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Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae

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Abstract

Despite the high amphibian biodiversity and increasing pesticide use in tropical countries, knowledge on the sensitivity of tropical amphibians to pesticides remains limited. The aim of this study was to evaluate the acute toxicity of the active ingredients of four of the main herbicides used in Brazilian sugarcane production to tadpoles of two tropical frog species: *Physalaemus cuvieri* and *Hypsiboas pardalis*. The calculated 96 h-LC50 (median lethal concentration; in mg a.s./L) values for *P. cuvieri* and *H. pardalis* were 4.4 and 7.8 (acetochlor); 15 and <10 (ametryn); 115 and 106 (glyphosate); and 85 and 68 (metribuzin), respectively. These toxicity values demonstrated little interspecies variation and the toxicity of the herbicides appeared to be at least partly related with the respective octanol-water coefficient. Published acute toxicity data of fish and amphibians for herbicides were also compiled from the US-EPA ECOTOX database. These data indicated little difference in herbicide sensitivity between tropical amphibians and both non-tropical amphibians and fish. These findings indicate that temperate (fish and amphibian) herbicide toxicity data are also protective for tropical amphibians. Constraints in such extrapolations and indications for future research are discussed.

Keywords Native species · Ttropics · Bioassays · Aquatic ecotoxicology · Herbicides · Amphibians

Introduction

Amphibians are important organisms for aquatic and terrestrial ecosystems, playing a key role maintaining ecosystem structure and functioning (Schiesari et al. 2009). In Neotropical ecosystems, for example, amphibians are essential for energy flow and nutrient cycling, in addition to aiding in controlling pest organisms (Valencia-Aguilar et al. 2013).

Declining amphibian populations have raised major concerns over the past decades. The International Union for Conservation of Nature (IUCN), for example, reported that approximately one-third (32%) of all amphibian species worldwide are threatened and that many others have their population numbers declining, which indicates that this situation is likely to continue or even aggravate in the near future (IUCN 2017). Several causes have been identified for this alarming trend, which include habitat loss and fragmentation, invasive species, overharvesting (for consumption), UV-B radiation, climate change, emerging infectious diseases and pollution (Araújo et al. 2014; Whitfield et al. 2016). Among these, pesticides have been considered important stressors (Alza et al. 2016; Ortiz-Santaliestra et al. 2018) and there are some compelling cases linking pesticide contamination from agricultural activities with amphibian population declines (Bionda et al. 2019; Davidson et al. 2002; Hayes et al. 2010; Pollo et al. 2019; Zhelev et al. 2018). Amphibians have indeed been indicated to possess traits that make them especially prone to pesticide stress, such as a highly permeable skin, shell-less eggs, biphasic life cycle with aquatic and terrestrial life stages, and a relatively rudimentary immune system (Bach et al. 2018; Kerby et al. 2010).

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Despite their worldwide decline and apparent high sensitivity to pesticides, relatively few studies have been conducted so far evaluating pesticide toxicity to amphibians as compared to other taxonomic groups (Ortiz-Santaliestra et al. 2018; Vasconcelos et al. 2016). This low research effort has been attributed to (1) animal welfare concerns; (2) the few existing standardized protocols for amphibian testing, but predominantly (3) the fact that amphibian testing is generally not required in ecological risk assessments (EFSA 2013; Ortiz-Santaliestra et al. 2018; Vasconcelos et al. 2016). The absence of mandatory toxicity tests with amphibians in pesticide risk evaluations is due to the assumption that the sensitivity of the standard fish test species also covers that of amphibians (EFSA 2018; Vasconcelos et al. 2016). Pesticide sensitivity comparisons between fish and amphibians have indeed demonstrated that toxicity thresholds for fish are typically also protective for amphibians (Fryday and Thompson 2012; Weltje et al. 2013). A recent review, however, indicated that this assumption is based on a very limited dataset and that this may not hold true for all pesticide mode of action groups (Ortiz-Santaliestra et al. 2018). Even though the number of studies into the sensitivity of tropical amphibians to pesticides has increased in the past decade (e.g., Attademo et al. 2016; Bionda et al. 2019; Lajmanovich et al. 2019), they remain underrepresented in sensitivity comparisons between amphibians and fish conducted so far (Egea-Serrano, Solé 2017; Ghose et al. 2014; Méndez et al. 2016; Sánchez-Domene et al. 2018).

The deficiency in research effort with native tropical amphibian species is unfortunate, given that amphibians have greater biodiversity and species decline in tropical regions (EFSA 2018; Kerby et al. 2015; Sánchez-Domene et al. 2018; Schiesari et al. 2007; Whitfield et al. 2016). In addition, tropical countries are among the largest consumers of pesticides in the world, some of which with poor regulation and enforcement, and using compounds that are banned in most temperate countries (Albuquerque et al. 2016; Ghose et al. 2014; Lewis et al. 2016). Life history and other important ecological traits also vary among temperate and tropical amphibians (Schiesari et al. 2007). Besides potentially altering their response to pesticide stress, this also allows Neotropical amphibians to exploit a wide variety of breeding habitats, including those most prone to pesticide contamination (Egea-Serrano and Solé 2017).

Sugarcane plantations in Southeastern Brazil—the most important sugarcane-producing region in the world—have expanded rapidly over the past decade as a result of increasing biofuel demand (Rudorff et al. 2010; CANASAT 2018). The conversion of Atlantic Forest and cerrado to pastures to sugarcane plantations has previously been demonstrated to result in an impoverished amphibian biodiversity (Schiesari and Corrêa 2016), and the conversion of pasture to sugarcane fields involves a variety of land management practices with potential adverse effects on amphibians, including pesticide application. Brazil is the largest consumer of pesticides in the world and sugarcane is the third largest market, responding to 12% of all pesticide sales in the country (Albuquerque et al. 2016; SINDAG 2012).

The aim of the present study was to evaluate the lethal toxicity of the active ingredients of four herbicides (acetochlor, ametryn, glyphosate and metribuzin) to tadpoles of two tropical frog species. These herbicides are among the most used herbicides in Brazilian sugarcane production (Armas et al. 2005; IBAMA 2014). To this end, acute laboratory bioassays were conducted with tadpoles of the native frog species *Physalaemus cuvieri* and *Hypsiboas pardalis*. These species are known to occur in or near sugarcane plantations (Schiesari and Corrêa 2016). The generated toxicity data were subsequently compared with published toxicity data for non-tropical amphibians and fish. In addition, published toxicity data were also compiled for other herbicides. This subsequently allowed a comparison of the acute herbicide sensitivity of tropical amphibians with that of non-tropical amphibians and fish for a wider range of compounds and taxa.

Materials and methods

Test species

Three or more egg masses from different parents of *Physalaemus cuvieri* and *Hypsiboas pardalis* were collected from ponds at the Estação Biológica de Boracéia in Salesópolis, South-East Brazil (23°37′59”S, 45°31′59”W), which is located within a non-polluted, protected watershed (Verdade et al. 2011). Egg masses were transported in sealed plastic bags containing water from the collection site to the laboratory of the School of Arts, Sciences and Humanities in the University of São Paulo. Hatched larvae were kept in 50-L plastic tanks filled with tap water filtered through an activated carbon granular filter. Tank water was renewed every other day. The temperature in the laboratory was controlled at 25 ± 2 °C with natural photoperiod. Larvae were fed daily with a 3:1 ground mixture of rabbit chow (Purina Mills, LLC, USA; ~16% protein) and Tetra Min Fish Flakes (Tetra Werke, Melle, Germany; ~45% protein) *ad libitum* until the beginning of the experiments. These conditions (temperature, light and food regime) were established in previous experiments to be adequate to maintain these organisms in healthy conditions in the laboratory (e.g., Jiquiriçá 2010; Schiesari 2004). The bioassays were conducted with Gosner stage 25 (Gosner 1960) tadpoles, which is the most common life stage used in amphibian ecotoxicological tests (Sparling et al. 2010). Only healthy

individuals, as judged by external morphology and behavior (Bantle et al. 1991), were selected for the experiments.

Lethality tests

Acute (96 h) bioassays were conducted to evaluate the sensitivity of *P. cuvieri* and *H. pardalis* to the pure active ingredients acetochlor (CAS Number 34256-82-1; Purity 96.8%; Sigma-Aldrich), amethryn (CAS Number 834-12-8; Purity 98.5%; Sigma-Aldrich), glyphosate (CAS Number 1071-83-6; Purity 99.2%; Sigma-Aldrich), and metribuzin (CAS Number 21087-64-9; Purity 99.8%; Sigma-Aldrich). A semi-static design was adopted, in which test solutions were renewed 48 h after the start of the experiment. DT50 (detection time 50%; in days) values available in the Pesticide Properties Database (PPDB 2018) indicate that this renewal assured stable test concentrations over the course of the experiment: acetochlor: 40.5; ametryn (no value available but considered stable to hydrolysis and photolysis); glyphosate: 9.9; and metribuzin: 41. To maximize comparability of test results with those obtained from the literature (c.f. sections "species sensitivity distributions" and "relative tolerance calculations"), internationally-adopted protocols for ecotoxicological tests with amphibians were followed (ASTM 2013; OECD 2015).

The tests were conducted under the same conditions as those described in section "Test species", except that animals were not fed during the test. Based on the results of range-finding tests, five logarithmically-spaced test concentrations (all in mg a.i./L) were determined:

Acetochlor: 1.0; 1.9; 3.5; 6.5; 12
Amethryn: 10; 13; 16; 20; 25
Glyphosate: 84; 97; 112; 130; 150
Metribuzin: 29; 47; 75; 121; 194

Test concentrations were prepared with stock solutions. Since all active ingredients tested other than glyphosate presented low water solubility, they had to be dissolved in ethanol (ametryn, metribuzin) or acetone (acetochlor). Subsequently, solvent controls were also included at the maximum solvent concentrations used (5 mL acetone/L and 5 mL ethanol/L), besides a culture water control. Each treatment was conducted in quadruplicate, in which each replicate consisted of a glass jar containing 10 tadpoles in 1-L test solution. Every 24 h, water quality parameters (pH, temperature, conductivity, DO) were recorded using a multi-parameter meter (YSI 556), and dead individuals counted and removed.

Species sensitivity distributions

Species sensitivity distributions (SSDs) were constructed to allow a comparison of the 96h-LC50 (median lethal concentration) values derived for the two native tropical frog species with those of fish and other amphibians. To this end, 96h-LC50 values were obtained from the US Environmental Protection Agency (US-EPA) ECOTOX database (https://cfpub.epa.gov/ecotox/). In case that more than one toxicity value was reported for the same compound and species, the geometric mean was calculated and used. SSDs were constructed using the ETX 2.1 software (Van Vlaardingen et al. 2004), by fitting log-normal distribution curves to the toxicity data. Log-normality of the constructed curves was confirmed with the Anderson–Darling test included in the ETX software package, which was evaluated at the 5% significance level. The HC5 and HC50 values (hazard concentration for 5% and 50% of the species assemblage included in the SSD, respectively), and their 95% confidence intervals, were calculated with this software following the method described in Aldenberg and Jaworska (2000).

Relative tolerance calculations

Besides the four herbicides tested, an effort was made to compare the sensitivity of tropical amphibians with that of fish and amphibians from subtropical and temperate regions for a greater range of herbicides. To this end, herbicide toxicity data for fish and amphibians was compiled from the US-EPA database indicated above. Subsequently, amphibians were attributed to a climatic region based on their (native) distribution range as indicated in the IUCN Red List of Threatened Species (IUCN 2017). Only LC50 values with a test duration of 2–21 days were considered and geometric means were taken if more than one toxicity values was available for the same species-compound combination (after Van den Brink et al. 2006; Maltby et al. 2009). To enable grouping toxicity values of different compounds and species, the LC50 values needed to be "normalized". For example, an LC50 value of a certain herbicide and species can not be directly compared with an LC50 value for a different species exposed to another herbicide. In previous studies, this has been solved by using the relative tolerance (Trel) approach, i.e., by dividing toxicity values of species with the toxicity value of a standard test species. In this way, the Trel approach has successfully been used to compare the sensitivity of aquatic macroinvertebrates and soil invertebrates using respectively *Daphnia magna* (Wogram and Liess 2001) and *Eisenia fetida* sensu lato (Daam et al. 2011) as standard test species. In the present study, the relative tolerance of fish and amphibians to herbicides was assessed using the standard fish test species *Oncorhynchus mykiss* (rainbow trout) as a reference.

Data analysis

The 96h-LC50, LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) were

calculated based on the % mortality rates in the different treatments using the statistical programs PROBIT 1.5 (EPA 2002) and TSK 1.5 (Hamilton et al. 1977). In all cases, the most appropriate statistical test was defined depending on the experimental design and the nature of the available data, following the recommendations of EPA (EPA 2002). To test for interspecies differences in sensitivity, LC50 values for each compound and species were compared with a *Z* test using the formula proposed by EPA (2002). Analyses of Variance (ANOVA) followed by post hoc tests were employed to test for treatment effects on physical-chemical variables (mean values for each treatment over the experimental period) using the software PAST (Hammer et al. 2001).

Results and discussion

Sensitivity of the two native frog tadpoles to the evaluated herbicides

Survival was 100% in all control treatments except in the ethanol solvent control conducted in the *P. cuvieri* test, in which one individual died (mortality rate 2.5%). Water quality parameters were comparable in control replicates with a coefficient of variation of less than 4% for all parameters (pH, temperature, conductivity and DO).

The 96h-LC50 values generated for the four herbicides are presented in Table 1, whereas the mortality levels of the individual treatments for *P. cuvieri* and *H. pardalis* are visualized in Figs. 1 and 2, respectively. *H. pardalis* was significantly more sensitive than *P. cuvieri* for ametryn, glyphosate and metribuzin and less sensitive to acetochlor (*Z* test; $p < 0.05$). However, the absolute difference in toxicity values for these herbicides between the two species was generally small (8–44%), considering that the variability in toxicity values for the same species and compound but generated in different laboratories may reach a factor of three or more (Sprague 1985; Baird et al. 1989). In line with this, the spread (i.e., the ratio of upper and lower limits of the 95% confidence interval; after Brock et al. 2008) in herbicide toxicity values in our dataset (see "Relative tolerance calculations") for the most tested fish (*Oncorhynchus mykiss*) and tropical amphibian (*Xenopus laevis*) were 3.0 ± 0.78 and 3.1 ± 1.9 (mean ± SE), respectively.

Table 1 LC50 (median lethal concentration; in mg/L) and their 95% confidence intervals as determined for larval *Physalaemus cuvieri* and *Hypsiboas pardalis* after 96-h exposure to glyphosate, ametryn, metribuzin and acetochlor. NP = not possible (lowest test concentration of 10 mg ametryn/L showed 90% mortality in *H. pardalis*)

	Physalaemus cuvieri	Figure	*Hypsiboas pardalis*	Figure
Acetochlor	4.4 (4.1–4.7)[a]	1A	7.8 (7.2–8.4)[a]	2A
Ametryn	15 (14–16)[b]	1B	NP (<10.0)	2B
Glyphosate	115 (112–119)[b]	1C	106 (103–109)[b]	2C
Metribuzin	85 (79–91)[a]	1D	68 (63–74)[b]	2D

[a]Trimmed Spearman–Karber test

[b]Probit test

Especially for ametryn, *H. pardalis* appeared to be clearly more sensitive than *P. cuvieri* (Table 1). For example, whereas the lowest ametryn concentration of 10 mg/L only caused 15% mortality in *P. cuvieri* (Fig. 1b), the same test concentration led to a 93% mortality of *H. pardalis* tadpoles (Fig. 2b). Interestingly, ametryn appeared to take a longer time to exert its full toxic action than the other three herbicides. The greatest part of the total tadpole mortality throughout the 96 h tests were elicited within the first 24 h: 95–97% (glyphosate), 79–83% (acetochlor) and 84–80% (metribuzin) for *P. cuvieri* and *H. pardalis*, respectively. For ametryn, however, corresponding values were 30–56%. Ametryn, and other triazine herbicides, are known to induce detoxification responses in fish and amphibians (Hostovsky et al. 2014; Moura et al. 2018; Van Der Kraak et al. 2014). Induced detoxification may thus counteract chemical stress until the system is not able anymore to cope with the ametryn-induced stress levels (Moura et al. 2018). In addition, when toxic effects start to emerge on the biochemical level, these do not translate immediately in effects of apical endpoints (Van Der Kraak et al. 2014). This may hence explain the delayed lethal effects observed in the ametryn-exposed tadpoles. In turn, potential differences in defense mechanisms between the two species may also explain the greater vulnerability of *H. pardalis*. Possibly, *P. cuvieri* has a better defense mechanism against ametryn toxicity than *H. pardalis*, which is supported by the discussed lower 24 h toxicity noted for this species (30%) as compared to *P. cuvieri* (56%).

Both species were more susceptible to acetochlor and ametryn than to metribuzin and glyphosate (Table 1; Figs. 1 and 2). The capacity of organic contaminants to enter the body of aquatic organisms has been demonstrated to increase with increasing hydrophobicity, which can be measured by the octanol–water partition coefficient (Kow; He et al. 2013 and references therein). In line with this, the log(Kow) of acetochlor (4.14) and ametryn (2.63) are considerably higher than those for metribuzin (1.65) and especially glyphosate (−3.2) (PPDB 2018). This may thus at least partly explain the relative sensitivity of the tadpoles to the herbicides. He et al. (2013) noted a significant correlation between Kow and generated LC50 values of the cladoceran *Daphnia carinata* for three chloroacetanilide herbicides. Although such a correlation also appeared to exist in the present study, this was just not significant

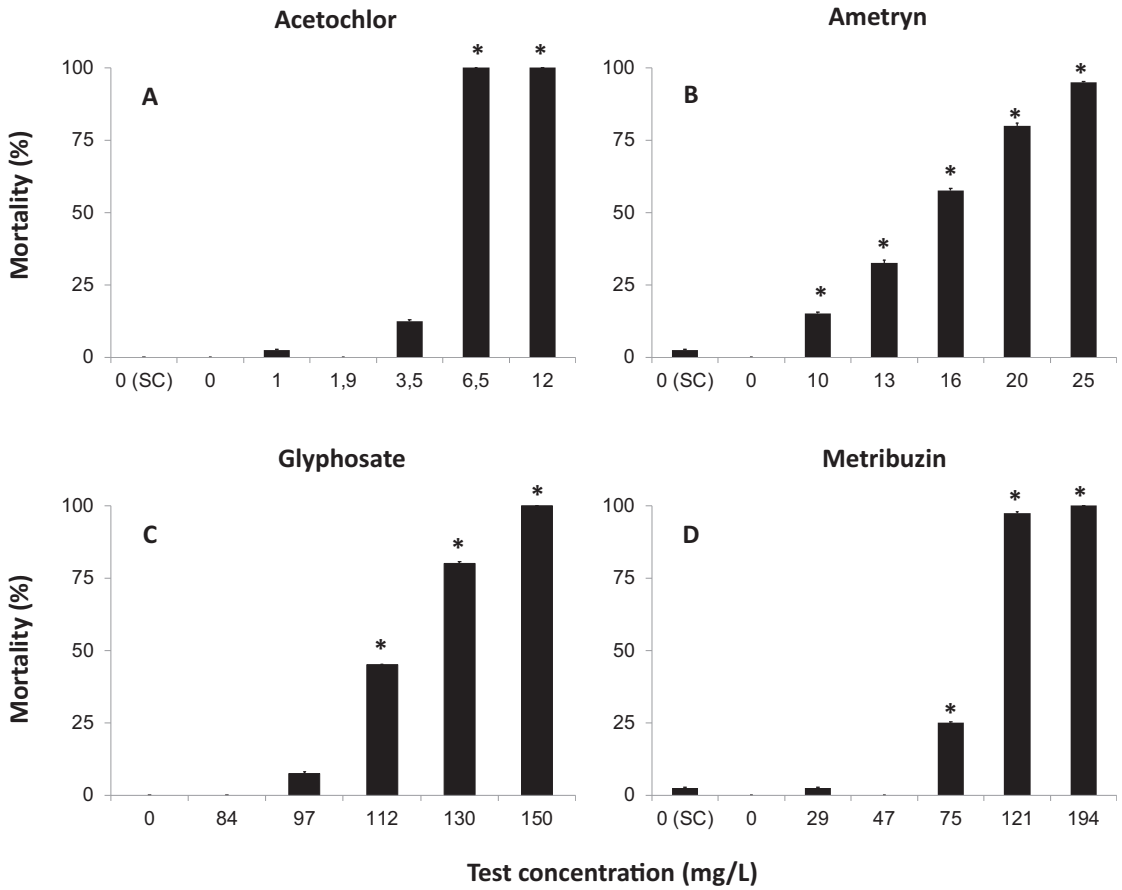

Fig. 1 Mortality (in %) of *Physalaemus cuvieri* at the end of the 96 h laboratory tests evaluating the toxicity of acetochlor, ametryn, glyphosate and metribuzin. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control

($r = 0.71$; $n = 7$; $0.05 < p < 0.1$), probably also due to the low number of generated toxicity values.

Relative tolerance of tropical amphibians to herbicides

The SSDs presented in Fig. 3 allow a comparison of the toxicity data of the four tested herbicides generated for the two frog tadpoles with those available for other fish and amphibian taxa. Only for glyphosate, separate SSD curves could be constructed for fish and amphibians. These SSD analyses indicate that both tadpole species were moderately sensitive to the herbicides, especially when compared to the sensitivity of fish. Overall, the standard fish test species *O. mykiss* (EFSA 2013) was more sensitive than the most sensitive amphibian test species (Fig. 3). Only for metribuzin, *H. pardalis* (gmLC50 = 68 mg/L) was slightly more sensitive than *O. mykiss* (gmLC50 = 76 mg/L). The assessment factor of 100 that is commonly applied to the acute LC50 of *O. mykiss* (e.g., EFSA 2013) would thus be sufficiently protective for all four herbicides tested. As can be deduced from Fig. 4, this also appears to be the case for a larger number of amphibian taxa and herbicide compounds. The range in toxicity data for twelve herbicides evaluating 23 tropical amphibian taxa includes cases where the toxicity of tropical amphibians is greater than *O. mykiss* and fish in general. However, this difference is generally lower than ten, which confirms the protectiveness of the assessment factor of 100 that is already applied to the acute fish toxicity value for tropical amphibians.

Implications for risk assessment and future research

Weltje and Wheeler (2015) compared the sensitivity of tadpoles of the tropical frog *Agalychnis callidryas* with fish for ten pesticides. Based on their findings, they concluded that fish are suitable surrogates for aquatic tropical amphibian stages and that the standard assessment factors cover the extrapolation to potentially more sensitive species and other uncertainties. In line with this, both the bioassays and the literature toxicity data analysis of the present study did not indicate a consistently large difference in sensitivity of tropical amphibian larvae with temperate amphibian larvae or fish.

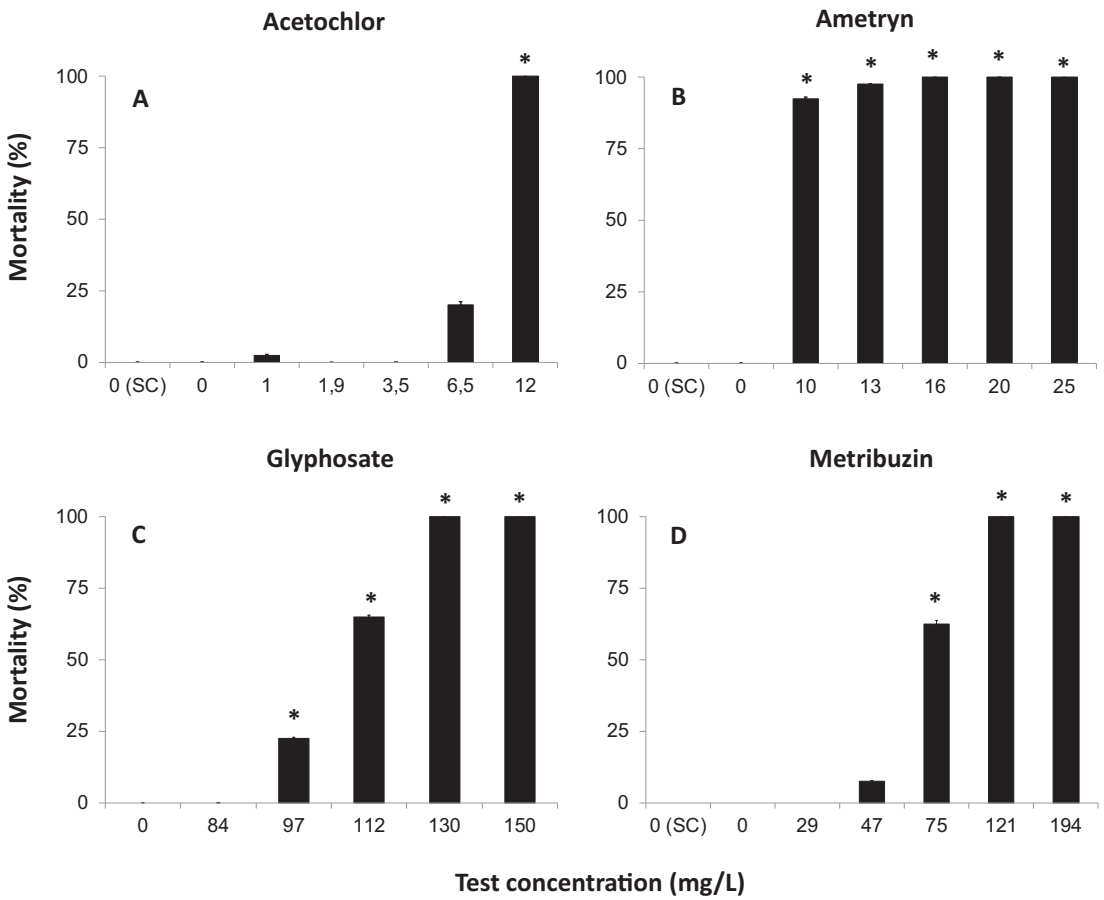

Fig. 2 Mortality (in %) of *Hypsiboas pardalis* at the end of the 96 h laboratory tests evaluating the toxicity of acetochlor, ametryn, glyphosate and metribuzin. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control

Several authors, however, have disputed the use of fish toxicity data to cover for the sensitivity of both temperate and tropical amphibians. In a recent review by Ortiz-Santaliestra et al. (2018), for example, the authors concluded that "there is still much information needed to reduce uncertainties and extract relevant conclusions on the overall protection of amphibians and reptiles by surrogate vertebrates". From a Legislative perspective, concerns have also been raised that the current risk assessment of pesticides may not sufficiently cover the risk to amphibians (e.g., EFSA 2018).

Such concerns may especially be imperative for the use of temperate toxicity data in tropical risk assessments (Schiesari et al. 2007). Ecological and life history traits (e.g., time to metamorphosis and use of waterbodies as habitat in the adult stage; Babini et al. 2018; Zhelev et al. 2018) as well as genetic characteristics (Pollo et al. 2019) are known to vary greatly among amphibians. This may be an additional factor affecting the way temperate and tropical amphibians are exposed and respond to pollutants (Egea-Serrano and Solé 2017; Méndez et al. 2016; Sparling et al. 2010). In addition, only few tropical taxa have been tested so far, although the diversity of amphibians is known to be greatest in tropical areas (Ghose et al. 2014; Lau et al. 2014; Méndez et al. 2016; Schiesari et al. 2007). This increases the chance of the existence of sensitive, yet untested, amphibian species in tropical areas. In addition, relatively few compounds have been evaluated and almost exclusively in acute laboratory toxicity tests. There is thus also an urgent need for chronic (life-cycle) toxicity tests and more environmentally-realistic (field) experiments with tropical amphibians (Schiesari et al. 2007; Vasconcelos et al. 2016). In-situ toxicity, biomarker and field population and community testing would be very useful to attain insights on the actual impact of laboratory-observed effects in real-word conditions (Attademo et al. 2016; Babini et al. 2018; Bionda et al. 2019; Lajmanovich et al. 2019; Pollo et al. 2019; Zhelev et al. 2018). For example, recent studies have indicated that certain amphibians may survive, adapt and breed despite of chemical-induced morphological changes and serious disorders in many physiological functions (Babini et al. 2018; Zhelev et al. 2018).

Like in the present study, most acute toxicity values in our database (on average about 70% for the four herbicides)

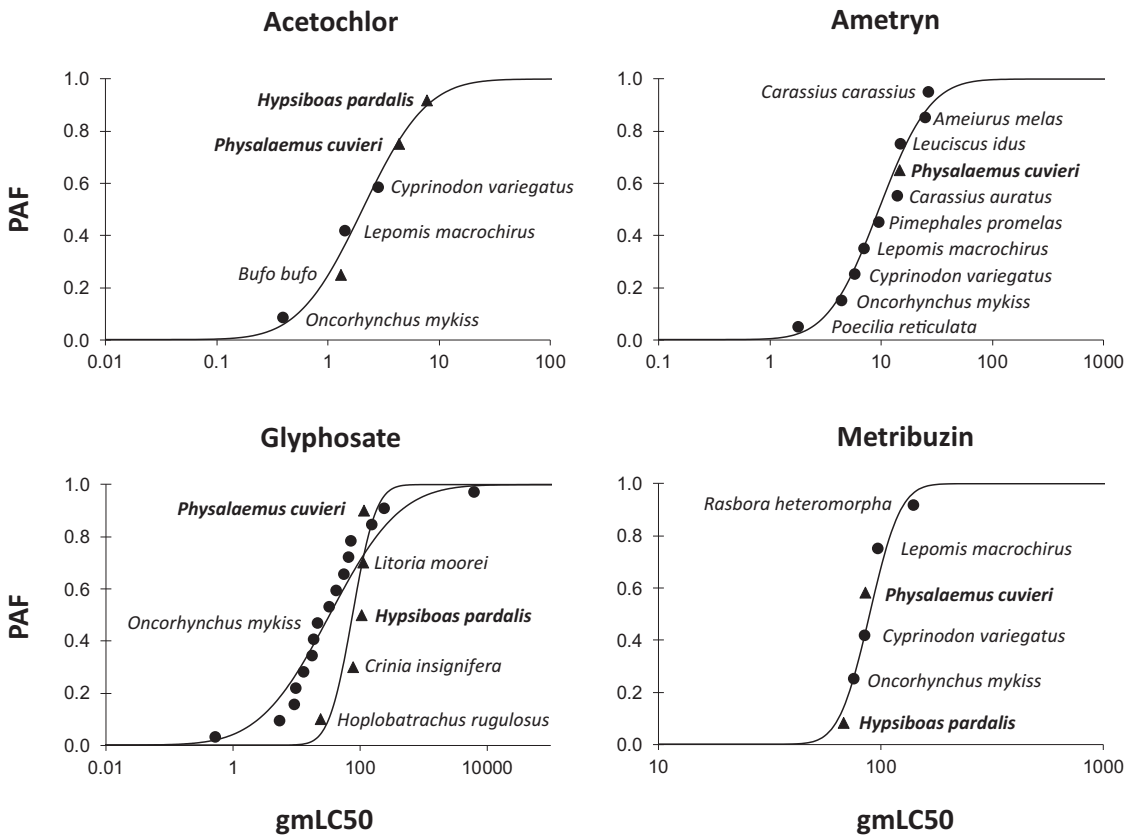

Fig. 3 Species sensitivity distribution (SSD) curves depicting the relative acute sensitivity of the two native frog larvae tested in the present study as compared to other amphibian and fish species

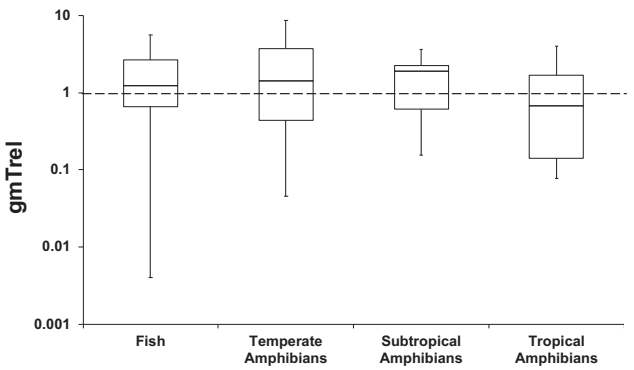

Fig. 4 Geometric means of relative tolerance (gmTrel) values for fish and temperate, subtropical and tropical amphibians based on acute toxicity data available in the US-EPA ECOTOX database. Trel values were calculated by dividing the median effect concentration (EC50) of each species with that of the rainbow trout *Oncorhynchus mykiss* (after Wogram and Liess 2001; For details, please see text)

were derived with the active ingredients. However, it is known that the toxicity of active ingredients may differ to a great extent from their formulated products. Mann and Bidwell (1999), for example, denoted that the 48h-LC50 values for tadpoles of the glyphosate-based herbicide Roundup® were significantly lower than those of its active ingredient. On the other hand, another formulation (Roundup® Biactive) appeared much less toxic than technical glyphosate (Mann and Bidwell 1999). The same conclusions were reached in follow-up studies by Mann et al. (2003) and Lajmanovich et al. (2011). The more since tadpoles in edge-of-field waterbodies are exposed to formulated products that are applied to agricultural crops, rather than solely their active ingredients, future studies evaluating the toxicity of (different) formulated products are also urgently needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The permit for collection, transport and storage of the test animals used in this study was provided by IBAMA/ICMBio (permit number 17559-1) and the tests conducted were approved by the ethical Commission of the Instituto de Biociências da Universidade de São Paulo (Protocol 039/2007).

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