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Effects of a commonly used glyphosate-based herbicide formulation on early developmental stages of two anuran species

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Abstract Environmental contamination, especially due to the increasing use of pesticides, is suggested to be one out of six main reasons for the global amphibian decline. Adverse effects of glyphosate-based herbicides on amphibians have been already discussed in several studies with different conclusions, especially regarding sublethal effects at environmentally relevant concentrations. Therefore, we studied the acute toxic effects (mortality, growth, and morphological changes) of the commonly used glyphosate-based herbicide formulation Roundup® UltraMax on early aquatic developmental stages of two anuran species with different larval types (obligate vs. facultative filtrating suspension feeders), the African clawed frog (*Xenopus laevis*) and the Mediterranean painted frog (*Discoglossus pictus*). While *X. laevis* is an established anuran model organism in amphibian toxicological studies, we aim to establish *D. pictus* as another model for species with facultative filtrating larvae. A special focus of the present study lies on malformations in *X. laevis* embryos, which were investigated using histological preparations. In general, embryos and larvae of *X. laevis* reacted more sensitive concerning lethal effects compared to early developmental stages of *D. pictus*. It was suggested, that especially the different morphology of their filter apparatus and the higher volume of water pumped through the buccopharynx of *X. laevis* larvae lead to higher exposure to the formulation. The test substance induced similar lethal effects in *D. pictus* larvae as it does in the teleost standard test organism used in pesticide approval, the rainbow trout (*Oncorhynchus mykiss*), whereas embryos of both species are apparently more tolerant and, conversely, *X. laevis* larvae about two times more sensitive. In both species, early larvae always reacted significantly more sensitive than embryos. Exposure to the test substance increased malformation rates in embryos of both species in a concentration-dependent manner, but not at environmentally relevant concentrations. However, the assumed field safety, based on calculated surface water concentrations of the active ingredient (glyphosate), should be validated with realistic field data and buffer strips have to be urgently regarded to any aquatic amphibian habitat.

Keywords Amphibia · Glyphosate · Pesticide · Roundup® UltraMax · *Xenopus laevis* · *Discoglossus pictus*

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Introduction

Worldwide, amphibian populations are declining (Houlahan et al. 2000; Stuart et al. 2008) and environmental contamination is suggested to be one out of six main reasons (Collins and Storfer 2003). Amphibian populations are often persisting in landscapes, which have been transformed for agriculture (Mann et al. 2009). Contamination of aquatic and terrestrial amphibian habitats with agrochemicals can occur by way of direct over-spraying (Brühl et al. 2013), wind drift (Davidson et al. 2002), run-off (Edwards et al. 1980), or drainage (Brown and van Beinum 2009). Indirect exposure routes for amphibians are contaminated food (McComb et al. 2008) or contaminated soil and plant material (Brühl et al. 2011). Even in protected areas, amphibian species can be at risk of pesticide

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exposure (Wagner et al. 2014a). Although causal relationships between population declines and increasing use of agrochemicals are widely lacking (Schmidt 2004; Wagner et al. 2013), the results of the field studies by Smalling et al. (2013, 2015) even suggested that higher pesticide amounts accumulate in frog tissues compared to water and sediment samples. Furthermore, in several laboratory and field studies, negative effects of environmentally relevant pesticide concentrations at the individual level have been observed (e.g., McCoy et al. 2008; Williams and Semlitsch 2010; Brühl et al. 2013; Wagner et al. 2015a). These include acute toxic, chronic, and delayed effects (e.g., Howe et al. 2004; Williams and Semlitsch 2010; Wagner et al. 2015b) and also indirect effects like avoidance of contaminated breeding sites (Takahashi 2007; Vonesh and Buck 2007; but see Wagner and Lötters 2013a). Although herbicides are applied in much higher amounts than other pesticides, for instance, many more insecticides than herbicides have been tested in amphibian toxicological studies (Weir et al. 2012). Glyphosate-based herbicides are dominating the world's herbicide market, mainly because they are complementary herbicides for crops with a genetically engineered tolerance to glyphosate (Duke and Powles 2008; Wagner and Lötters 2013b). However, glyphosate-based herbicides are also used in no-tillage farming in conventional agriculture, viticulture, forest management, non-cultivated areas and private gardening (Dill et al. 2010). The impact of glyphosate-based herbicides on amphibians has been found to be formulation, taxon and life-stage specific and abiotic and biotic co-stressors mainly increased adverse effects in conducted amphibian toxicological studies (e.g., Mann and Bidwell 1999; Edginton et al. 2004; Howe et al. 2004; Relyea 2005a, b; Jones et al. 2010; Williams and Semlitsch 2010).

Early embryonic development includes basic steps of organogenesis. Larval development of amphibians before onset of metamorphosis is characterized by increased metabolism and growth. Because of these differences and the suggestion that the larvae are more sensitive due to their enhanced metabolism (see Wagner et al. 2013 and references therein), we investigated the effects of a commonly used glyphosate-based herbicide formulation on both embryos and early stage larvae of two anuran species: the African clawed frog (*Xenopus laevis*, family Pipidae) and the Mediterranean painted frog (*Discoglossus pictus*, family Alytidae). Selected endpoints were growth, mortality, and morphological changes.

Phylogeny, morphology, and life history are different in these larval species (Wells 2007). On the basis of morphological characters, Orton (1953) allocated *Xenopus* to the larval type I (i.e., obligate filtrating feeders) and *Discoglossus* to type III (i.e., facultative filtrating feeders). Both species are suspension feeders, but *X. laevis* has obligate filtrating and *D. pictus* facultative filtrating larvae based on different morphology and efficiency of their filter apparatus including the volume of water pumped through their buccopharynx (Seale and Wassersug 1979). Water volume pumped by *Xenopus laevis* is around 2 to 14 times higher than in *Epidalea (Bufo) calamita*, *Rana temporaria*, and *Bufo bufo* at comparable larval stages as demonstrated by the different ingestion and filtering rates (Viertel 1990, 1992). *D. pictus* is more closely related to *E. calamita*, *R. temporaria*, and *B. bufo* than to *X. laevis*. The filter apparatus of *D. pictus* compares with *E. calamita*, *R. temporaria*, and *B. bufo* but not with *X. laevis*. So it is concluded that the water volume in *D. pictus* is lower than in *X. laevis* and in consequence also the exposure to the test item. The contact of the larvae with the compound increases with the water volume ingested. Therefore, a connectivity of the pumped water volume rate and the exposure to a xenobiotic is very likely (see also Wagner et al. 2015c).

For the aforementioned reasons and based on preceding studies of toxic effects of herbicides on embryos and early larvae of *Xenopus* and *Discoglossus*, we hypothesized that

(1). in general, early developmental stages of *X. laevis* react more sensitive to the test compound than those of *D. pictus* because aquatic developmental stages of *X. laevis* were found to be the most or at least among the most sensitive species in other studies (e.g., for the glyphosate-based herbicide Vision®: Edginton et al. 2004; for different agricultural surfactants: Mann and Bidwell 2001; for the cycloxydim-based herbicide Focus® Ultra: Wagner et al. 2015b, c);

(2). the compound induces only in embryos and larvae of *D. pictus* similar lethal effects as it does in aquatic standard test organisms while early developmental stages of *X. laevis* are more sensitive because this was also observed after exposure to the cycloxydim-based herbicide Focus® Ultra (Wagner et al. 2015b, c).

In accordance with studies that compared the responses of different developmental stages of anurans to glyphosate-based herbicides, we furthermore hypothesized that

(3). in both species early larvae react more sensitive than embryos (for glyphosate-based herbicides, for instance: Edginton et al. 2004; Howe et al. 2004; for the cycloxydim-based herbicide Focus® Ultra, Wagner et al. 2015b, c).

(4). The teratogenicity of glyphosate-based herbicides is discussed, especially regarding environmentally relevant concentrations (Perkins et al. 2000; BVL 2010; Paganelli et al. 2010). For a definition of teratogenicity see Wilson and Warkany (1965).

Our (4) hypothesis was that the formulation under research significantly increases malformations in embryos of both species in a concentration-dependent manner, but not at environmentally relevant concentrations.

Material and methods

Test organisms and test substance

The African clawed frog (*Xenopus laevis*) is a member of the anuran family Pipidae and originates from the southern part of Africa (Nieuwkoop and Faber 1956). The developmental stages of *X. laevis* are often used in amphibian toxicological studies as surrogates for other aquatic organisms (including other amphibian larvae) because of their high sensitivity to pesticides and their availability as a laboratory species (ASTM 1998; Bantle et al. 1998, 1999; Wagner et al. 2015a). Developmental biology of *X. laevis* is well known, which was the reason to include the species into the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX: ASTM 1998). Reproduction was initiated by injection of human chorionic gonadotropin into the dorsal lymph sac of both genders.

The Mediterranean painted frog (*Discoglossus pictus*) is distributed in northern Algeria and Tunisia and eastern Morocco (Vences et al. 2014) and on the islands of Sicily (Italy), Malta and Gozo (Malta). Furthermore, it is introduced to southern France and northeastern Spain (Girona Province), where it is expanding its range (Bosch et al. 2009). It inhabits a variety of aquatic and terrestrial habitats including cultivated landscapes (Bosch et al. 2009). The eggs of *D. pictus* are available over the whole year—which is not the case in, for instance, Central European species. To obtain eggs from *D. pictus*, females and males with visible nuptial pads at the forelimbs were placed (from terraria with an aquatic and a terrestrial part and ca. 23 °C) into small plastic terraria containing about 1 L of ca. 15 °C cold water over night. Aquatic life stages are relatively fast developing (in the closely related *D. scovazzi* about 1 month at ca. 23 °C from egg deposition until completed metamorphosis: Wagner et al. 2015b). These are benefits for its use in laboratory work.

We tested the effects of the commonly used glyphosate-based herbicide Roundup® UltraMax (RU-UM) on early developmental stages of both anuran organisms. According to its safety data sheet, RU-UM contains about 51 wt% of glyphosate isopropylamine salt (CAS 38641-94-0) as active ingredient (a.i.), which corresponds to 450 g a.i./L. The added surfactant (about 7.5 wt%) is not POEA (=polyethoxylated tallow amine as in several other glyphosate-based herbicides: Dill et al. 2010) but ether amine ethoxylate (CAS 71486-88-9). RU-UM is a non-selective, broad-spectrum foliar herbicide and, for instance, has been approved for 10 years in Germany (2/17/2004 to 12/31/2014, to use up 6/30/2016). RU-UM is not approved for use in private gardening, but it is commonly used for controlling weeds on agricultural fields (e.g., cultivation of corn, rape, cereals, or vegetables), grassland, non-cultivated areas, orchards, paths, railway tracks, in forests, and vineyards. This large field of applications increases the risk that non-target organisms like amphibians are exposed to RU-UM.

Test procedures

All experiments were conducted in a climate chamber at 23 ± 1 °C and 12:12-h light–dark cycle. All test solutions were freshly prepared with FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) solution (see ASTM 1998). Ammonium, nitrate, nitrite, dissolved oxygen (all mg/L), and pH were measured at the beginning and end of the experiments. For quality assurance, water samples for glyphosate analysis of stock solution and each test concentration were taken and stored at -20 °C in stainless steel containers. Samples were shipped on ice to an external, DIN-certified laboratory (Eurofins SOFIA GmbH, Berlin) for liquid chromatography-mass spectrometry (LC-MS/MS).

X. laevis embryo tests started with normally developed eggs at NF (Nieuwkoop and Faber 1956) stages 8–11, likewise *D. pictus* embryo tests with eggs at Gosner (Gosner 1960) stages 8–9 (=blastula to early gastrula). Glass petri dishes (60 mm in diameter) contained 10-mL test solution and 25 embryos. Experiments were terminated after 96 h when *X. laevis* individuals had reached NF stage 46 (*D. pictus* Gosner stage 22–23). The FETAX protocol (ASTM 1998) was applied, i.e., four controls were used, other test concentrations were duplicated (i.e., a total of 350 embryos), and solutions were renewed every 24 h (static renewal). The FETAX protocol was slightly modified regarding the jelly coating of the eggs and positive controls. In accordance with Yu et al. (2013), jelly coats of eggs were not removed because of concerns that the dejelling L-cysteine would induce teratogenic effects and to study a more natural development. On the one hand, leaving the jelly coat on could have changed the toxicity seen compared to the studies that used L-cysteine; on the other hand, it was found that the jelly coat did not totally protect the embryo from xenobiotics (Greulich and Pflugmacher 2003). Furthermore, no positive control was used to prohibit cross-contamination with 6-aminonicotinamide in the climate chamber and to reduce the amount of test animals. Based on prior range finding tests (unpublished data), nominal concentrations for *X. laevis* embryo tests were 0, 4.5, 9, 18, 36, 45, and 90 mg a.i./L and 0, 45, 90, 135, 180, and 225 mg a.i./L for *D. pictus* embryo tests. The results are represented in Fig. 1. At the end of the *X. laevis* experiment, we furthermore selected three malformed individuals and two control animals for pathohistological investigation. Photographs were taken under a Leica S8APO stereo microscope with the digital camera DFC290 (see Figs. 2 and 3). For the tests with early larvae, embryos were hatched in petri dishes (94 mm in diameter) containing 25 mL FETAX solution and 25 embryos each. In accordance with the standard protocol of the ASTM (2002), the tests with larvae started at NF stage 47 (*X. laevis*) and Gosner stage 25 (*D. pictus*), respectively (= free-swimming larvae). Only non-malformed larvae with normal swimming and feeding
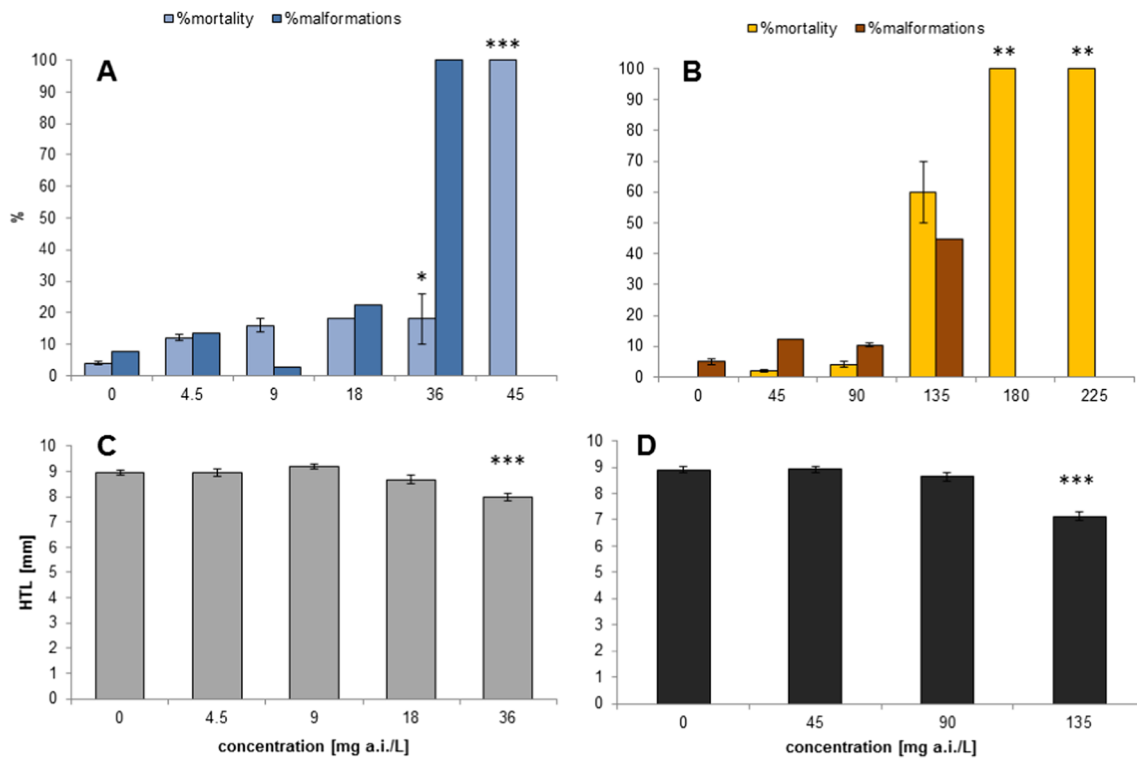
Fig. 1 Mortality and malformation rates in *X. laevis* (**a**) and *D. pictus* embryos (**b**) and growth inhibition due to exposure to Roundup® UltraMax in *X. laevis* (**c**) and *D. pictus* embryos (**d**). All values are given ±standard error. *Asterisks* indicate significant differences to the control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). *HTL* total length, head to tail length

behavior were introduced. The larvae tests were performed using 5-L full glass aquaria containing 1 L of test solution and 10 larvae each (which were randomly assigned to the experiment). Test concentrations were triplicated (i.e., a total of 180 larvae were tested). The non-renewal trials were terminated after 96 h when the larvae had reached NF stage 48 (*X. laevis*) and Gosner stage 26 (*D. pictus*). Based on prior range finding tests, nominal concentrations for *X. laevis* larvae

tests were 0, 0.9, 1.8, 3.6, 4.5, and 9 mg a.i./L and 0, 4.5, 9, 18, 27, and 36 mg a.i./L for *D. pictus* larvae.

Histology and staining of cartilage

Embryos selected for clearing and staining were double stained for bone and cartilage following Taylor and van Dyke (1985). Specimens selected for serial sections were dehydrated,
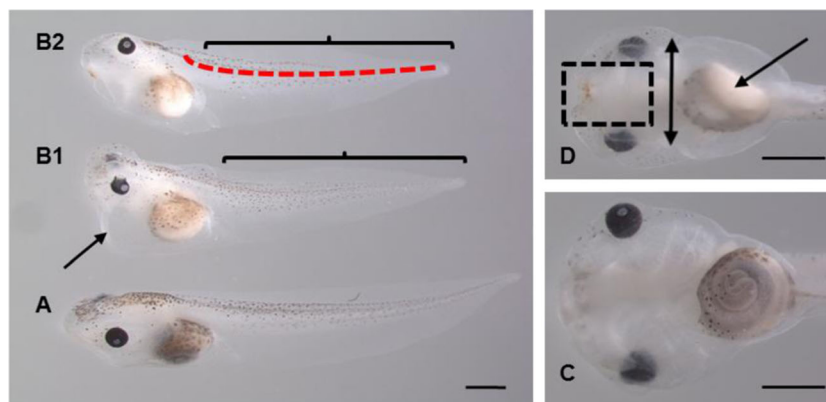

Fig. 2 *X. laevis*: *A* NF stage 46 from the control group (lateral view), *B1* and *B2* of the same developmental age as in *A* from the 36 mg a.i./L concentration group with developmental retardation and different grades of pathological changes (lateral view). They comprise changed body proportions (*curly brackets*), curved body axis and tail (*red dashed line*), edema (*arrow*), and malformations of the head-pharynx region (see *D*). *C* individual from *A* (ventral view), *D* individual from *B1* (ventral view) with malformed head-pharynx region (*dashed rectangle*), narrow body (*double arrow*) and changed intestinal convolution (*arrow*); *scale bars* = 1 mm

Fig. 3 *X. laevis*: chondrocranial development in a control larva of NF stage 46 in lateral (**a**) and ventral (**b**) view, and larva of the same developmental age from the 36 mg a.i./L concentration group in lateral (**c**) and ventral (**d**) view, with cartilage stained blue, (**c**) changed proportions of the chondrocranium (*curly brackets*), (**d**) changed position and shape of *chy*, *mc*, and *ir* (*arrows*) and of *cb* (*double arrow*); *scale bars* = 200 μm. *cbI* ceratobranchial I, *cbII* ceratobranchial II, *chy* ceratohyal, *ir* infrarostral cartilage, *mc* Meckel's cartilage, *oc* otic capsule, *ocr* otic capsule region
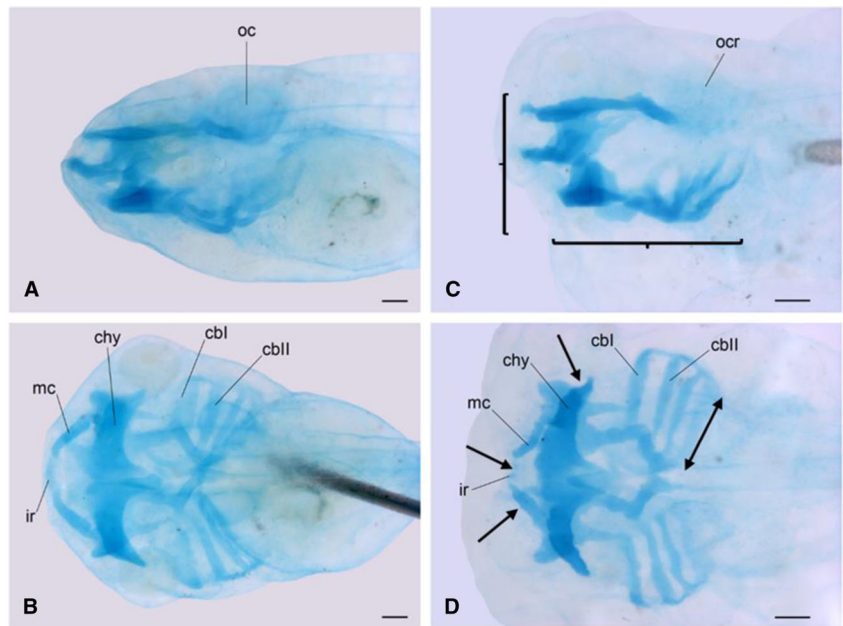
embedded in Histoplast S (Serva GmbH), and serially sectioned transversely at 7 μm using a Microm HM355 microtome. Sections were stained with azocarmine-red and anilin-blue (AZAN) and all histological preparations followed standard procedures (Böck 1989). Photographs of cleared and stained specimens were taken with a Zeiss SteREO Discovery V12 equipped with a Zeiss AxioCam Icc 1 digital camera.

All morphological changes including malformations were identified according to the tables of Bantle et al. (1998).

Considered endpoints and statistical analysis

Mortality, malformations (according to Bantle et al. 1998), and growth inhibition were monitored after 96 h. Embryos and larvae were photographed after euthanization with 150 to 200 mg/L MS-222 (OECD 2009) and fixation in 5 % formalin. The software "ImageJ" (National Institute of Health) was used to measure head–tail-length (or total length) (HTL) in embryos and larvae.

Ninety-six-hour LC50 and ninety-six-hour EC50 (malformation) values (median lethal and effect concentration, respectively) were calculated with probit analyses. Significant differences were examined by overlap tests of 95 % confidence intervals. Differences in mortality and malformation rates, and HTL (indicating growth inhibition) between groups were checked using one-way ANOVA (some data had to be Box-Cox transformed prior analysis), followed by Bonferroni-corrected post hoc tests (for small sample sizes). If normal distribution and homogeneity of variances could not be reached by data transformation, Kruskal-Wallis rank sum test followed by Wilcoxon rank sum test with continuity corrections were

conducted. The software R and the package MASS were applied for statistical analyses (R Developmental Core Team).

Results

Water analysis

In embryo tests, ammonium, nitrate, and nitrite were not measurable (i.e., 0 mg/L) at beginning and end of experiments. Dissolved oxygen and pH level remained constant through the experiments (8 and 6.8 mg/L, respectively). In the larvae tests, water parameters at beginning of the experiments (fresh test solutions) were the same as for embryo tests. At the end of the *X. laevis* tests (after 96 h), average values for ammonium were 0.4 mg/L, for nitrate 6.67 mg/L, and for dissolved oxygen 7 mg/L. At the end of the *D. pictus* tests, average values for ammonium were 0.4 mg/L, for nitrate 3.33 mg/L, and for dissolved oxygen 7 mg/L. At termination of both larvae experiments, nitrite was not measurable (0 mg/L) and pH level did not change (6.8). The slightly increase in ammonium and nitrate concentrations and decrease of dissolved oxygen were most probably caused by the increased metabolism of the larvae producing feces and by amounts of unconsumed food. Missing mortality demonstrated the validity of the study.

Glyphosate concentration of the measured stock solution was confirmed by the analysis of the DIN-certified laboratory (450 mg a.i./L). Due to strong deviation, the fifth test concentration in *D. pictus* larvae test (27 mg a.i./L) was excluded from further analysis. Remaining measured test concentrations were in average 100.49 ± 3.07 % of the calculated

concentrations (Table 1) and did not significantly differ (Wilcoxon-Mann-Whitney-Test: $W = 182$, $P = 0.98$).

Acute toxic effects of RU-UM on embryos

Because lack of overlap of the 95 % confidence intervals, *X. laevis* embryos were significantly more sensitive than *D. pictus* embryos; about fivefold more sensitive regarding lethal effects and about 3.5-fold more sensitive regarding the induction of malformations (Table 2). Because already overlap tests of the 83–84 % confidence intervals seem to be enough to give an approximate $\alpha = 0.05$ test (Payton et al. 2003), our comparisons can be even seen as conservative results. Starting at 36 mg a.i./L, mortality significantly increased in *X. laevis* embryos ($F_{1,14} = 44.75$, $P < 0.001$; Fig. 1a) and starting at 180 mg a.i./L in *D. pictus* embryos ($F_{1,12} = 42.99$, $P < 0.001$; Fig. 1b). NOEC for mortality in *X. laevis* embryos was 18 mg a.i./L, but 135 mg a.i./L in *D. pictus* embryos (Fig. 1).

Congenital malfomations occurred in the control group of *X. laevis* and *D. pictus*. They comprised edema ($n = 6$) and one time a combined malformation (axial and edema) in *X. laevis* and axial ($n = 4$) and edema ($n = 1$) in *D. pictus* (Table 3). The findings were understood as the basic spontaneous incidence.

Table 1 Calculated and measured glyphosate concentrations in first dilution series. Measured concentrations were in average 100.49 ± 3.07 % of the calculated concentrations and did not significantly differ ($P > 0.05$). All values are given in mg a.i./L

Experiment	Calculated test concentration	Measured test concentration
X. laevis embryos	4.5	6
	9	12
	18	15
	36	32
	45	37
D. pictus embryos	45	48
	90	84
	135	140
	180	180
	225	220
X. laevis larvae	0.9	0.92
	1.8	1.9
	3.6	3.7
	4.5	4.3
	9	9
D. pictus larvae	4.5	4.2
	9	7.6
	18	18
	36	37

Incidence of malformations increased concentration dependently in *X. laevis* ($F_{1,9} = 24.77$, $P < 0.001$; Fig. 1a). Also, in *D. pictus*, incidence of malformations increased with the concentration (49, 90, and 135 mg a.i./L, $F_{1,7} = 8.98$, $P < 0.05$; Fig. 1b). However, for both embryo experiments, post hoc tests were not possible due to low survival in the replicates exposed to the highest test concentration with surviving embryos. Hence, no NOEC values can be stated, but for both species, Teratogenic Indices <1.5 (Table 2) indicate low teratogenic potential of the herbicide formulation (Bantle et al. 1999; Fort and Rogers 2005). The goal of the Teratogenic Index (TI = LC50 values divided by the EC50 values) is to separate the lethal action of a compound from his teratogenic potential. TI values <1.5 should indicate low teratogenic potential of a substance because little or no separation exists between lethal and malformation inducing concentrations (according to Bantle et al. 1999; Fort and Rogers 2005).

To describe the morphological changes after 96-h exposure of *X. laevis*, out of 16 fixed malformed embryos from the 36 mg a.i./L concentration group, three were selected for pathological investigations, and additionally, for comparison to individuals from the control. The external morphological changes are depicted in Fig. 2. Changed proportions of body and tail, further the curved tail axis, the puffy body, and the small intestines are typical traits. They forecast internal deviations. At histopathological examination, edema was confirmed in the puffy body parts. Staining of cartilage revealed developmental retardation and deformation of elements of the viscerocranial skeleton (Fig. 3c, d). The most profound findings were the rather small and poorly differentiated infrarostralia (Fig. 3d). In addition, Meckel's cartilages (lower jaw), in ventral view, were more concave as opposed to having a convex shape in the control. The ceratohyalia of the hyoid arch and the ceratobranchial arches 1 and 2 were narrower compared to the control. Most cartilages seemed furthermore less developed and showed more irregular outlines. Additionally, the otic capsules were only indicated by slight chondrification in the mesenchymal condensations (for comparison, see also Kotthaus 1933). No chondromalacia or other histopathological changes of the cartilage were seen.

Incidence of morphological changes are summarized in Table 3. They comprised in the control group 7 embryos out of 96 surviving embryos (7 %) demonstrating the spontaneous incidence. In the 4.5 mg a.i./L dose group, 6 out of 44 surviving embryos (14 %) showed edema ($n = 5$) and one embryo a combination of edema and head malformations. In the 9 mg a.i./L dose group in one out of 41 surviving embryos (2 %), edema was seen. In the 18 mg a.i./L dose group in 9 out of 40 surviving embryos (23 %) head malformations ($n = 3$) and in four embryos, a combination of head malformation and edema in the lateral and ventral of the head-pharynx region were diagnosed. In each one, a combination of axial and head malformation and axial malformation and edema was

Table 2 Lethal and teratogenic median concentrations of Roundup® UltraMax on embryos and early larvae of *X. laevis* and *D. pictus*. All values are given in mg a.i./L and were calculated using probit analyses with 95 % confidence limits stated

Species	Developmental stage	96-h LC50	96-h EC50	TI
X. laevis	Embryos	25.82 (22.94; 28.70)	37.35 (28.42; 46.28)	0.69
D. pictus	Embryos	128.20 (121.51; 134.88)	173.24 (114.62; 231.89)	0.74
X. laevis	Early larvae	7.04 (6.24; 7.84)	[a]	–
D. pictus	Early larvae	18.29 (14.93; 21.65)	–	–

TI values < 1.5 indicate low teratogenic potential

TI Teratogenic Index (LC50/EC50)

[a] Only two malformed individuals

recorded. In the 36 mg a.i./L dose group, all 16 surviving embryos (100 %) had a combination of head malformation and edema in the lateral and ventral of the head-pharynx region (Figs. 1a and 2a–d). It has to be underlined that these combinations of head malformation and edema in the lateral and ventral of the head-pharynx region were observed in the

two highest test concentrations which did not induce 100 % mortality (i.e., 18 and 36 mg a.i./L).

The morphological changes in the *D. pictus* embryos are summarized in Table 3. They comprised in the control group 5 out of 100 surviving embryos (5 %) demonstrating the spontaneous incidence. In the 45 mg a.i./L dose group, 6 out of 49

Table 3 Number of malformed embryos and proportion of different malformation types in the control groups and due to exposure to Roundup® UltraMax concentrations, which did not induce total mortality after 96 h

Species	Malformation type[a]	Test concentration[b]	Number (proportion)
X. laevis	Edema	Control	6 out of 96 (6.25 %)
X. laevis	Axial and edema	Control	1 out of 96 (1.04 %)
X. laevis	**Total**	**Control**	**7 out of 96 (7.29 %)**
X. laevis	Edema	4.5	5 out of 44 (11.36 %)
X. laevis	Head and edema	4.5	1 out of 44 (2.27 %)
X. laevis	**Total**	**4.5**	**6 out of 44 (13.64 %)**
X. laevis	Edema	9	1 out of 41 (2.44 %)
X. laevis	**Total**	**9**	**1 out of 41 (2.44 %)**
X. laevis	Head	18	3 out of 40 (7.5 %)
X. laevis	Head and edema	18	4 out of 40 (10 %)
X. laevis	Axial and edema	18	1 out of 40 (2.5 %)
X. laevis	Axial and head	18	1 out of 40 (2.5 %)
X. laevis	**Total**	**18**	**9 out of 40 (22.5 %)**
X. laevis	Head and edema	36	16 out of 16 (100 %)
X. laevis	**Total**	**36**	**16 out of 16 (100 %)**
D. pictus	Edema	Control	1 out of 100 (1 %)
D. pictus	Axial	Control	4 out of 100 (4 %)
D. pictus	**Total**	**Control**	**5 out of 100 (5 %)**
D. pictus	Edema	45	1 out of 49 (2.04 %)
D. pictus	Axial	45	5 out of 49 (10.20 %)
D. pictus	**Total**	**45**	**6 out of 49 (12.25 %)**
D. pictus	Axial	90	1 out of 48 (2.08 %)
D. pictus	Axial and head	90	4 out of 48 (8.33 %)
D. pictus	**Total**	**90**	**5 out of 48 (10.42 %)**
D. pictus	Head	135	3 out of 20 (15 %)
D. pictus	Head and edema	135	2 out of 20 (10 %)
D. pictus	Head and eye	135	4 out of 20 (20 %)
D. pictus	**Total**	**135**	**9 out of 20 (45 %)**

[a] According to Bantle et al. (1998)

[b] In mg a.i./L

surviving embryos (12 %) had axial malformations ($n = 5$) and one embryo edema. In the 90 mg a.i./L dose group in 5 out of 48 surviving embryos (10 %), a combination of axial and head malformations ($n = 4$) and in one a singular axial malformation was diagnosed. In the 135 mg a.i./L dose group, 9 out of 20 surviving embryos (45 %) showed malformations, which included head malformations ($n = 3$), combinations of edema and head malformations ($n = 2$) and combinations of head and eye malformations ($n = 4$) (Figs. 1b and 4).

Similar to increased mortality rates, the minimum concentration that inhibits growth (MCIG) in *X. laevis* embryos was 36 mg a.i./L and the NOEC for growth inhibition 18 mg a.i./L ($F_{1,228} = 9.61$, $P < 0.001$; Fig. 1c). In *D. pictus*, MCIG was 136 mg a.i./L and consequently, the NOEC 90 mg a.i./L ($F_{1,215} = 27.72$, $P < 0.001$; Fig. 1d).

Acute toxic effects of RU-UM on early larvae

Mortality was significantly increased in early *X. laevis* larvae ($X^2 = 13.16$, $df = 5$, $P < 0.05$; Fig. 5a), but Wilcoxon rank sum test between the controls and the highest concentrations did not reach the level of significance (W = 0, $P = 0.059$). Starting at 36 mg a.i./L, mortality of early *D. pictus* larvae was significantly different from the control ($F_{1,14} = 14.45$, $P < 0.01$; Fig. 5b). Like in embryos, *X. laevis* larvae were significantly more sensitive to exposure to RU-UM if compared to

Fig. 4 *D. pictus*: *A* Gosner stage 23 from the control group (lateral view), *B1*, *B2*, and *B3* of the same developmental age as in *A* from the 135 mg a.i./L concentration group with developmental retardation and different grades of pathological changes (*B1* and *B2* lateral view, *B3* anteriolateral view). They comprise changed body proportions (*curly brackets*), edema (*arrow*), head malformations (*quadrat*) and small eyes; *scale bars* = 1 mm

D. pictus larvae (Table 2). NOEC for mortality was 4.5 mg a.i./L in *X. laevis* and 18 mg ai./L for *D. pictus* (Figs. 4a, b).

Only one *X. laevis* larva with edema was observed in the control and at 4.5 mg a.i./L one axial malformation. Due to missing concentration dependency, these changes were understood as incidental. No malformed *D. pictus* larvae were found in the control or in the concentration groups.

The MCIG in both *X. laevis* ($F_{1,148} = 16.67$, $P < 0.001$; Fig. 5c) and *D. pictus* larvae ($F_{1,94} = 171.40$, $P < 0.001$; Fig. 5d) was 4.5 mg a.i./L. Consequently, NOEC for growth inhibition in *X .laevis* was 3.6 and <4.5 mg a.i./L in *D. pictus* larvae (Figs. 4c, d).

Discussion

Lethal effects on embryos and early larvae

Applying the FETAX protocol, a 96-h LC50 value of 9.4 mg a.e./L (a.e. = acid equivalents) of Roundup® Original has been found for *X. laevis* embryos (Perkins et al. 2000). Based on the conversion factor 0.75 of Giesy et al. (2000), the LC50 value from Perkins et al. (2000) is approximately 12.5 mg a.i./L, so that RU-UM can be considered as half as acute toxic for *X. laevis* embryos as the original formulation (Table 2). As for many other pesticides too, the added substances (surfactants) should mainly be responsible for adverse effects (Puglis and Boone 2011; Wagner et al. 2013, 2015b). This is underpinned by the fact that Perkins et al. (2000) have found for *X. laevis* embryos a 96-h LC50 value of the glyphosate-based herbicide Rodeo® without a surfactant system of >5000 mg a.e./L, but a 96-h LC50 value of polyethoxylated tallow amine (POEA, the surfactant in Roundup® Original) of only 2.7 mg/L. Likewise, exposure of *X. laevis* embryos to a cycloxydim-based herbicide formulation (Focus® Ultra) resulted in significantly higher mortality rates compared to the a.i. (Wagner et al. 2015b). Edginton et al. (2004) found that a higher pH level of 7.5 nearly doubled the lethal effect of another glyphosate-based herbicide (Vision®) to *X. laevis* embryos compared to individuals exposed to the same concentrations at pH 6.0 (96-h LC50 values of 7.9 mg a.e/L ≈ 10.5 mg a.i./L vs. 15.6 mg a.e./L ≈ 20.8 mg a.i./L). The latter LC50 value is comparable with the results from the present study (see Table 2), but it has also to be taken into account that our average pH level in test solutions was intermediate (= pH 6.8) compared to the pH levels applied by Edginton et al. (2004). It was supposed that, at high alkalinities, accumulation of the compound in the gills would be accelerated due to higher proportion of the nonionized form of the POEA surfactant of the Vision® formulation (Edginton et al. 2004). With regard to the embryos of other, non-pipid anuran species, our results indicate that
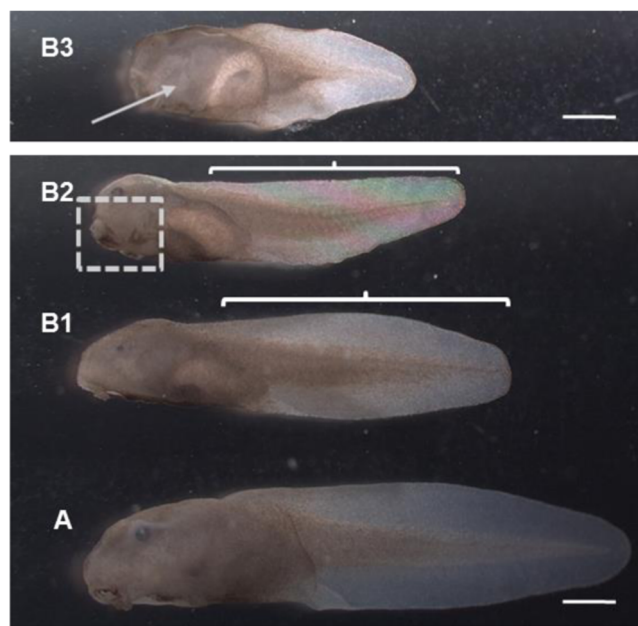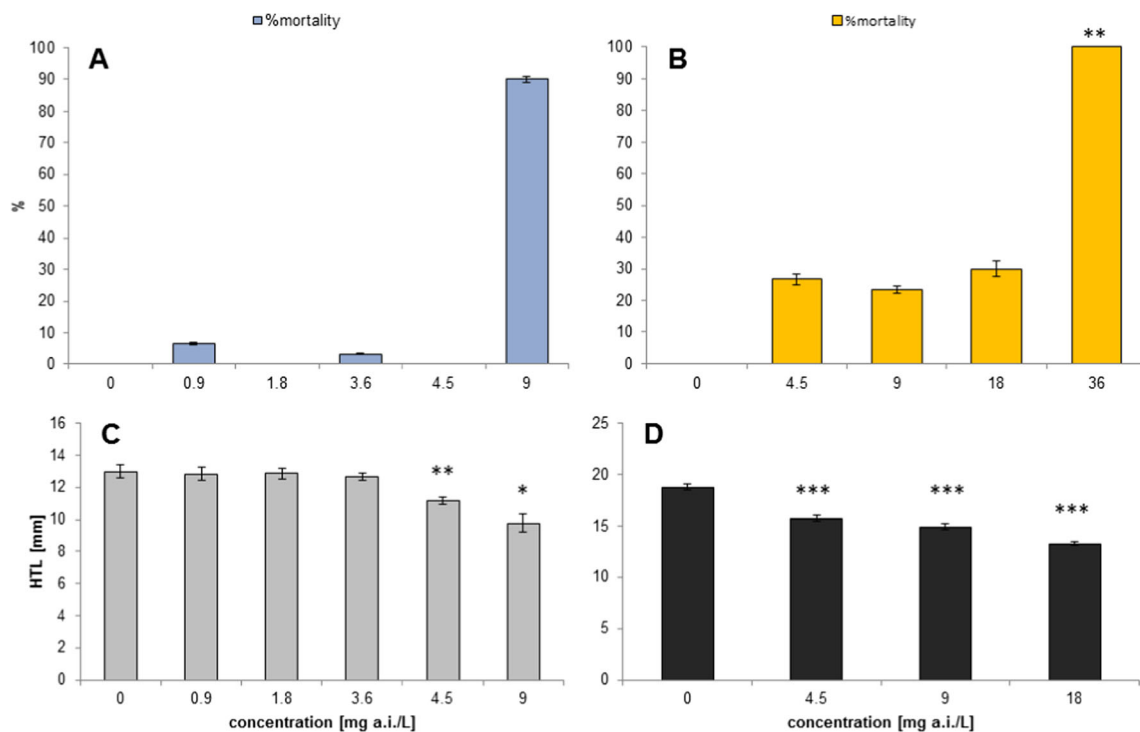
Fig. 5 Mortality rates in early *X. laevis* (**a**) and *D. pictus* larvae (**b**) and growth inhibition due to exposure to Roundup® UltraMax in *X. laevis* (**c**) and *D. pictus* larvae (**d**) .All values are given ±standard error. *Asterisks* indicate significant differences to the control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). *HTL* total length, head to tail length

D. pictus embryos were significantly more tolerant to exposure to the formulation under research than *X. laevis* embryos (Table 2). However, *X. laevis* embryos are not per se more sensitive than those of other anurans. Edginton et al. (2004) found embryos of *Lithobates clamitans* and *Anaxyrus americanus* to be even more sensitive than *X. laevis*, whereas *Lithobates pipiens* embryos (at both tested pH levels) compare to *X. laevis*. After all, it has to be suggested that *D. pictus* is a relatively tolerant anuran test species.

Lajmanovich et al. (2011) exposed early larvae of *Rhinella arenarum* to RU-UM and found a 48-h LC50 value of only 2.42 mg a.e./L ≈ 3.23 mg a.i./L. This was up to now the only study on toxic effects of RU-UM on anuran larvae. In the present study, *X. laevis* larvae were found to be twofold and *D. pictus* larvae more than fourfold more tolerant (Table 2) than *R. arenarum*, taken into account that the LC value from Lajmanovich et al. (2011) probably would be even lower after 96-h exposure. Although the effects of glyphosate-based herbicides have been found to vary species specifically (Wagner et al. 2013 and references therein), the differences to *R. arenarum* are not clearly related to species-specific properties. Besides of the differences in the laboratory specific-test procedures, the composition of RU-UM in Argentina differs from the RU-UM applied in the present study (http://www.monsanto.com/global/ar/productos/documents/roundup-ultramax-mon-79840.pdf).

Growth inhibition in embryos and larvae

Starting at 1.85 mg a.e./L (≈2.46 mg a.i./L) and increased at 3.75 mg a.e./L (≈4.99 mg a.i./L), Lajmanovich et al. (2011) found inhibition of different B-esterases and glutathione S-transferases in *R. arenarum* larvae after sublethal exposure to RU-UM for 48 h indicating general stress. This could be an explanation for growth inhibition observed in both *X. laevis* and *D. pictus* larvae at low concentrations in the present study (Fig. 5c, d), probably also for growth inhibition and developmental retardation in the embryos possibly induced in their late developmental phase. Conversely to our results, growth inhibition in the study by Perkins et al. (2000) using Roundup® Original already started at 10 mg a.e./L (≈13.33 mg a.i./L) in *X. laevis* embryos, i.e., in an about three times lower concentrations as MCIG for RU-UM in the present study (Fig. 1c). It is also notable that growth inhibition in larvae of both species started at 4 mg a.i./L, but this was the lowest test concentration in the *D. pictus* experiments, so that it remains unknown if larvae were also unaffected at lower concentrations like in *X. laevis* (Figs. 5c, d).

It is notable that other sublethal effects but growth inhibition and alterations of enzymatic activities have been observed in anuran larvae after exposure to glyphosate-based herbicides. For example, the glyphosate-based herbicide formulation Credit® alone as well as in a binary mixture with the

dicamba-based herbicide formulation Banvel® induced genotoxic effects in larval *R. arenarum* after 96-h exposure (Soloneski et al. 2016).

Malformations

The present study demonstrated systemic effects of the formulation on embryonic development of *X. laevis* and *D. pictus*. Mortality and malformations were accompanied by decrease of body size. Developmental effects in *X. leavis* started at a concentration of 18 mg a.i./L (malformations in 22.5 ± 0.5 % of surviving embryos with malformations, Fig. 1a). All surviving *X. laevis* embryos showed head and eye malformations after exposure to 36 mg a.i./L (Figs. 2 and 3). Concentration dependency and incidences allow to attribute the result to the action of the compound. The malformations at 4.5 and 9 mg a.i./L were in connection with the basic spontaneous incidence of the species. Pathological investigations demonstrated an effect of the compound on the development of the viscerocranial skeleton (developmental retardation and slight deformation).

Also Paganelli et al. (2010) observed an increase of head defects and craniofacial malformations after exposure of *X. laevis* embryos to a one five-thousandth dilution of Roundup® Classic equaling 72 mg a.e./L (BVL 2010), i.e., about 96 mg a.i./L (conversion factor 0.75: Giesy et al. 2000). In *X. laevis* embryos exposed to Roundup® Original, no significant increases in malformation rates could be observed at any sublethal concentration (Perkins et al. 2000). In yet another study, only the highest test concentration of Roundup® Original (5 mg/L, i.e., about 18 mg a.i./L as 360 mg a.i./L are in 100 mg of formulation) increased incidence of malformed gastrointestinal tract in NF stage 41 *X. laevis* when exposed for 48 h (Lenkowski et al. 2010).

In *D. pictus,* the 135 mg a.i./L concentration clearly affected the development of the head region including the development of smaller eyes (Fig. 4). Incidences of malformations at 45 and 90 mg a.i./L were close to the basic spontaneous incidence of the species. The effect of the compound remained questionable.

No treatment related malformed larvae were observed in the present test with early stage larvae. Conversely, internal and external damages (craniofacial and mouth malformations, eye abnormalities, and bent, curved tails) were observed in surviving *Scinax nasicus* larvae after acute exposure to Glyfos® at a concentration already lethal to some conspecifics (Lajmanovich et al. 2003). Increased tail damages have been observed in *L. pipiens* after chronic exposure to POEA, Roundup® Original and Roundup® Transorb (Howe et al. 2004). Likewise, chronic exposure to Roundup® Original increased fluctuating asymmetry in *Physalaemus cuvieri* larvae (Costa and Nomura 2016). In acute toxicity testing, a cycloxydim-based herbicide formulation (Focus® Ultra) significantly increased axial malformations in *Discoglossus* larvae, starting at a concentration free of lethal effects (Wagner

et al. 2015c). However, no increase in malformation rates has been found in *Xenopus* and *Discoglossus* larvae and metamorphs after exposure to low, environmentally relevant concentrations Focus® Ultra (Wagner et al. 2015a). All these results show that malformations in anuran larvae are particularly depending on the formulation, exposure time and concentration. Apparently, environmentally relevant concentrations are often not high enough to significantly increase malformation rates in the wild (Wagner et al. 2014b).

In contradiction to the amphibian monitoring guideline of Böll et al. (2013), developmental retardation, enzymes and blood biomarkers may be in many cases more suitable to detect effects of agrochemicals on wild amphibian populations than survival and malformation rates alone (Peltzer et al. 2013; Attademo et al. 2014; Wagner et al. 2014b).

Comparison with standard test organisms and environmental concentrations

In the safety data sheet (http://s3.nuuspace.com/monsanto/roundup/wp-content/uploads/2015/09/Roundup-UltraMax-13427CLPde-de.pdf),the 96-h LC50 value of RU-UM for the rainbow trout (*Oncorhynchus mykiss*) is given as 28 mg formulation/L, which corresponds to about 12.6 mg a.i./L (450 mg a.i./L are in 100 mg of RU-UM). The 48-h EC50 value (sign of immobilization) for the crustacean *Daphnia magna* reads even 69 mg formulation/L ($\approx$31.1 mg a.i./L).

Hence, concerning acute lethal effects embryos of both species are more tolerant to RU-UM exposure and *D. pictus* larvae compare well to the aquatic standard test organism *O. mykiss* (Table 2). This may be seen in accordance to the review by Weltje et al. (2013), which suggested fish to be adequate surrogates for amphibian larvae. However, *X. laevis* larvae reacted about two times (1.8-fold) more sensitive towards exposure to RU-UM (Table 2). Furthermore, the statement by Weltje et al. (2013) is only based on simple rank correlations and even if acute lethal effects of chemicals on fish and amphibian larvae are often in a similar range, more subtle effects of chemicals on amphibian larvae like endocrine effects, effects on the metamorphosis cannot be assessed using fish as surrogate species (Wagner et al. 2015b, c).

Compared to other pesticides, a relatively large amount of amphibian toxicological studies with glyphosate-based herbicides is available, especially on aquatic anuran larvae (see Wagner et al. 2013 and references therein or the ECOTOX database from the U.S. Environmental Protection Agency: http://cfpub.epa.gov/ecotox/). However, need for clarification remains in the field of teratogenic action at environmentally relevant concentrations (e.g., Relyea 2006 vs. Thompson et al. 2006 concerning real world concentrations; see also Paganelli et al. 2010 vs. BVL 2010 concerning teratogenic effects at environmentally relevant concentrations). Although glyphosate-based herbicides are

dominating the worldwide herbicide market, pesticide residue analysis data for glyphosate (and especially surfactants) in the field are rather scarce. Environmental surface water samples reach a maximum concentration of 0.7 mg a.i./L. Parameters concerning the degradation state (like period since application, occurrence of heavy rainfalls) are usually unknown (Wagner et al. 2013) reducing the validity of the data. Hence, like with other pesticides expected environmental concentrations (EEC) are of interest. Though glyphosate-based herbicides can reach up to 7.6 mg a.i./L in EEC (see Wagner et al. 2013 and references therein), worst-case EEC for surface waters in Europe next to agrarian use of glyphosate-based herbicides calculated by authorities range only from 1.2 to 1.7 mg a.i./L for applications without buffer strips and drift reduction (ECB 2000; BVL 2010). However, these calculations do not apply for aerial applications, which are common in, for instance, forestry in Canada (see Thompson et al. 2004). A buffer strip of five meters width is supposed to reduce the EEC to values under 7 µg a.i./L (BVL 2010). According to the safety data sheet, no buffer strips are required for RU-UM use, but usually national laws foresee buffer strips for surface waters, for instance, 5–10 m in the different German federal states. However, it has to be mentioned that many aquatic breeding habitats of amphibians are not regarded as "surface waters" (e.g., ditches, drainage channels, vernal pools: Battaglin et al. 2009). To cope with this particular deficiency, we first used the worst-case EEC (1.7 mg a.i./L) to calculate Toxicity to Exposure Ratios (TER = effect concentration divided by expected worst-case concentration). Regarding a safety factor of 10 (standard in pesticide approval) for acute toxicity tests (96-h LC50 values), TERs are 75.4 for *D. pictus* embryos and 15.2 for *X. laevis* embryos, but ≤ 10 for larvae of both species (10.8 for *D. pictus* larvae and only 4.1 for *X. laevis* larvae). However, a buffer strip of 5 m width (even without drift reduction) should reduce the EEC to 6.84 µg a. i./L (according to the BVL 2010). This results in TER values of >18,000 and >3000 for *D. pictus* and *X. laevis* embryos, respectively, and >2000 and >1000 for *D. pictus* and *X. laevis* larvae, respectively. In conclusion, buffer strips have to be required for any aquatic amphibian habitat when RU-UM is applied in the field. It has to be mentioned that this apparent field safety is merely based on EEC of the a.i. and not on the added substances. Finally, dermal uptake by terrestrial life stages from soil or plant material and direct over spraying are important exposure routes (Quaranta et al. 2009; Brühl et al. 2013; Van Meter et al. 2015) and there is an especially high coincidence observed for the application of glyphosate-based herbicides and migrating amphibians in Germany (Berger et al. 2013). Up until now, this type of exposure has not been assessed for the European RU-UM formulation, but for the Argentinian one. Lajmanovich et al. (2015) found signs of neurotoxicity, oxidative stress, and immunological depression due to dermal uptake in adult *R. arenarum*.

Conclusions

Regarding our hypotheses (according to hypothesis number):

(1). In general, *X. laevis* embryos and larvae reacted more sensitive to RU-UM exposure than early developmental stages of *D. pictus*. It was suggested, that beside of unknown properties the different morphology of their filter apparatus and the higher volume of water pumped through the buccopharynx of *X. laevis* lead to higher exposure of the later species to the compound.

(2). RU-UM induced similar lethal effects in *D. pictus* larvae as it does in the teleost standard test organism used in pesticide approval, the rainbow trout (*Oncorhynchus mykiss*), whereas embryos of both species are more tolerant concerning acute lethal effects and, conversely, *X. laevis* larvae about two times more sensitive. Based on calculated surface water concentrations of the a.i., there is, however, an apparent field safety, but buffer strips have to be urgently regarded to any aquatic amphibian habitat.

(3). In both species, early larvae always reacted significantly more sensitive than embryos. Increased metabolism demonstrated by increased growth was understood as the source.

(4). Exposure to RU-UM increased malformation rates in embryos of both species in a concentration-dependent manner, but not at environmentally relevant concentrations. However, the apparent field safety data, based on calculated surface water concentrations of the a.i., should be validated with realistic field data.

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