl_2$ 15, $CaSO_4$-$2H_2O$ 60, and $MgSO_4$ 70, pH 7.5–8.5 (Dawson and Bantle, 1987).

2.3. Animals

Adult *X. laevis* were purchased from Centre de Ressources Biologiques *Xénopes* (Université de Rennes 1, Rennes Cedex), housed in aquariums with dechlorinated tap water at a 22 ± 2 °C and alternating

12-h light/dark cycles. The animals were fed a semi-synthetic diet (Mucedola S.r.L., Settimo Milanese, Italy) three times a week.

2.4. Frog Embryo Teratogenesis Assay-Xenopus (FETAX)

Embryotoxicity tests were conducted according to the standard guide for the Frog Embryo Teratogenesis Assay –*Xenopus* (FETAX) (ASTM, 1998) with minor modification.

2.4.1. Breeding and embryo collection

Embryos were obtained as previously described (Bonfanti et al., 2015). Briefly, to obtain natural breeding, pairs of adult *X. laevis* previously injected with HCG into the dorsal lymph sac (females: 300 IU; males: 150 IU), were placed in false-bottom breeding tanks filled with well-aerated FETAX solution. Amplexus normally ensued within 2–6 h, and the egg fertilization occurred from 9 to 12 h after injection. After breeding, adults were removed, embryos collected and the jelly coat was removed by swirling the embryos for 1–2 min in a 2.25% L-cysteine solution (pH 8.1).

2.4.2. Treatment groups

An initial range finding test has been set up for RU-PW in the range of 1–100 mg a.e./L to identify the best approximation of the 96 h LC50 and EC50 for definitive testing. In order to compare the embryotoxic effects of RU-PW and pure glyphosate, at least three replicate definitive tests in the same experimental conditions were performed. Each test was conducted using embryos from a different male/female pair of *X. laevis* (n = 4 for RU-PW, in this case the additional test was performed for control and concentrations of 5, 7.5 and 20 mg a.e./L, and n = 3 for glyphosate). Normally-cleaved embryos at the midblastula stage (Stage 8), 5 h post-fertilization (hpf) (Nieuwkoop and Faber, 1956), were selected for testing and groups of 25 embryos from each male/female pair were randomly placed in covered 6.0 cm Petri dishes containing 10 mL of control or test solution. Three replicate dishes were used for each test concentration (RU-PW 1–25 mg a.e./L and pure glyphosate 7.5–50 mg/L freshly prepared in FETAX solution), while for control group four replicate dishes were used.

Control (not exposed) embryos were incubated in standard FETAX medium. Since the acidic nature of glyphosate changed pH value of the FETAX solution at the highest RU-PW concentration tested (100 mg a.e./L), a group of embryos was exposed to FETAX solution adjusted to the lowest pH measured (pH 6.8) with addition of HCl.

All of the Petri dishes were incubated in a thermostatic chamber at 23 ± 0.5 °C until the end of the test (96 hpf). Exposure solutions were changed daily, and dead embryos were recorded and removed.

2.4.3. Data collection and statistical analysis

At the end of the assay, surviving embryos of each experimental group were anaesthetized with MS-222 at 100 mg/L and screened for single morphological abnormalities by examining each embryo under a dissecting microscope (Zeiss, Germany). Surviving normal embryos were formalin fixed to estimate the growth retardation by measuring head–tail length with the digitizing software AxioVision.

The data were tested for homogeneity and normality. When these assumptions were met, one-way analysis of variance (ANOVA) was performed; otherwise, the non-parametric Kruskal–Wallis test was applied. The significance level was set at $p < 0.05$. The incidence of specific malformations was investigated by chi-square method, using Yates's correction for continuity ($\chi 2$ test) or Fisher's exact tests (FE test). Mortality and malformation percentages were used to calculate the 96 h LC50 (concentration causing 50% lethality) and 96 h EC50 (concentration inducing teratogenesis in 50% of surviving embryos) for each experimental group. These values were obtained following the elaboration of the lethality and malformation percentages by the Probit analysis (Finney, 1971), using the U.S. EPA Probit Analysis Program, Version 1.5. The Teratogenic Index (TI), useful in estimating the teratogenic risk associated with the tested compounds, is represented by the LC_{50}/EC_{50} ratio (ASTM, 1998).

2.5. Morphological analysis of stage 46 embryos

2.5.1. Whole mount cartilage staining

To clearly observe the cartilage structures, randomly selected stage 46 control and treated embryos (n = 15 for each experimental group) were fixed in 10% buffered formalin overnight at room temperature, bleached and stained overnight with Alcian Blue 8GX and then cleared in a mixture of benzyl alcohol:ethanol 70%:glycerine (1:2:2). The cartilage has resulted stained in blue while skin and the other embryo tissues have become almost transparent. Each embryo was examined under a dissecting microscope (Zeiss, Germany) equipped with digitizing software AxioVision.

2.5.2. Histopathological analysis

For light microscopy analyses, stage 46 embryos (n = 15 for each experimental group) were randomly selected, fixed in Bouin's solution and processed for paraffin embedding. The samples were transversely cut from eye to proctodeum into serial sections 6 µm thick, then mounted on glass slides and stained with hematoxylin and eosin (H&E). The sections were finally examined by a Zeiss Axioplan light microscope, equipped with an Axiocam MRc5 digital camera. Ten specimens for each experimental group were histologically screened.

3. Results

3.1. Comparative embryotoxicity of Roundup® Power 2.0 and glyphosate

In the initial range finding test, we found that exposure to 100, 50 and 30 mg a.e./L of RU-PW affected severely the survival rates during FETAX test, causing the death of 100% of the embryos within 24, 48 and 72 hpf respectively (Fig. 1). At the concentration of RU-PW 25 mg a.e./L, the mortality occurred significantly only at the end of exposure reaching values close to 50%. The calculated 96 hpf LC50 for RU-PW was indeed 24.78 mg a.e./L (Table 1).

Instead, at the end of exposure in the range of concentrations 1–22.5 mg a.e./L the mortality rate stood at values comparable to those of control groups (Fig. 2a). Concurrently, the malformation rate increased in a concentration dependent manner with a sharp trend. The threshold effect appeared at 5 mg a.e./L of RU-PW, and at 20 mg a.e./L, in spite of a low mortality, the malformation rate was 100% (Fig. 2a).

The median 96 h EC50 for RU-PW was 7.28 mg a.e./L and Teratogenic Index (TI) value calculated as the ratio LC50/EC50 was 3.4 (Table 1). According to ASTM guidelines (1998), TI values greater than 1.5 indicate increasing developmental hazard and TI values greater than 3.0 indicate concern. Since TI value for RU-PW is greater than 3, we can attribute to this compound a high teratogenic hazard.

In contrast to the formulation of RU-PW, glyphosate did not result embryolethal at any of the concentrations tested and a significant increase in the incidence of malformations was recorded only at 50 mg/L (Fig. 2b). Comparing the malformation incidence between RU-PW and glyphosate, we observed that the rate of malformed embryos exposed to glyphosate 50 mg/L (17.78%) is comparable to the rate recorded in embryos exposed to a concentration 10 times lower of RU-PW (15.28%). Since glyphosate was not embryolethal and was only slightly teratogenic in the range of concentrations tested, it was not possible to calculate LC50 and EC50 values (Table 1).

As last FETAX endpoint, we measured the head-tail length of embryos. Significant growth inhibition was observed after exposure to RU-PW starting from 5 mg a.e./L compared to the unexposed control group (Fig. 3). No significant reduction in length was observed in embryos exposed to glyphosate (data not shown).

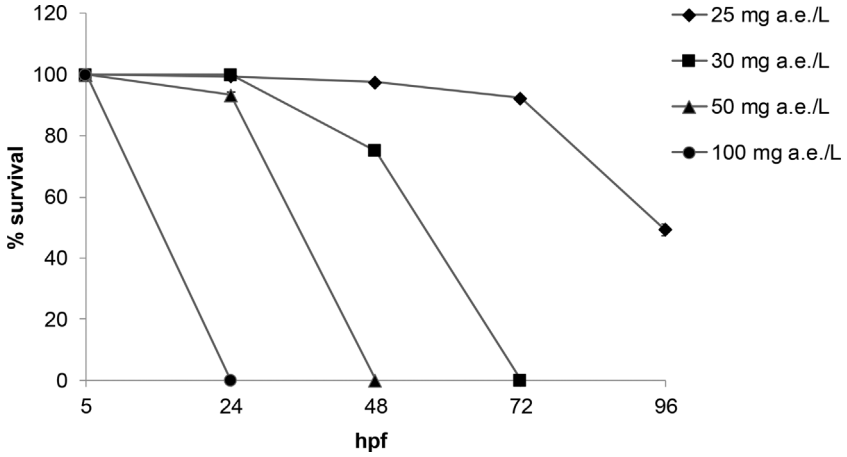

Fig. 1. Daily survival rate in embryos exposed to Roundup® Power 2.0 during FETAX test. Exposure from blastula stage (5 hpf) to the herbicide in the range of 25–100 mg a.e./L, caused 100% mortality in a time-dependent manner starting from 24 hpf. All values are given as mean ± SE of three independent assays.

Table 1
Comparative toxicity of Roundup® Power 2.0 and pure glyphosate in 96 hpf *Xenopus laevis* embryos during FETAX.

Treatment	LC_{50} [a]	EC_{50} [b]	TI [c]
Roundup	24.78 (24.54–25.04)	7.8 (4.24–8.62)	3.4
Glyphosate	n.d.	n.d.	n.d.

LC_{50} and EC_{50} were determined by US EPA Probit Analysis Program (version 1.5, with 95% confidence interval in parenthesis) and are expressed on an acid equivalent basis in mg/L.

[a] LC_{50} = Median lethal concentration.
[b] EC_{50} = Median teratogenic concentration.
[c] TI = Teratogenic index (LC_{50}/EC_{50}).

3.2. Morphological analysis of stage 46 embryos

3.2.1. Gross malformations

In order to describe malformations related to RU-PW and glyphosate exposure, stage 46 embryos were observed at the dissection microscope and each malformation quantitatively scored on a standard score sheet and tallied according to the ASTM International Guide (ASTM, 1998). Only the recurrent and not those sporadic malformations were reported in Table 2. In comparison to the normal morphology observed in control embryos, the multiple malformations registered in RU-PW exposed embryos appear in a concentration dependent manner starting from 1 mg a.e./L where gut miscoiling and cardiac edema had a significant incidence (Table 2a). Miscoiling of the gut included different degrees of deviation from the normal arrangement in a spiral pattern of the gastrointestinal tract, which ranged from a looser coiled or few looped to a sigmoid or an almost straight tube. Cardiac edema appeared as swollen fluid filled area in the cardiac region. On the contrary, in glyphosate exposed embryos, the only malformation resulted to be statistically significant was the cardiac edema at 30 and 50 mg/L (Table 2b).

Starting from RU-PW 5 mg a.e./L, the most common induced phenotypes included, besides the uncorrected gut coiling and cardiac edema, facial and abdominal edemas, craniofacial anomalies such as small, narrowed and flattened head with rounded brow and prominent oral sucker, and eyes defects such as oval shape, monolateral or bilateral microphthalmia and narrowing (Table 2a; Fig. 4). The large edemas that characterize the embryos exposed to RU-PW 7.5 and 10 mg a.e./L were reduced at the higher concentrations leaving the place to epidermal blisters (Fig. 4).

The craniofacial anomalies consisted mainly in a size concentration dependent reduction of head region, accompanied by a decrease of the distance between eyes and by a truncation of the anterior dorsal part (Figs. 4 and 5).

Paying attention to the brain, the loss of delineated structures in the forebrain was observed in embryos exposed to RU-PW 20 mg a.e./L where the telencephalon with olfactory bulbs and diencephalon, clearly visible in control embryos, were indistinguishable (Fig. 5, lower panel). Moreover, in lateral views, treated embryos showed an abnormal sliding and folding of the forebrain toward the rostral region in comparison to the control. Likewise, the eyes were located in a more dorsal and rostral region. In contrast, midbrain and hindbrain seemed to remain fairly delineated also in RU-PW embryos. This altered phenotype is well evident in the histological sections (Fig. 7).

3.2.2. Whole mount cartilage staining

To better characterize the anomalies of the cephalic region, the cartilages were stained with Alcian blue (Fig. 6).

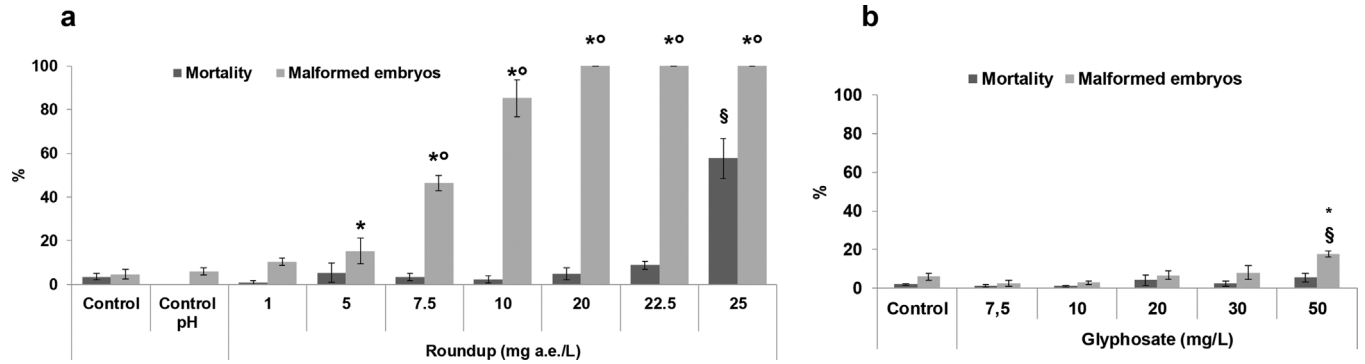

Fig. 2. Comparative embryotoxicity of Roundup® Power 2.0 and glyphosate, evaluated by FETAX. Mortality and malformation rates in 96 hpf embryos after exposure to RU-PW 1–25 mg a.e./L (a) and 7.5–50 mg/L glyphosate (b). Control pH group was exposed to FETAX solution with the pH adjusted to 6.8 value (see materials and methods). All values are given as mean ± SE of three independent assays. (*) statistically different from control, (°) malformation and (§) mortality rates statistically different from those registered at the lower concentrations. ($p < 0.01$, ANOVA + Fisher LSD Method).

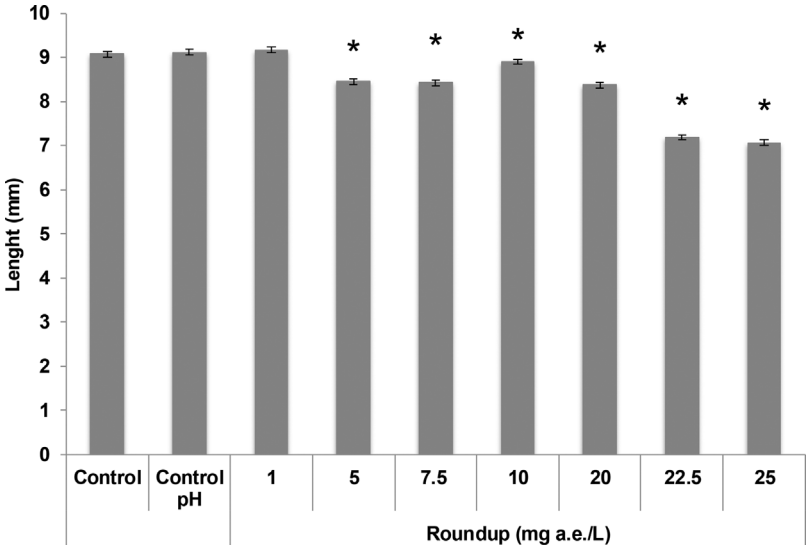

Fig. 3. Head-tail length of stage 46 embryos. Error bar represents ± SE of the mean length of embryos at the end of Roundup® Power 2.0 FETAX experiments. (*) indicates significant differences compared with controls ($p < 0.01$, ANOVA + Fisher LSD Method).

The analysis at the dissection microscope of control embryos showed the organization of the cartilaginous elements derived from cranial neural crest cells that, after detachment from mid-hindbrain region of neural tube, have colonized the branchial arches (Fig. 6). In particular, Meckel's and palatoquadrate cartilages, constituting the lower and upper jaw elements respectively, are evident as well as ceratohyal cartilages and gill basket (Fig. 6, upper panel). Moreover, the ethmoid-trabecular cartilage and the cartilage supporting the otic vesicles are visible in lateral view (Fig. 6, lower panel). RU-PW treatment interferes with development of cranial cartilage structures in concentration dependent manner, up to almost complete disappearance of upper and lower jaws in embryos exposed to 20 mg a.e./L. Only some sketches of ceratohyal remain detectable.

3.2.3. Histopathological analysis

In order to supplement the gross morphological observations, serial transverse E&E stained histological sections were performed in 46 stage embryos and examined with a light microscope (Fig. 7).

In control embryos, section passing through olfactory bulbs (Fig. 7, line A) showed the pharynx cavity bordered by cartilaginous structures

Table 2
Pattern of malformations in *Xenopus laevis* embryos exposed to Roundup® Power 2.0 and pure Glyphosate.

a	Control	Control pH 6.8	Roundup® Power 2.0 (mg a.e./L)						
			1	**5**	**7.5**	**10**	**20**	**22.5**	**25**
Total embryos	**521**	**220**	**224**	**300**	**303**	**225**	**304**	**228**	**172**
Living embryos	**503**	**220**	**222**	**283**	**293**	**220**	**289**	**208**	**78**
Severe	3 (0.6)	1 (0.5)	2 (0.9)	2 (0.7)	3 (1.0)	1 (0.5)	4 (1.4)	13 (6.3)*	5 (6.4)*
Gut miscoiling	12 (2.4)	8 (3.6)	17 (7.7)*	27 (9.5)*	98 (33.4)*	153 (69.5)*	262 (90.7)*	195 (93.8)*	73 (93.6)*
Edema Multiple	2 (0.4)		1 (0.5)	2 (0.7)	30 (10.2)*	218 (99.1)*	152 (52.6)*	111 (53.4)*	23 (29.5)*
Cardiac	2 (0.4)	2 (0.9)	6 (2.7)*	1 (0.4)	5 (1.7)	22 (10.0)*	61 (21.1)*	35 (16.8)*	4 (5.2)*
Abdominal	1 (0.2)		3 (1.4)		8 (2.7)*	36 (16.4)*	23 (8.0)*		22 (28.2)*
Facial	2 (0.4)	1 (0.5)	3 (1.4)		15 (5.1)*	18 (8.2)*	4 (1.4)	38 (18.3)*	36 (46.2)*
Blisters							25 (8.7)*	44 (21.2)*	15 (19.2)*
Craniofacial defects	8 (1.6)	6 (2.7)	8 (3.6)	25 (8.8)*	76 (25.9)*	149 (67.7)*	242 (83.7)*	194 (93.3)*	72 (92.3)*
Eye defects	8 (1.6)	2 (0.9)	6 (2.7)	9 (3.2)	36 (12.3)*	81 (36.8)*	263 (91.0)*	195 (93.8)*	61 (78.2)*
Hemorrhage		1 (0.5)			7 (2.4)*	31 (14.1)*	29 (10.0)*	20 (9.6)*	17 (21.8)*

b	Control			Glyphosate (mg/L)		
		7.5	**10**	**20**	**30**	**50**
Total embryos	**228**	**226**	**226**	**225**	**219**	**225**
Living embryos	**224**	**224**	**224**	**216**	**214**	**213**
Severe					2 (0.89)	
Gut miscoiling	7 (3.13)		2 (0.89)	7 (3.13)	5 (2.23)	7 (3.13)
Edema Multiple	3 (1.34)		1 (0.45)	1 (0.45)		
Cardiac			1 (0.45)	7 (3.13)	9 (4.02)*	32 (14.3)*
Abdominal	1 (0.45)				1 (0.45)	
Facial						1 (0.45)
Blisters	1 (0.45)					1 (0.45)
Craniofacial defects	2 (0.89)		2 (0.89)	1 (0.45)		2 (0.89)
Eye defects	4 (1.78)		2 (0.89)	1 (0.45)		3 (1.4)
Hemorrhage			1 (0.45)			1 (0.45)

Percentages based on number of malformations/number of those living.
Bold was used to differentiate malformation incidence from total number of embryos used and from living embryos.
* chi square test: $p < 0.001$ vs Control.

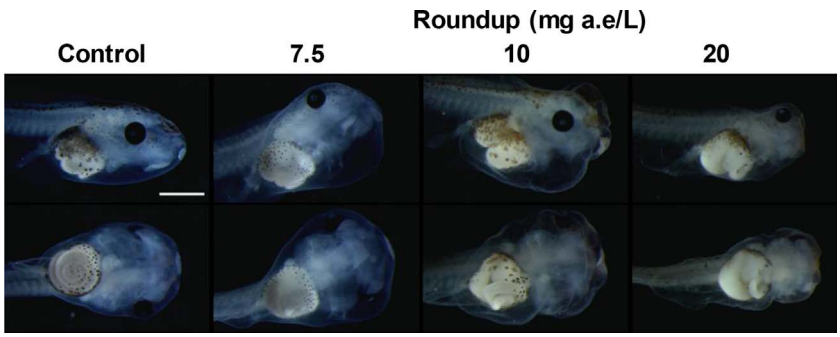

Fig. 4. Lateral (upper panel) and ventral (lower panel) views of representative *X. laevis* embryos at 96 hpf exposed to Roundup® Power 2.0 (mg a.e./L). In comparison to control phenotype, exposed embryos were severely affected by edemas, gut miscoiling and alteration in shape and size of head structures. Scale bar = 500 µm.

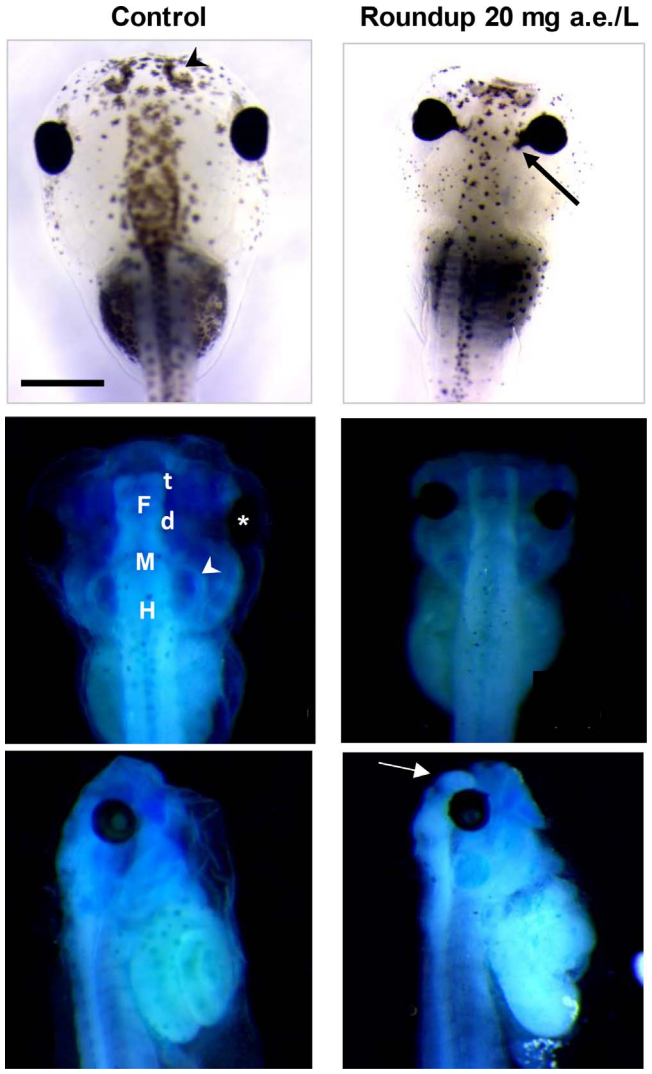

Fig. 5. Representative dorsal (upper, middle panels) and lateral (lower panel) views of cephalic region of *X. laevis* embryos at 96 hpf. Embryos exposed to Roundup® Power 2.0 20 mg a.e./L show eye abnormal phenotype such as microphthalmia and eyes narrowing towards the midline of the body (upper panel). The eyes are located close to the brain and the optic tract is covered by pigment (black arrow, upper panel). In control embryos, a clear delineation of the telencephalic (t) and diencephalic (d) portions of the forebrain (F) as well as the midbrain (M) and hindbrain (H) are evident (middle panel). The treatment seems to prevent the regionalization of the forebrain (telencephalon with olfactory bulbs and diencephalon are indistinguishable, middle panel) and caused its folding and sliding forward (white arrow, lower panel). (*) eye; (white arrowhead) otocyst. Scale bar = 500 µm.

and levator mandibular muscle fibers well organized. In sheer contrast, treated embryos showed a concentration dependent damage of craniofacial cartilages and associated muscles arrangement that culminate with an unarticulated and reduced mouth opening. Furthermore, the treatment with the highest concentration of RU-PW altered the morphology of the telencephalon, which lacks of the paired olfactory bulbs.

In section passing through eye level (Fig. 7, line B), control embryos showed differentiated eyes characterized by *tapetum nigrum*, retina and crystalline lens well apart from diencephalon. Moreover, the velar plate occupied the pharynx cavity ventrally. In treated embryos, the above-mentioned structures were progressively affected showing a range of phenotypes from mild to severe in relation to dose. In particular, the eyes appeared to be modified in shape, close to the neural tube and showed a disorganization of the retina multipolar cell layers at RU-PW 10 and 20 mg a.e./L (Fig. 8).

In section passing through rhombencephalon and otic vesicles (Fig. 7, line C), control embryos were characterized by the notochord and parachordal cartilages and by velar plate that divides the pharynx into two lateral branchial cavities. Ventrally, the three-chambered heart was well evident. In RU-PW treated embryos, velar plate and branchial chamber phenotype were progressively damaged. The treatment did not hinder the formation of the three cardiac chambers, but the atria have appeared more widened and the ventricular myocardial wall seemed thinner and provided by few trabeculae, especially in embryos exposed to 20 mg a.e./L. In addition, the cardiac region of all treated embryos is affected by the presence of edema. Cross-sections of control embryos at abdominal level (Fig. 7, line D) show the appearance of the first intestinal loops and near liver, pancreas and stomach. In the dorsal side, the nothocord, bordered by somitic musculature and the spinal cord are well evident. In embryos exposed to RU-PW 7.5 and 10 mg a.e./L, the abdominal region presents mild changes in organ distribution. More severe injuries are evident in embryos treated with 20 mg a.e./L, where morphology and localization of primitive organs are affected and the intestinal tract is still characterized by abundant vitelline platelets and by almost complete closure. In addition, only few pronephric tubules can be seen and somitic musculature, surrounding notochord and spinal cord, is poorly organized.

4. Discussion

The present study was planned to investigate the response of *X. laevis* embryos to a relatively new formulation of Roundup (Roundup® Power 2.0), currently authorized in Italy (http://www.fitosanitari.salute.gov.it/fitosanitariswsWeb_new/FitosanitariServlet), and for which no toxicological data are available so far.

Since very few data relate to GBHs exposure during early development, the FETAX test was used to follow the early phases of *X. laevis* development, when important morphogenetic movements and primary organogenesis occur. As shown in previous studies, FETAX is a reliable and sensitive test to detect developmental toxicants, in particular pesticides toward which *X. laevis* embryos were often found to be highly sensitive (Bonfanti et al., 2004; Colombo et al., 2005; Di Renzo et al., 2011).

Unlike other Roundup formulations (Roundup® Original, Vision® and Roundup® UltraMax) previously tested in *X. laevis* embryos (Perkins

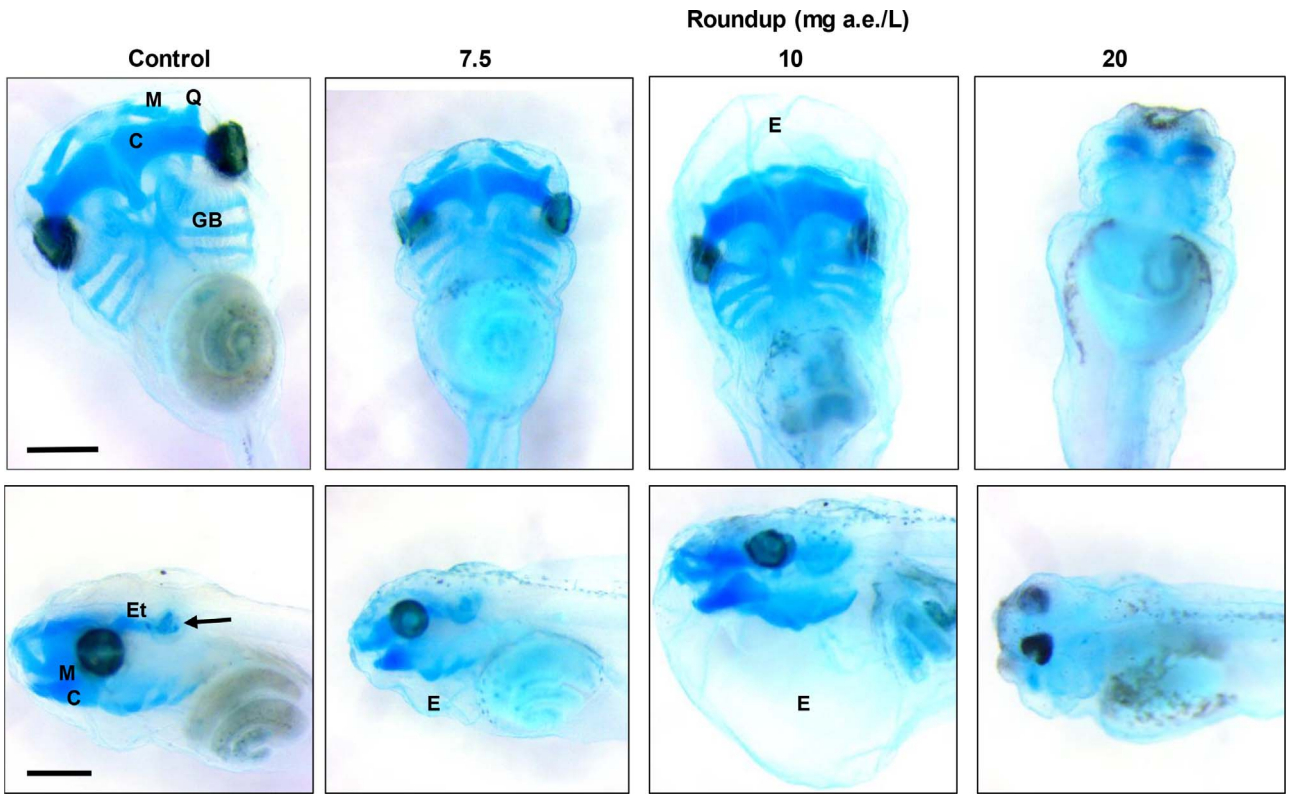

Fig. 6. Representative chondrocranial alterations resulting from Roundup treatment of *X. laevis* embryos at 96 hpf. In control embryos, Meckel's (M) and palatoquadrate (Q) cartilages derived from the first branchial arch, the ceratohyal (C) cartilages derived from the second branchial arch and the gill basket (GB) derived from the more posterior arches are well evident (upper panel, ventral view). In lateral view (lower panel), ethmoid-trabecular cartilage (Et) and the cartilage supporting the otic vesicles (arrow) can be observed. In comparison to control, RU-PW treatment caused a progressive reduction until the almost complete disappearance of the cartilaginous elements in embryos exposed to Roundup® Power 2.0 20 mg a.e/L. E = edema. Scale bars 500 µm.

et al., 2000; Edginton et al., 2004; Wagner et al., 2016), RU-PW contains glyphosate potassium salt instead of IPA salt, and a little percentage of a particular ethoxylated ether alkyl amine surfactant, declared as having high affinity with leaf cuticle waxes.

Beside lethality, a high incidence of malformations during early development is an important issue to be considered in amphibian decline because it can impair the success of metamorphosis and increase the natural population losses due to predation, competition and parasitism (Collins, 2010).

The main finding of this study is that RU-PW has a TI of 3.4 and thus is to be classified as highly teratogenic according to the ASTM guide (1998). This is the first time that a teratogenic potential is reported for a GBH, because other formulations tested during embryonic development (i.e. Roundup® Ultramax) were reported to have a TI < 1.5, although concentration dependent increments in malformation rates were evidenced both for *X. laevis* and *Discoglossus pictus* embryos (Wagner et al., 2016).

On the contrary, glyphosate did not show any embryolethality and only at the highest concentration (50 mg a.e./L) embryos showed diffused edemas statistically different from controls. The difference between the toxicity observed with our commercial formulation compared to the glyphosate is in agreement with most studies previously performed in amphibians and other aquatic organisms. In one of the first Roundup® acute toxicity studies performed on four aquatic invertebrate and four fish species, Folmar et al. (1979) had already evidenced that the toxicity of the surfactant POEA was similar to those of Roundup® formulation, while technical grade glyphosate was considerably less toxic. Perkins et al. (2000) confirmed these findings in *X. laevis* embryos, demonstrating that Rodeo®, a commercial surfactant free-formulation, was 575 times less toxic than Roundup® with POEA. Similarly, studies in different anuran larvae established that surfactants appear to be primary responsible for the acute toxicity of GBH formulations (Mann and Bidwell, 1999; Howe et al., 2004).

Unfortunately, in this work we did not have the chance to test the surfactant toxicity because not commercially available and, to the best of our knowledge, there were not previous toxicity test for a comparison. Nevertheless, based on 96 h LC50 values reported in previous FETAX studies, we can state that RU-PW is not as lethal (96 h LC50 24.78 mg a.e./L) as other GBH formulations for *X. laevis* embryos. In detail, it results less toxic than Roundup® original (96 h LC50 9.3 mg a.e./L) of 2.66 times (Perkins et al., 2000), than Vision (considering 96 h LC50 7.9 mg a.e./L obtained at pH 7.5) of 3.14 (Edginton et al., 2004) and than Roundup® Ultramax (96 h LC50 19.36 mg a.e./L) of 1.3 (Wagner et al., 2016). Assuming that the embryolethality is caused mainly by the surfactant, we can hypothesize that the lower embryotoxicity of RU-PW is due to the type of surfactant added as well as to its lower percentage content (6% by volume versus 15% of POEA in Roundup® Original and Vision® and 7.5 wt% of ether amine ethoxylate in Roundup® Ultramax). However, the amounts of surfactant calculated in all the above-mentioned GBH solutions at the concentrations corresponding to the respective LC50 values are comparable (about 4 µL/L). This indicates that the reduced embryolethality of RU-PW is related to the lower surfactant percentage rather than to the kind of surfactant present in the formulation. At the highest tested concentration (100 mg a.e./L), the embryotoxicity was so severe as to prevent embryonic development already after 24 hpf, likely being the result of alterations in lipid composition and fluidity of cell membranes and subsequent loss of osmotic stability caused by the surfactant (Cardellini and Ometto, 2001).

On the other hand, sublethal RU-PW concentrations induced dose dependent teratogenic effects (96 h EC50 7.8 mg a.e./L) with phenotypes including abnormal gut coiling, craniofacial and eye defects

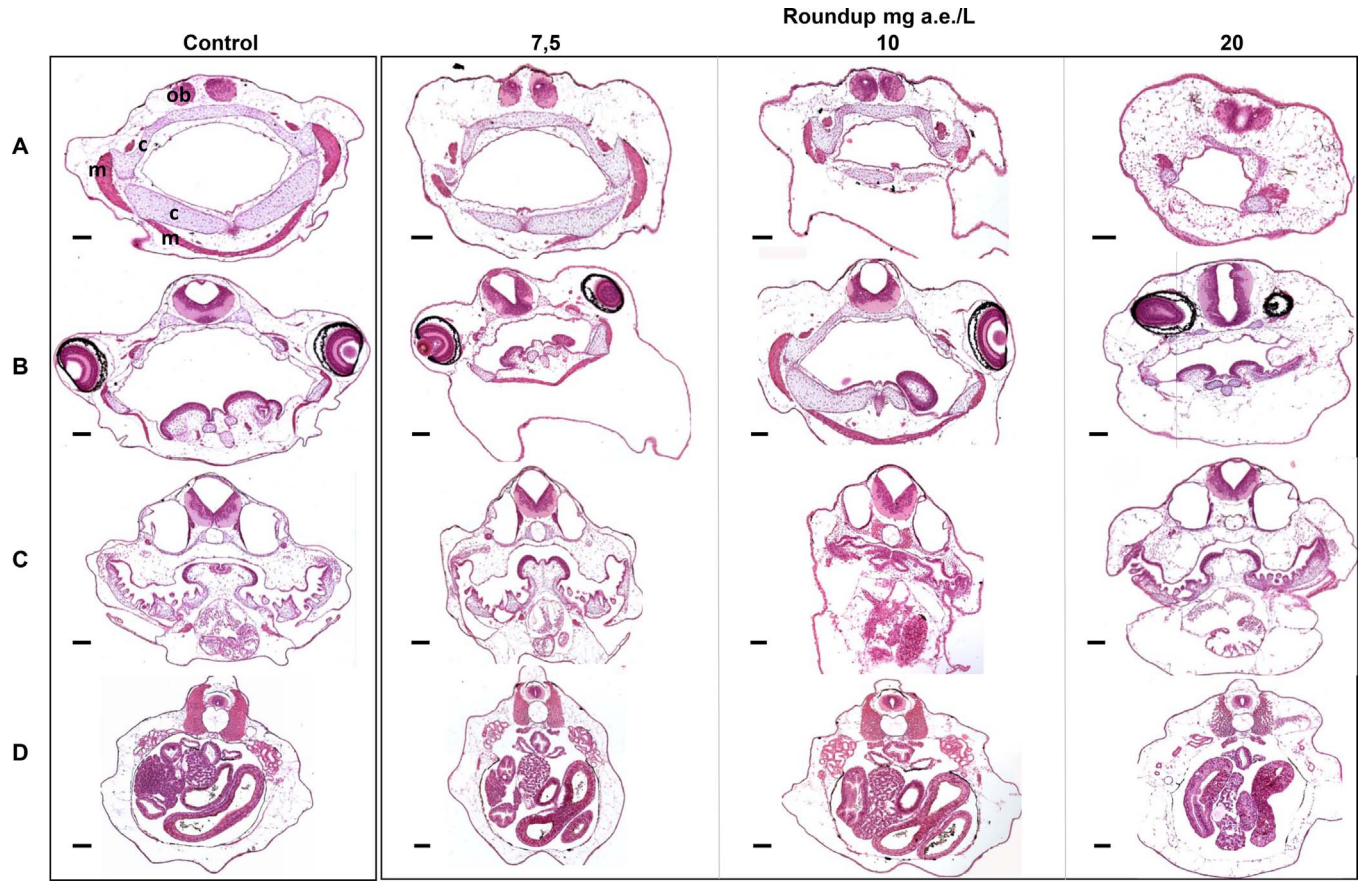

Fig. 7. Histological transversal sections of stage 46 *X. laevis* embryos at level of olfactory bulbs (line A), eyes (line B), rhombencephalon (line C), abdominal region (line D). Note the altered morphology and localization of primitive organs in the treated embryos compared to control. ob = olfactory bulbs; c = cartilage; m = muscle. Scale bar = 100 μm.

starting from 5 mg a.e./L. Moreover, at 10 mg a.e./L cardiac, abdominal and multiple edemas in almost all surviving embryos were detected. While improper gut coiling, edemas and ocular abnormalities (reduced size and oval shape) were previously described as malformations occurring in *X. laevis* embryos exposed to different non-ionic surfactants, which however have a TI < 2.0 (Presutti et al., 1994; Mann and Bidwell, 2000), cephalic and chondrocranial alterations are relevant to GBH exposure during early development (Paganelli et al., 2010; Wagner et al., 2016). Since stage 46 *X. laevis* embryos, developing after injection of pure glyphosate at 2-cell stage, displayed the same phenotypes of GBH exposed embryos, Paganelli et al. (2010) conclude that glyphosate and not the adjuvant could be responsible for the onset of these malformations. In the same paper, they argued that glyphosate itself causes an increase of endogenous retinoic acid activity, consistent with the observed decrease of sonic hedgehog (*shh)* signalling from the embryonic dorsal midline, especially from the prechordal mesoderm, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development. The narrowing of the eyes towards the midline of the embryos, the loss of olfactory bulbs and the forebrain regionalization we observed in our samples are similar to the holoprosencephalic syndrome cited by Paganelli, coherent with inhibition of anterior *shh* signalling and reduced *otx2* domain, which impair the brain separation into two hemispheres, the eye field subdivision and the craniofacial development.

In the light of the above discussed, we hypothesize that the RU-PW malformations induced in *X. laevis* embryos can be attributed only minimally to the surfactant, while a significant contribution to the appearance of specific phenotypes is given by the active ingredient glyphosate.

Since in our study glyphosate does not display teratogenicity, the

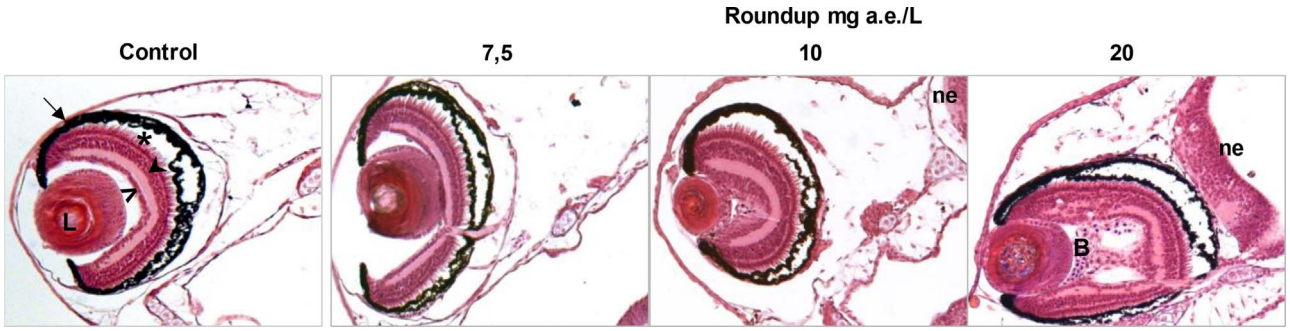

Fig. 8. Representative transverse sections of eyes at higher magnification. In Roundup® Power 2.0 treated embryos, a disorganisation of retina layers is evident at 10 and 20 mg a.e./L, worsened by the presence of haemorrhagic areas (B) in 20 mg a.e./L. Note the closeness of eye to neural epithelium (ne). (→) pigmented layer of the retina; (*) fotoreceptor layer; (◀) bipolar cell layer; (>) multipolar cell layer; (L) lens.

teratogenic potential of RU-PW observed for the first time in formulation with this brand can be related to the typology of its characteristic components. In particular, the smaller size of glyphosate potassium salt associated with a high-affinity adjuvant for cuticle waxes, as highlighted in data sheet, may be the reason for more efficient glyphosate penetration through biological barriers of non-target organisms. This hypothesis is supported by the DLS results which have shown that the RU-PW formulation consists of a micro-emulsion, being constituted by micelles with hydrodynamic diameter of about 4 µm, a feature that facilitates their interaction with cells.

Also a recent zebrafish early development study supports the idea that glyphosate itself is a developmental toxicant, being able to induce cephalic and eye reductions and to exert neurotoxicity with loss of brain ventricle delineation comparable to those of commercial formulation Roundup® classic (Roy et al., 2016a). Moreover, in an additional study Roy et al. (2016b) demonstrated cardiotoxicity of glyphosate in zebrafish embryos, which recalls the malformations to cardiac chambers appreciated in our histological sections of 20 mg a.e./L exposed embryos, evidencing another developmental target of glyphosate.

To the best of our knowledge, the mechanism underlying retinoic acid signalling disruption by glyphosate has not yet been elucidated, although the CYP26 enzyme, essential for the catabolism and homeostasis of retinoic acid during development, is suspected of being a good candidate. In fact, several cytochrome P450 members resulted to be targets of glyphosate. For example, hepatic level of cytochrome P-450 and monooxygenase activities in the rat are decreased by glyphosate (Hietanen et al., 1983). Moreover, activity and mRNA levels of aromatase, a CYP450 that converts testosterone to estrogen, are disrupted by exposure to glyphosate in human cell lines (Richard et al., 2005: Gasnier et al., 2009). In amphibians, a study showed gonadal abnormalities in *Rana pipiens* tadpoles chronically exposed to environmentally relevant concentrations of glyphosate formulations, even if they were related in part to disruption of thyroid hormone signalling (Howe et al., 2004).

In addition to CYP26, it would be interesting to explore if other mechanisms of action of glyphosate and GBH, well documented in amphibian and fish larvae, could contribute to the onset of observed toxic effects on *Xenopus* embryos. As suggested by numerous studies, oxidative stress is one of the mechanisms of glyphosate toxicity (Annett et al., 2014). In different larval anuran species, including *Xenopus*, glyphosate exposure can cause alteration in the activity of antioxidant enzymes such as glutathione S-transferase (GST) and glutathione reductase (Lajmanovich et al., 2011; Güngördü, 2013). The unregulated generation of reactive oxygen species (ROS) derived by the unbalance of antioxidant systems can initiate oxidative damage to nucleic acids, lipids, and proteins compromising cellular integrity and inducing apoptosis. Since apoptosis has a crucial role in a variety of morphogenetic events during development, its increase may compromise normal development. The link between the appearance of malformations and apoptosis caused by ROS abundance was highlighted in zebrafish embryos where glyphosate exposure caused an inhibitory effect of carbonic anhydrase enzyme (Sulukan et al., 2017). Moreover, although glyphosate is not classified as an acetylcholinesterase (AChE) inhibitor, some studies have reported that exposure to GBH causes inhibition of this enzyme activity in aquatic organisms (Lajmanovich et al., 2011; Modesto and Martinez, 2010; Sandrini et al., 2013). On the contrary, glyphosate caused an increase in AChE activity in tadpoles of three different anuran species, of which *Xenopus laevis* resulted the most sensitive (Güngördü, 2013). Since it has been demonstrated that AChE is required for normal muscle and neuron development in fish and amphibians (Behra et al., 2002; Bonfanti et al., 2004), the alteration of its activity may be a contributing factor to the teratogenic effects induced by RU-PW.

Considering that RU-PW has a low embryolethality and a high teratogenic potential in *Xenopus* embryos, it would be important to evaluate its impact on wildlife anuran embryonic development. It has been shown that amphibians have variable sensitivity to GBHs depending on the species and stages of development (Howe et al., 2004; Fuentes et al., 2011). Even if larval stages are more susceptible to GBHs than embryos mainly because of surfactant action on gills epithelia (Wagner et al., 2016), a teratogenic effect during morphogenetic process of embryonic development could lead to larvae unable to eat and to accomplish the delicate phase of metamorphosis, with long-term effects on species survival.

As the 96 h EC 50 value of 7.8 mg a.e./L is much higher than the background values measured in surface water, the RU-PW application does not seem to pose any apparent risk. However, it is important to consider that this value approaches the expected environmental concentrations (EECs) in worst-case scenarios, that as reported by Wagner et al. (2013) can reach up to 7.6 mg active ingredient/L. Considering that, land area treated with GBHs rose rapidly, with the consequent appearance of weed phenotype less sensitive to glyphosate, the rate and the number of applications continue to increase (Benbrook, 2016). Therefore, concentrations as high as EECs could easily be reached in lentic and ephemeral waters close to agricultural areas, where amphibians prefer to breed. Unfortunately, there is a lack of accurate monitoring data on these waters, necessary for an adequate risk assessment (Battaglin et al., 2009). It should also be noted that near the agricultural areas, lentic and ephemeral waters become collectors of pesticide mixtures, which could synergistically affect amphibian development. It has been shown for example that triazole fungicides interfere with RA pathway, causing craniofacial malformations in *Xenopus* embryos similar to those induced by GBHs (Di Renzo et al., 2011).

Another important consideration is that FETAX constitutes an efficient development toxicity alert test, not only to evaluate environmental toxicants for potential effects on amphibian populations, but also to predict human teratogens with a 75% accuracy (Fort and Robbin, 2002). RU-PW teratogenic potential is manifested with specific malformations, which appear recurrent when exposure to different GBHs occurs during amphibian early developmental stages, even though at different concentrations depending on the type of formulation. As mentioned above, the onset of these specific malformations was correlated with the increase of RA, a known morphogen involved in morphogenetic processes common to all Vertebrates (Paganelli et al., 2010). It would be important to expand the knowledge on these mechanistic aspects even during the development of mammals to assess whether or not a risk for human embryo development exists following chronic or accidental exposure during pregnancy. This is even more important since it has been shown that glyphosate can cross the placenta (Poulsen et al., 2009).

5. Conclusion

In the present study, we have characterized for the first time the teratogenic hazard of a commercial GBH. Although not embryolethal, RU-PW induced a dose-dependent increase of craniofacial and eye malformations on *Xenopus* embryos from a concentration of 5 mg a.e./L and marked neural defects at 20 mg a.e./L. In addition, this study has highlighted that slight differences in GBH formulation, in terms of active ingredient and surfactant, can modulate sub-lethal and/or long-term effects (e.g. teratogenicity) and thus should be carefully considered by the authorities. Finally, further research concerning mechanistic toxicity of glyphosate and GBHs are needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2017.12.007.

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