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Acute Toxic Effects of the Herbicide Formulation and the Active Ingredient Used in Cycloxydim-Tolerant Maize Cultivation on Embryos and Larvae of the African Clawed Frog, *Xenopus laevis*

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Abstract Most genetically engineered herbicide-tolerant crops are still awaiting approval in Europe. There is, however, a recent trend for the cultivation of cycloxydim-tolerant maize hybrids for use in maize production. We studied the acute toxic effects of the complementary herbicide Focus® Ultra and its active ingredient cycloxydim on embryos and early-stage larvae of the African clawed frog (*Xenopus laevis*). The results indicate that the herbicide formulation is significantly more toxic than the active ingredient alone. Therefore, it is suggested that the added substances either solely or in a synergistic action with the active ingredient are responsible for adverse effects. The formulation was found to be moderately toxic to embryos but highly toxic to early larvae. Based on calculated teratogenic indices, both cycloxydim and Focus® Ultra seem to be non-teratogenic and also the minimum Focus® Ultra concentration to inhibit growth in embryos and larvae was close to the LC50 values. The data suggest that tests with the rainbow trout are not in all cases appropriate to assess the risk in aquatically developing anurans. This is demonstrated by 96-h LC50 values, which are for rainbow trout more than 50- to 20-fold higher than for early *X. laevis* larvae. However, based on worst-case predicted environmental concentrations for surface waters, there is apparently a large safety margin in field use of Focus® Ultra if buffer strips between the farm land and the amphibian habitats are regarded.

Keywords Focus® Ultra · FETAX · Amphibian decline · Pesticides · Corn

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Amphibian populations are declining globally at alarming rates (Stuart et al. 2008). Different factors, often operating in tandem, such as habitat destruction, invasive species but also environmental contamination are often cited to play a role (Collins and Storfer 2003). In particular, pesticides reach the habitats of amphibians by various ways. These substances are known to cause toxic effects on all stages of amphibian development in both aquatic and terrestrial habitats (Mann et al. 2009). In Europe, maize cultivation has increased markedly in the last few decades, especially for fodder crop production. For example, in Germany the cultivation area for maize increased >20 % in relatively short time, from 21,111 km^2 in 2009 to 25,642 km^2 in 2012 (Federal Bureau of Statistics: https://www.destatis.de). Today, land use changes for biofuel production are among the main concern for conservationists, as this type of agriculture is expanding in fallow wasteland or former mining landscapes (Dauber et al. 2012). These lands are, among others, necessary secondary habitats and prerequisites for amphibian survival after the tremendous anthropogeneous changes of the landscape. In the USA and many other countries, maize cultivation is dominated by genetically modified (GM) crops and their specific herbicides. In Europe, GM cultivation is marginal, because such crops are still undergoing approval (Böll et al. 2013). Instead, since the beginning of the millennium, cycloxydim-resistant maize hybrids (CTM) have gained growing importance (Vancetovic et al. 2009) enabling repeated applications of the cycloxydim-based pesticide Focus® Ultra (BASF), which is also used by foliar spraying against perennial grasses in rape, sugar beet, potato, green bean, and field bean cultivations (EFSA 2010). For example, in 2012 the domestic sales for cycloxydim in Germany have been 10–25 tons of active ingredients (a.i.) and the export has been 100–250 tons of a.i. (BVL 2013). The effects of

this herbicide formulation on amphibians remained unstudied. We conducted the first experimental trials to investigate the toxicity of Focus® Ultra on embryos and early larvae of the African clawed frog (*Xenopus laevis*). The developmental stages of *X. laevis* can be used as surrogates for other aquatic organisms because of their high sensitivity to pesticides and their availability as a laboratory species (ASTM 1998; Bantle et al. 1998, 1999; Wagner et al. 2013).

Materials and Methods

African clawed frog (*X. laevis*) is a pipid anuran amphibian from southern Africa. The larvae are obligate suspension feeders pumping high amounts of water through their buccopharynx with the suggested consequence of tremendously increased contact to the compounds (Viertel 1990, 1992). Reproduction was initiated by injection of human chorionic gonadotropin into the dorsal lymph sac of *X. laevis*.

Focus® Ultra is a selective herbicide containing 100 g/L (=10.8 %) of cycloxydim (CAS 101205-02-1) as a.i. Cycloxydim is a cyclohexene oxime herbicide, i.e. it inhibits acetyl-CoA carboxylase in grasses while dicotyle plants and CTM are not affected (Burton et al. 1989). Added vehicles to the formulation Focus® Ultra include 50 % of Solvent Naphtha (a flammable liquid mixture of hydrocarbons, CAS 64742-95-6) and 2.4 % of dioctyl sodium sulfosuccinate (CAS 577-11-7). For further information on the a.i. and both added substances, see the Pesticide Properties DataBase (PPDB) of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

All experiments were conducted in a climate chamber at 23 ± 1°C and 12:12-h light–dark cycle. Embryo tests started with eggs at NF (Nieuwkoop and Faber 1956) stages 8–11 and were terminated after 96-h when the embryos had reached NF stage 46. 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. 100 mg of the pure a.i. (=cycloxydim) were predissolved in 0.0001 % (v/v) ethanol. Because the NOEC for *X. laevis* is 0.00005 % (v/v) ethanol (Coady et al. 2005), the test concentrations have been 0, 1, 5, 10, 25, and 50 mg a.i./L. For Focus® Ultra, based on prior dose range-finding studies, concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 1.5 mg a.i./L were selected for embryo testing, which corresponds to 0, 1, 2.5, 5, 10, and 15 mg formulation (=Focus® Ultra) per litre. The embryo test procedure according to the FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) protocol (ASTM 1998) was applied, i.e. four controls and four solvent controls with 0.00005 % (v/v) ethanol were used, test concentrations were duplicated, and the solutions were renewed every 24-h (static renewal).

The tests with larvae started at NF stage 47 in accordance with the standard protocol of the ASTM (2002). The trials were terminated after 96-h when the larvae had reached NF stage 48. Only non-malformed larvae with normal behaviour were introduced. Because prior dose range finding studies indicated that larvae were 5–6 times more sensitive than embryos, test concentrations of 0, 0.5, 1, 2.5, 5, 10 mg cycloxydim/L and 0.00001 % (v/v) were selected and ethanol was used as solvent and in the solvent controls. For Focus® Ultra, based on prior dose range-finding studies, nominal concentrations of 0, 0.001, 0.01, 0.1, 0.25, and 0.5 mg a.i./L were selected corresponding to 0, 0.01, 0.1, 1, 2.5, and 5 mg formulation/L. Tests with early larvae were conducted using 5-L all glass aquaria, each containing 1 L of test solution and 10 larvae. Test concentrations were triplicated and the solutions were renewed every 24-h (static renewal). Larvae were fed with 2.5 mg Sera Micron®/animal/day.

All test solutions were freshly prepared with FETAX solution (see ASTM 1998). Conductivity, dissolved oxygen, and pH ranged from 1,482 to 1,492 µS/cm, 5 to 8 mg/L, and 6.5 to 7.2, respectively. The average ammonia concentration was 0.15 mg/L with a range of 0.1–0.2 mg/L. For quality assurance, cycloxydim stock concentration of 100 mg a.i./L was measured using HPLC (Thermo Finnigan® TSP with UV2000 detector, P4000 pump, and AS3000 Auto sampler). 200 mL of solution were enriched on SPE columns (OASIS® HLB 6 cc), conditioned with 3 mL of methanol and 3 mL of water, eluted with 4 mL of methanol, evaporated until dryness, and eventually absorbed into 100 µL of methanol:water (1:1). Sample injection was 20 µg/L. For higher nominal concentrations (i.e. 1.5, 1.0, 0.5, 0.25, and 0.1 mg a.i./L), 15, 10, 5, 2.5, and 1 mL of stock solution were filled with FETAX solution in a Duran® graduated flask to obtain 1 L of test solution. For lower nominal concentrations (i.e. 0.01 and 0.001 mg a.i./L), 10 and 1 mL of previously diluted 1 mg a.i./L test solution were filled with FETAX solution in a Duran® graduated flask to obtain 1 L of test solution.

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 (HTL). 96-h LC50 and 96-h TC50 values (median lethal and teratogenic concentration, respectively) were calculated with probit analyses. Significant differences were examined by overlap tests of 95 % confidence intervals. Differences in mortality, malformations and HTL between

groups were checked using one-way ANOVA (some data had to be Box-Cox transformed prior analysis to account for normal distribution and homogeneity of variances), followed by Bonferroni-corrected post hoc-tests (for small sample sizes). The software R and the package MASS were applied for statistical analyses (R Developmental Core Team).

Results and Discussion

Duplicate determination of the stock concentration of 100 mg a.i./L revealed only 86 and 89 mg a.i./L. These lower measured values are most probably based on the poor solubility of cycloxydim in water (53 mg/L in purified water at 20°C: EFSA 2010). Hence, diluted concentrations are expected to be lower than the calculated concentrations, but because only the stock solution has been measured, calculated concentrations were used to determine the endpoints. Survival of controls and solvent controls was always ≥90 %. In the FETAX with cycloxydim, no significant mortality was observed at any concentration. In consequence, a 96-h LC50 value of >50 mg/L was assigned for embryos (Fig. 1a). In the larvae

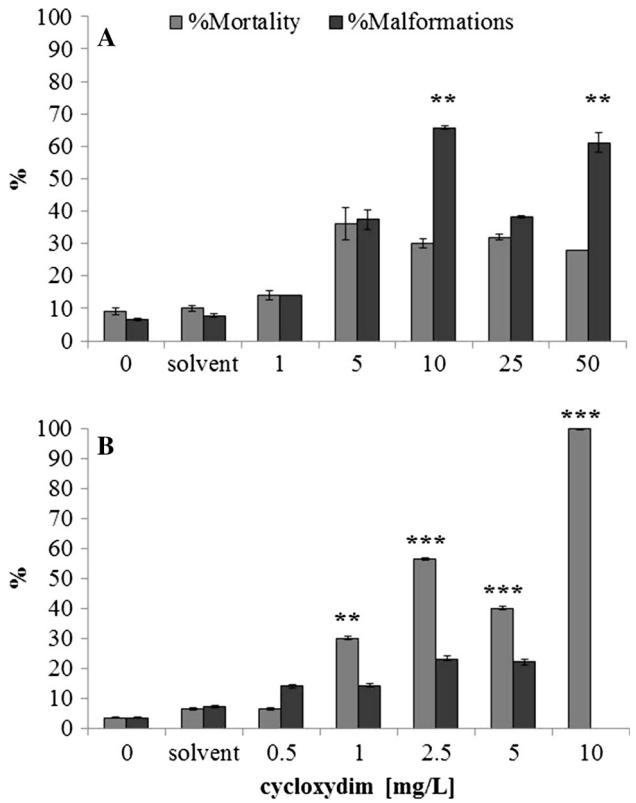

Fig. 1 Influence of cycloxydim on the 96-h mortality and malformation rates of *X. laevis* embryos (**a**) and early larvae (**b**). *Asterisks* indicate significant differences to the control. All values are given ± standard error

experiment with cycloxydim, mortality was significantly increased starting at 1 mg/L (ANOVA: $F = 85.73$, $df = 1$, $p < 0.001$; Fig. 1b). The 96-h LC50 value allows to suggest moderate toxicity (1 mg/L < LC50 < 10 mg/L) of cycloxydim for early larvae (Table 1). In the FETAX with Focus® Ultra, total mortality of embryos started at 1.0 mg a.i./L (i.e. 10 mg formulation/L) (ANOVA: $F = 29.96$, $df = 1$, $p < 0.001$). In the larvae experiment with Focus® Ultra, mortality was significantly increased at concentrations higher than 0.05 mg a.i./L (ANOVA: $F = 70.79$, $df = 1$, $p < 0.001$; Fig. 2a–b). The 96-h LC50 values for the herbicide formulation demonstrated high toxicity of Focus® Ultra (0.1 mg/L < LC50 < 1 mg/L) for embryos and very high toxicity (LC50 < 0.1 mg/L) for early larvae if the values were calculated for the amounts of a.i./L. If they were calculated using the amounts of formulation/L, moderate toxicity of Focus® Ultra (1 mg/L < LC50 < 10 mg/L) was demonstrated for embryos and high toxicity (0.1 mg/L < LC50 < 1 mg/L) for early larvae (Table 1). Similar low LC50 values are rarely found in literature for *X. laevis* larvae, e.g. 0.04–0.05 mg/L for the insecticide dieldrin (Schuytema et al. 1991). Overlap tests of 95 % confidence intervals in the present study revealed that embryos were significantly more resistant than early larvae (Table 1). This finding is in accordance with Edginton et al. (2004) and probably caused by the absence of target organs in embryos such as functional external gills where the surfactants of pesticides can accumulate. It is suggested that the exposure of larvae is higher than in embryos due to the ventilation of the buccopharynx (see below). Furthermore, embryos and larvae were significantly more sensitive to the formulation (Focus® Ultra) than to the a.i. (cycloxydim) alone (Table 1). The 96-h LC50 for cycloxydim on the rainbow trout (*Oncorhynchus mykiss*), a teleost fish, is tenfold higher than for the formulation (220 mg/L vs. 20.4 mg formulation/L: European Chemical Agency http://echa.europa.eu/documents/10162/cd799762-dd1a-4bb0-a772-bcd2d34da0b4; Safety Data Sheet of Focus® Ultra). Likewise, the 96-h LC50 for cycloxydim on *X. laevis* embryos is at least tenfold higher than for Focus® Ultra and about fivefold higher for early larvae (Table 1). Also in other studies, added substances to pesticide formulations significantly increased adverse effects on anuran larvae (Puglis and Boone 2011).

No observed effect concentration (NOEC) for mortality in embryos was >50 mg cycloxydim/L but 0.5 mg a.i./L (5 mg formulation/L) and in larvae 0.5 mg cycloxydim/L but 0.05 mg a.i./L (0.5 mg formulation/L) (Figs. 1, 2).

Malformation rate in control and solvent control embryos was always ≤7 %, confirming validity of the present developmental toxicity test (ASTM 1998). In the FETAX with cycloxydim, malformation rates were increased in the dose groups 10 and 50 mg/L, but not at 25 mg/L (Fig. 1a). Though a clear dose-dependency was

Table 1 Lethal and teratogenic concentration values of the active ingredient (a.i.) cycloxydim alone (in the left column) and the herbicide formulation Focus® Ultra (in the right column) on embryos and early larval stages of *X. laevis*

Life stage	Lethal and teratogenic concentration values		
	Cycloxydim	Focus® Ultra	
Embryos	96-h LC50 (mg/L)	96-h LC50 (mg a.i./L)	96-h LC50 (mg formulation/L)
	>50.0	0.6 (0.5, 0.7)	5.9 (5.1, 6.7)
	96-h TC50 (mg/L)	96-h TC50 (mg a.i./L)	96-h TC50 (mg formulation/L)
	32.2 (23.4, 41.9)	0.6 (0.4, 0.9)	6.3 (4.1, 8.5)
Early larvae	96-h LC50 (mg/L)	96-h LC50 (mg a.i./L)	96-h LC50 (mg formulation/L)
	4.0 (3.1, 4.8)	0.1 (0.07, 0.11)	0.9 (0.7, 1.1)
	96-h TC50 (mg/L)	96-h TC50 (mg a.i./L)	96-h TC50 (mg formulation/L)
	>5.0	0.1 (0.04, 0.22)	1.3 (0.4, 2.2)

All values were calculated using probit analyses with 95 % confidence limits stated

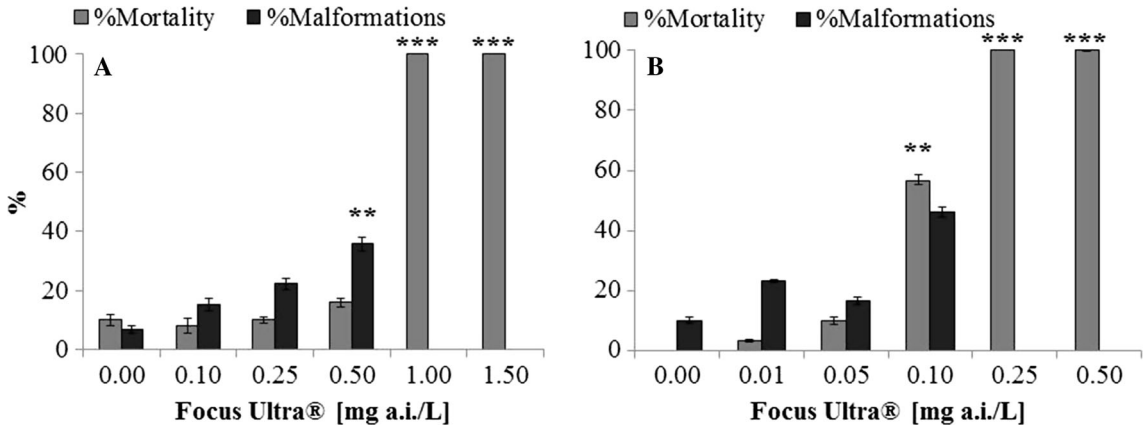

Fig. 2 Influence of Focus® Ultra on the 96-h mortality and malformation rates of *X. laevis* embryos (**a**) and early larvae (**b**). *Asterisks* indicate significant differences to the control. All values are given ± standard error

missing increased malformation rates were attributed to the compound. Starting at 0.5 mg a.i./L (i.e. 5 mg formulation/L), malformation rates in embryos were significantly increased in the FETAX with Focus® Ultra (ANOVA: $F = 38.06$, $df = 1$, $p < 0.001$) (Fig. 2a). This increase of morphological changes was exclusively observed at concentrations with increased mortality (LC50). Therefore, the calculated Teratogenic Index (TI = 96-h LC50/96-h TC50) was 0.94. According to Bantle et al. (1999) the compound can be considered as non-teratogenic. However, it has to be taken into account that mortality may camouflage teratogenic effects at an early stage before they are visible as diagnosable morphological changes. Therefore, the calculation of a TI may be generally misleading for some substances.

No significant dose-dependent increase in malformation rates in larvae has been observed after exposure to cycloxydim or Focus® Ultra (Figs. 1b–2b). Malformation rate in controls in the Focus® Ultra experiment was relatively high (3 out of 30 animals = 10 %), but the highest malformation rate (6 out of 13 surviving larvae = 46.15 %) occurred in the highest concentration in the surviving larvae (Fig. 2b). The TI was even lower than for embryos (0.69). Cycloxydim and Focus® Ultra can be considered to cause no developmental effects in larvae, however, with the restriction mentioned above for embryo toxicity. NOEC for teratogenicity in embryos was 5 mg cycloxydim/L and 0.25 mg a.i./L (2.5 mg formulation/L) and in larvae >5 and >0.10 mg a.i./L (1.0 mg formulation/L) (Fig. 2a–b).

The a.i. cycloxydim alone did not affect growth in embryos and larvae (Fig. 3a–b); however, Focus® Ultra significantly reduced growth in embryos at 0.5 mg a.i./L (i.e. 5 mg formulation/L) and higher (ANOVA: $F = 38.1$, $df = 1$, $p < 0.001$) (Fig. 4a). Growth inhibition in larvae started at 0.1 mg a.i./L (i.e. 1 mg formulation/L) (ANOVA: $F = 21.0$, $df = 1$, $p < 0.001$) (Fig. 4b). The

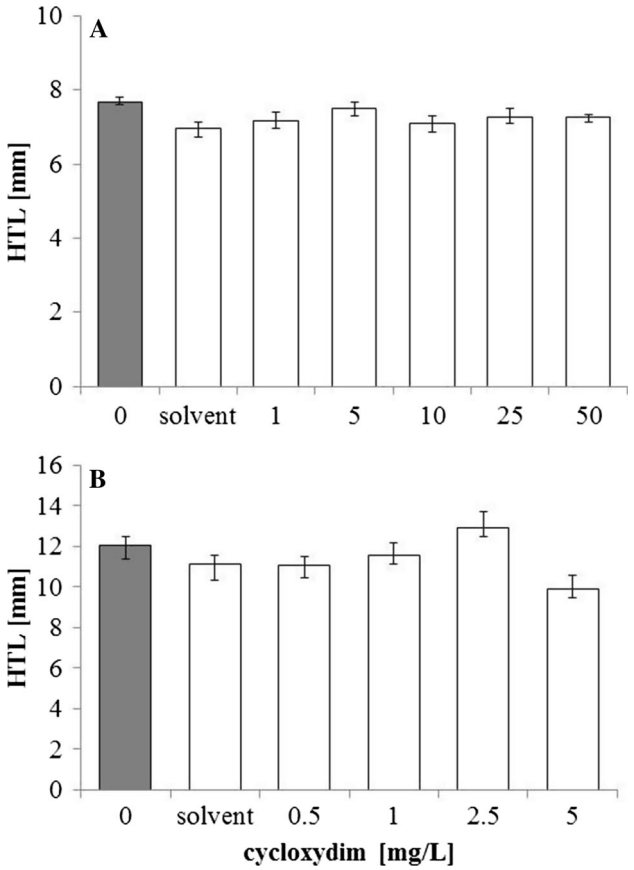

Fig. 3 No effects of cycloxydim on the 96-h growth of *X. laevis* embryos (**a**) and early larvae (**b**). HTL = Head-tail-length. All values are given ± standard error

minimum concentration to inhibit growth (MCIG) in embryos and larvae was close to the LC50 values. NOEC for growth retardation in embryos was >50 mg cycloxydim/L but 0.25 mg a.i./L (2.5 mg formulation/L) and in larvae >5 mg cycloxydim/L but 0.05 mg a.i./L (0.5 mg formulation/L) (Fig. 4a–b).

Our results confirm the findings of previous studies (Puglis and Boone 2011; Wagner et al. 2013) that the added substances to pesticide formulations significantly increase adverse effects. In consequence, pesticide risk assessment and approvals should always be based on the whole formulation that is applied in the field and not only on the active ingredient.

Due to the low 96-h LC50 values for Solvent Naphtha for *O. mykiss* (1.03 mg/L: USEPA 2011 http://www.epa.gov/chemrtk/hpvis/hazchar/Category_Gasoline%20Blending%20Streams_December_2011.pdf) and dioctyl sodium sulfosuccinate (lowest 120-h LC50 of 0.4 mg/L for *O. mykiss*; Goodrich et al. 1991), these compounds are understood to induce adverse effects. The most interesting finding in the present study is that the 96-h LC50 values for Focus® Ultra on *O. mykiss* are more than 20-fold and for cycloxydim more than 50-fold higher than for early *X. laevis* larvae.

It is suggested, that the different biology of anuran larvae if compared with *O. mykiss*, such as morphological properties, food and mode of food ingestion is responsible for this result. The body surface of teleosts is completely covered by scales or other dermal bones functioning as a barrier to the environment. The gills play a unique role in gas and ion exchange (Feder and Burggren 1985; Fenwick 1989; Evans et al. 1999, 2006). It does not wonder that toxic compounds are transported mainly via the fish gills (Evans 1987; Erickson and McKim 1990). In contrary, the body surface of anuran larvae including their large tail fins play a role in gas exchange (Boutilier et al. 1992; Ultsch et al. 1999). Additionally high amounts of water are in close contact with the epithelia of the oral cavity, the filter apparatus and the gills during ventilation (Gradwell 1972a, b, c, 1975; Wassersug and Hoff, 1979, 1982; Viertel 1990, 1992). It has to be suggested that anuran larvae are much more exposed to their aquatic environment and as

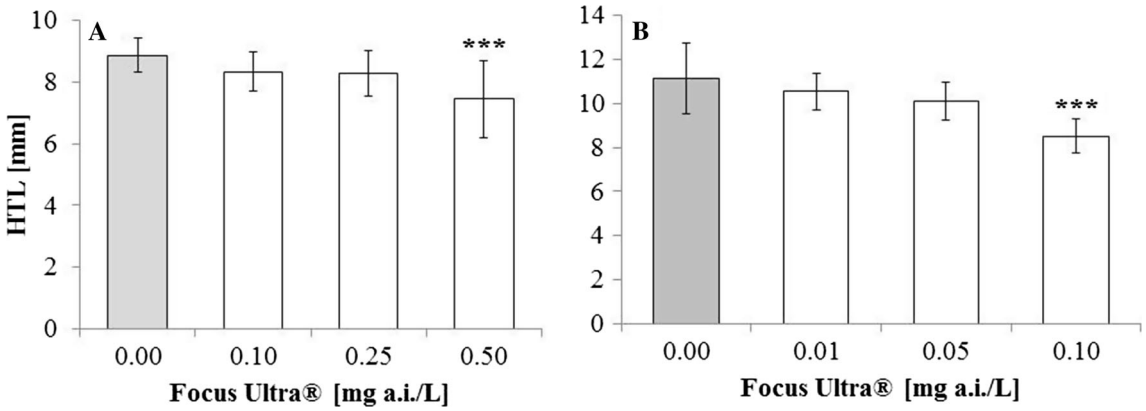

Fig. 4 Influence of Focus® Ultra on the 96-h growth of *X. laevis* embryos (**a**) and early larvae (**b**). *Asterisks* indicate significant differences to the control. HTL = Head–tail-length. All values are given ± standard error

consequence to compounds than teleosts. So the toxic effects described are the result of both the compound and the specific properties of *Xenopus* larvae.

The data suggest that *O. mykiss* is not a sufficient surrogate organism to assess the risk of pesticides such as cycloxydim and its formulation Focus® Ultra to anuran larvae. A 10-fold safety factor is appropriate only for anuran embryos, but not for the early larvae. For the later, a 100-fold safety factor has to be postulated. Due to the specific properties of anuran larvae, surrogate species are perhaps not sufficient for proper risk assessment of all pesticides and at least one anuran model organisms should be used. However, for most of the active ingredients such as organophosphates or carbamates (Aldrich 2009) and some formulations such as most glyphosate-based herbicides (Wagner et al. 2013) standard aquatic test organisms seem to be sufficient for pesticide risk assessment, at least if a 10-fold safety factor is applied.

The EFSA (2010) states a worst-case foliar application scenario of Focus® Ultra of two times 400 g a.i./ha for sugar beets and beans (=dicot plants), which should be comparable with its use in CTM cultivations. The (simple) step 1 of the official surface water models of FOCUS (FOrum for the Coordination of pesticide fate models and their USe) states a worst-case predicted environmental concentration of cycloxydim for surface waters (PECsw; global maximum) of 0.26 mg a.i./L after two applications of 400 g a.i./ha (EFSA 2010). However, in the more realistic FOCUS step 2 and 3 scenarios, the worst case PECsw is reduced to 0.007 mg a.i./L after one application of 600 g a.i./L (unfortunately, no more precise models for two applications are available) in the northern European Union (EU), 0.009 mg a.i./L for the same application in the southern EU (step 2), and similarly 0.003 mg a.i./L for shallow bodies of water (ditches) (step 3) (EFSA 2010). Hence, there is apparently a large safety margin in field use for the herbicide, but more field data on real contamination levels and effects on autochthonous amphibians are necessary. Furthermore, all the calculated values are based on the a.i. (cycloxydim), but the formulation that is applied in the field, i.e. the toxicity of the added substances is not included in the risk assessment.

Although Focus® Ultra is already labelled to be harmful to aquatic organisms, instructions and safety information foresee no buffer strips. For example in Germany, depending on the federal state, 5–10 m buffer strips are recommended, but critical amphibian breeding habitats in cultivated landscapes like vernal pools are not protected by no-spray buffer zones, as it is also the case in other countries like the U.S. (Battaglin et al. 2009), so that, in these areas, the step 1 worst-case PECsw could be possible.

All autochthonous anurans in Europe are of the non-pipid type. The morphology of their larval buccopharynx is quite different from *Xenopus*. They are also suspension feeders, however, exploiting a much broader range of food sources than *Xenopus*. Some of them are bottom feeders ingesting sediment and detritus, some are facultative macro feeders. Experimental data demonstrate that they pump less water through their buccopharynx than *X. laevis* (Viertel 1990, 1992). The influence of these preconditions on toxic action of Focus® Ultra will be investigated separately.

In conclusion, (1) studies with *O. mykiss* are not appropriate to assess the risk of pesticides to anuran development. (2) The added substances alone or their synergistic action with the active ingredient are mainly responsible for adverse effects. By contrast, the role of the pure active ingredient, cycloxydim, seems to be negligible. (3) Although the present data from laboratory experiments demonstrate high toxicity of Focus® Ultra to *X. laevis* larvae, there is apparently a large safety margin in field use for the herbicide (based on the EEC). However, these EEC are only calculated for amounts of a.i./L, but the more toxic added substances are not considered leading to an inappropriate risk assessment. (4) Buffer strips between the farm land and the habitats of amphibians are recommended to prevent direct over-spraying of small ponds, which could cause high local concentrations of the compound and result in adverse consequences for amphibian reproduction. (5) Most anurans in contact with farming areas are non-pipids. The larvae are different from pipids especially in some aspects of exploitation of food sources and food ingestion. Because the later are beside of ventilation of the buccopharynx the main gates for compounds to enter the larval organism the effects of cycloxydim and Focus® Ultra on aquatic life stages of non-pipids have to be investigated.

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