



# Lethal and Sublethal Effects of the Herbicide Atrazine in the Early Stages of Development of *Physalaemus gracilis* (Anura: Leptodactylidae)

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## Abstract

Water sources used as reproductive sites by crying frog, *Physalaemus gracilis*, are extensively associated with agroecosystems in which the herbicide atrazine is employed. To evaluate the lethal and sublethal effects of atrazine commercial formulation, acute and chronic toxicity tests were performed in the embryonic phase and the beginning of the larval phase of *P. gracilis*. Tests were started on stage 19 of Gosner (*Herpetologica* 16:183–190, 1960) and performed in 24-well cell culture plates. Acute tests had a duration of 96 h with embryo mortality monitoring every 24 h. Chronic assays contemplated the transition from the embryonic to larval stages and lasted 168 h. Every 24 h the embryos/larvae were observed for mortality, mobility, and malformations. The LC<sub>50</sub> of atrazine determined for *P. gracilis* embryos was 229.34 mg L<sup>-1</sup>. The sublethal concentrations did not affect the development of the larvae but were observed effects on mobility and malformations, such as spasmodic contractions, reduced mobility, malformations in mouth and intestine, and edema arising. From 1 mg L<sup>-1</sup> atrazine, the exposed larvae began to have changes in mobility and malformations. The atrazine commercial formulation has caused early life effects of *P. gracilis* that may compromise the survival of this species but at higher concentrations than recorded in the environment, so *P. gracilis* can be considered tolerant to this herbicide at environmentally relevant concentrations.

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) has been extensively used in crops, such as rice, wheat, maize, and sorghum (Zhang et al. 2014), because this can be found in water sources adjacent to agroecosystems (Miltner et al. 1989; Graymore et al. 2001). Atrazine has been found in high concentrations both in Brazilian natural waters (Casara et al. 2012; Chicati et al. 2012; Sousa et al. 2016), including drinking waters (Montagner et al. 2014). Due to the great persistence in water and environmental consequences, the use of this active ingredient has been banned in Germany since 1991 and the European Union since 2004 (Sass and Colangelo 2006). In Brazil, atrazine is the third best-selling herbicide, ranking seventh among the most traded pesticides (IBAMA 2014).

Atrazine concentrations tend to be higher in lentic environments and after the first rains (Cerejeira et al. 2003; Rohr and McCoy 2010). These lentic environments are the breeding sites of several amphibian species (Knutson et al. 2004), which are exposed to atrazine throughout the different stages of development. Atrazine may be a factor in the decline of amphibians worldwide (Hayes et al. 2002). Exposure to atrazine may lead to physiological and biochemical changes, such as reduced testosterone hormone and demasculinization of adult males (Hayes et al. 2002; Ezemonye and Tongo 2009), delayed timing and reduced size and weight at metamorphosis (Larson et al. 1998), and malformations (Lenkowski et al. 2008).

The life cycle of most amphibians occurs in two phases: aquatic and terrestrial (Semlitsch 2003). Especially during the aquatic phase, there are important morphological, physiological, ecological, and behavioral changes (Duellman and Trueb 1994). At this stage embryos and larvae are susceptible to various environmental interferences. During the permanence in water, the thin permeable layer surrounding the egg, the permeable skin, and the small displacement of the larvae contribute to the vulnerability to contaminants present

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in aquatic environments (Schiesari et al. 2007). Contact with contaminants can be detrimental to the embryos and, during their development, cause changes in DNA and chromosomes, leading to mortality, anatomical malformations, biochemical or functional defects, and delayed growth. These effects are characterized as sublethal effects or teratogenic effects (Freitas et al. 2001; Newman and Unger 2003). The sensitivity of amphibians to damage caused by contaminant, due to their life cycle characteristics and the permeability of eggs and skin, places them as good indicators of environmental quality (Simon et al. 2012; Gonçalves et al. 2014).

*Physalaemus gracilis* (Anura: Leptodactylidae), popularly known as the crying frog, is an amphibian species widely distributed in southern Brazil, Uruguay, Paraguay, and Argentina (Frost 2017). Reproduction occurs in lentic environments, such as puddles and edges of vegetated ponds, where eggs are deposited in foam nests (Lingnau 2009). The species is found in water sources close to agroecosystems and is tolerant of heavily disturbed and polluted habitats (Lavilla et al. 2010).

The objective of this study was to evaluate the lethal and sublethal toxicity of the atrazine commercial formulation in the early stages of development of *P. gracilis*, a species nontarget of the pesticides, which is in natural contact with contaminants in its habitat. We examined mainly the influence of atrazine on survival and mobility and the occurrence of teratogenic effect in this amphibian species.

## Materials and Methods

### Spawning Collection and Acclimatization

Foam nests of *P. gracilis* were collected from unpolluted water bodies in the Federal University of Fronteira Sul - campus Erechim (Latitude:—27.728681°; Longitude:—52.285852°), Rio Grande do Sul State, Brazil, area considered as a reference. Spawning collected between October 2015 and March 2016, all of them prior to 24 h after oviposition and transported to the laboratory; then they were placed in 15-liter aquariums containing artesian well water, following the standards: 23 °C ( $\pm 1$ ), dissolved oxygen 5.0 ( $\pm 1.0$  mg L<sup>-1</sup>), turbidity < 5, conductivity 160 ( $\pm 10$ )  $\mu$ S cm<sup>-1</sup>, and alkalinity 9.74 mg CaCO<sub>3</sub>/L<sup>-1</sup>. This same water was used in all tests and to dilute atrazine. Organisms were maintained at 20 °C  $\pm$  2 °C and 12:12 light/dark photoperiod.

### Toxicity Bioassays

Toxicity testing was performed using a commercial formulation with 50% active ingredient of atrazine, Siptran®SC 500 g L<sup>-1</sup>. For toxicity bioassays, we adapt protocol

of the fish embryo acute toxicity (FET) test (OECD 2013), and 24 embryos of *P. gracilis* were selected (randomly) using a stereo microscope and transferred to 24-well plates filled with 2 mL of freshly prepared test solutions and controls per well. Embryos were distributed to well plates, with 1 embryo per well, whit 20 embryos on one plate for each test concentration and 4 embryos in water (negative control) as internal plate control (the same plate). Then, we combined the embryos from the different clutches to distribute potential genetic effects. All tests were performed at the same time in duplicates, totaling 40 exposed embryos for each concentration and 8 internal plate controls (negative controls). We used two plates with 40 embryos in the negative control. The internal plate control was used only for the limit test, because if more than one dead embryo was observed in the internal plate control, the plate should be rejected and the test must be repeated. The 40 embryos in the negative control were examined for comparison with exposed embryos and verified percentage of lethality, which may not exceed 10%. This protocol was used for acute and chronic test.

This study was performed within the developmental stages 19–25, considered the end of embryo stage [S.19—stage (S.) according to Gosner 1960] and within hatchling stage (S.20–25). For determination of median lethal concentrations (acute test, LC50s), the larvae were exposed during 96 h to 10 atrazine concentrations: 45, 65, 85, 115, 145, 195, 245, 295, 355, and 475 mg L<sup>-1</sup>. Lethal effects were evaluated every 24 h. The embryos entered the acute test in S.19, with the heart beat and external gill buds, and finished in S.23, with the opercular fold cover base of gills and teeth at the beginning of differentiation (Gosner 1960). For the acute test, we adopted the name “embryos” to refer to these phases.

The chronic (sublethal) test lasted 168 h, with continuous exposure of embryos from stage 19 (S.19) up to late complete operculum stage, with larvae in S.25 (Gosner 1960). Due to the different names that can be used, such as larvae, tadpoles, and post hatching larvae, we only use the term “larvae” for the chronic test. From S.23, larvae were fed daily, ad libitum, with fish flake feed, composed of at least 45% crude protein.

In the chronic test, 16 sublethal concentrations were tested: 0.45, 1.0, 1.7, 8.5, 10, 25, 50, 60, 70, 80, 115, 125, 135, 145, 155, and 165 mg L<sup>-1</sup>. Lethal and sublethal effects were evaluated each 24 h. Larvae were observed by stereo microscope for mobility. Mobility was recorded according to the following endpoints: (1) movement equal to the control; (2) movement reduced in relation to the control; (3) increased movement in relation to control (more active); (4) spasmodic contraction. We chose the term mobility to describe the first movements performed by *P. gracilis*, shortly after hatching.

After chronic test, the larvae (S.25) were analyzed for morphological changes. To identify these alterations, the exposed larvae were compared with those of control. The observed morphological alterations were as follows: (1) changes in the mouth, identified as absence of keratodonts and parts of the upper and lower lip; (2) shape of the intestine, when the intestine presented a straight or different pattern from the folding of the control; (3) edema, identified as swelling in the larvae body, visible mainly in the abdominal region. Morphological changes were considered as malformations.

### Data Analysis

Median lethal concentration (LC50) values and their respective 95% confidence intervals were statistically estimated by Trimmed Spearman–Kärber method. We used a one-way analysis of variance (ANOVA) to evaluate lethal and sublethal effects and Dunnett (different from the control treatment) Tukey (from each other) post hoc test when  $p < 0.05$ . For mobility and malformations, we calculated the no observable-effect concentration (NOEC) and the lowest observable-effect concentration (LOEC) by analysis of variance with mean comparison made by Dunnett's test. The maximum acceptable toxicant concentration (MATC) was calculated from NOEC and LOEC and expressed mathematically as the geometric mean of the NOEC and LOEC. This analysis was performed with the use of the Statistica software 8.0.

### Results

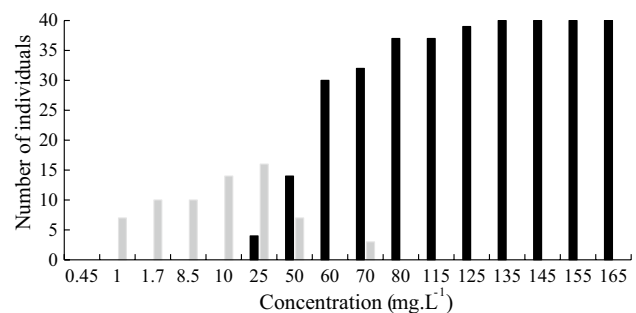
In acute toxicity tests, embryos mortality occurred especially within the first 48 h of exposure. LC50 values at 96 h was  $229.43 \text{ mg L}^{-1}$  (range  $215.94\text{--}243.75 \text{ mg L}^{-1}$ ). Total lethality occurred only for the concentrations of 295, 355, and  $475 \text{ mg L}^{-1}$  (Table 1). In the chronic test, most concentrations presented larval mortality after 120 h of exposure. Complete larvae mortality (S.25) occurred in 144 test hours, at concentrations 135, 145, 155, and  $165 \text{ mg L}^{-1}$ . The exposure time had a significant influence on lethality ( $F_{6,112} = 4.77$ ;  $p < 0.01$ , being significant at 120 test hours, Tukey test,  $p < 0.05$ ) the opposite of concentrations ( $F_{16,102} = 0.99$ ;  $p = 0.47$ ).

### Mobility

Most exposed larvae showed mobility alterations in the  $25 \text{ mg L}^{-1}$  concentration (Fig. 1). Reduced mobility occurred in 10.5% of larvae, and spasmodic contractions were observed in 55.2%. Reduced mobility occurred in larvae exposed to concentrations from 1 to  $70 \text{ mg L}^{-1}$  and spasmodic contractions in  $25 \text{ mg L}^{-1}$  (Fig. 1). There was a

**Table 1** Mortality of embryos and larvae of *Physalaemus gracilis* exposed to commercial formulation of atrazine 96 h

Concentration $\text{mg L}^{-1}$	No. exposed	No. alive	No. dead	Percent mortality
0	40	39	1	2.5
45	40	37	3	7.5
65	40	38	2	5.0
85	40	40	0	0
115	40	38	2	5.0
145	40	36	4	10
195	40	17	23	57.5
245	40	38	2	5
295	40	0	40	100
355	40	0	40	100
475	40	0	40	100



**Fig. 1** Changes in the mobility of larvae of *Physalaemus gracilis* exposed to the commercial atrazine formulation. Gray bars: reduced mobility; Black bars: Spasmodic contractions

significant difference in the mobility of the exposed larvae in relation to the control ( $F_{16,94} = 23.66$ ;  $p < 0.01$ ; significant for all concentrations above  $25 \text{ mg L}^{-1}$ , Dunnett test,  $p < 0.05$ ). In total, 65.62% of the larvae presented changes in movement.

Larval movements (any type of movement, altered or not) can be observed in most larvae ( $n = 40$ ) from 48 h, S.21. The length of exposure was not significant ( $F_{6,104} = 0.96$ ;  $p = 0.45$ ). The NOEC for mobility was  $10 \text{ mg L}^{-1}$  and LOEC was  $25 \text{ mg L}^{-1}$ , and MATC was atrazine  $17.5 \text{ mg L}^{-1}$ .

### Malformations

Malformations were detected at all tested concentrations  $> 0.45 \text{ mg L}^{-1}$  (Table 1). Malformations were found in the three observed endpoints: in the mouth, intestine shape, and edemas (Table 2). From the exposed embryos, 68% presented malformation a significant difference in comparison with the control ( $F_{16,34} = 13.46$ ;  $p < 0.01$ ; all

**Table 2** Malformations observed for the respective concentrations of the commercial atrazine formulation

Concentration (mg L <sup>-1</sup> )	Larvae number without malformation	Larvae number with malformation	Endpoint		
			Mouth	Intestine	Edema
0.00	40	0	0	0	0
0.45	40	0	0	0	0
1	37	3	3	0	0
1.7	19	21	16	5	0
8.5	22	18	18	8	0
10	14	26	20	6	0
25	11	29	24	5	8
50	18	22	7	22	22
60	2	38	38	36	36
70	3	37	37	37	27
80	7	33	27	33	25
115	12	28	28	28	28
125	11	29	26	29	29
135	0	40	20	40	40
145	0	40	30	40	40
155	0	40	26	40	40
165	0	40	28	40	40
NOEC (mg L <sup>-1</sup> )		1	1	25	25
LOEC (mg L <sup>-1</sup> )		1.7	1.7	50	50
MATC (mg L <sup>-1</sup> )		1.35	1.35	37.5	37.5

Some larvae presented more than one type of malformation. At each concentration, 40 larvae were analyzed

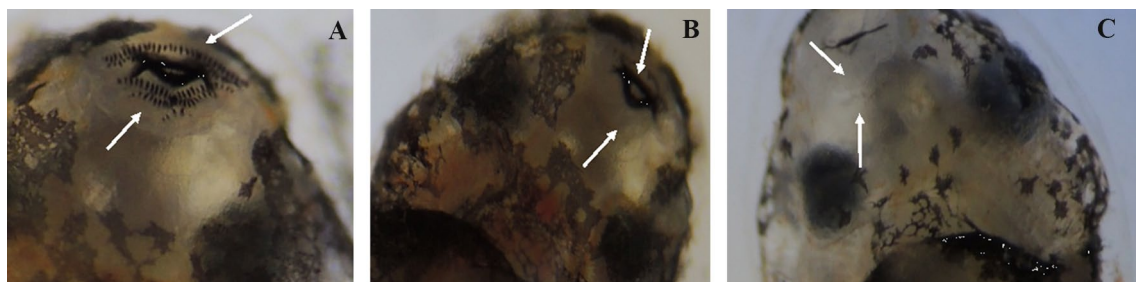
NOEC no observable-effect concentration, LOEC lowest observable-effect concentration, MATC maximum acceptable toxicant concentration

concentrations were significant in relation to the control from 1.7 mg L<sup>-1</sup>, Dunnet test,  $p < 0.05$ ).

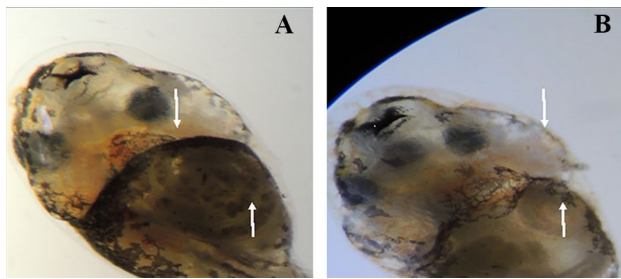
The absence of keratodonts and of parts from the upper and lower lip (Fig. 2) occurred in 54.3% of the exposed larvae. Regarding the intestine shape, 57.6% of the exposed larvae presented intestine with a straight line format, very distinct from the normal spiral shaped (Fig. 3). These changes appeared from the concentration of 1.7 mg L<sup>-1</sup>. Edema was found in 52.3% of the exposed larvae.

## Discussion

In this study, the herbicide atrazine presented low acute toxicity for *P. gracilis* at S.19–23. The LC50 for *P. gracilis* is above the indicative levels of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS Criteria, GHS 2011; where low toxicity is considered LC50 > 10 mg L<sup>-1</sup>) and also above the LC50 recorded for other amphibians (e.g., 27.16 mg L<sup>-1</sup> in *Rhinella arenarum*, Brodeur et al. 2009; 100 mg L<sup>-1</sup> for *Xenopus*, Morgan et al.



**Fig. 2** Malformations in the larvae mouth of *Physalaemus gracilis*, S.25. **a** Mouth of an individual from the negative control. **b** After exposure to the concentration of 80 mg L<sup>-1</sup>. **c** After exposure to the concentration of 125 mg L<sup>-1</sup>



**Fig. 3** Normal spiral intestine (a) and malformed intestine from *Physalaemus gracilis* larvae, S.25, after exposure to the commercial formulation of atrazine (b)

1996). However, chronic concentrations of the commercial formulation caused effects on mortality, mobility, and malformation in the early stages of development (S.19–25, Gosner 1960). In the first 24 h of testing, larvae were still protected by the embryo membrane (S.19), and until S.23 they used yolk supplies (development data, see McDiarmid and Altig 1999). This was observable in the sublethal dosages (chronic test), where the mortality started more markedly after 120 h, when larvae reached the S.24 already ingesting external food, with gill respiration and permeable skin, facilitating the absorption of contaminants (Bridges 2000; Ortiz-Santaliestra et al. 2006).

Changes in mobility can hamper or preclude swimming during the larval phase of amphibians (Peltzer et al. 2013). Atrazine caused changes in the movement capacity of *P. gracilis* exactly when swimming activity began. Development S.21–25 mark the transition from a relatively immobile embryo to a free-swimming tadpole (Duellman and Trueb 1994). At this life point, mobility is an important factor for the future tadpole, which needs to swim to find food and escape from predators. Regarding the mobility, the maximum tolerated atrazine concentration was  $17.5 \text{ mg L}^{-1}$ , a level where spasmodic contractions began, which may indicate neurotoxic effects on amphibians (Svartz et al. 2012). Effects on mobility were observed from the  $1\text{-mg L}^{-1}$  atrazine concentration. The effects caused by atrazine on the mobility of *P. gracilis* have been observed in other amphibians exposed to herbicides, such as *Ptychadena bibroni* (Ezeonye and Tongo 2009), *R. arenarum* (Svartz et al. 2012), *Ambystoma barbouri* (Rohr et al. 2003), *Rhinella schneideri*, and *Physalaemus nattereri* (Pérez-Iglesias et al. 2015). If these are neurotoxic effects, the changes in mobility are worrisome, especially because they have already been demonstrated in at least six species.

The development stage of larvae exposed to atrazine in the chronic test in the present study coincides with the mouth formation. Mouthparts begin their development in S.23, and they are fully developed on S.25 (Duellman and Trueb 1994); therefore, it is expected that part of the body would

be affected. However, there are other explanations about oral malformation. It has been demonstrated that the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) is responsible for oral changes (Knapp and Morgan 2006; Venesky et al. 2010). Because no mouth changes were found in the control group or in the lowest tested concentration ( $0.45 \text{ mg L}^{-1}$ ), *Bd* effect on this malformation may be excluded in the present study. This hypothesis also can be ruled out by the fact that atrazine reduces zoospore abundance of *Bd* in culture and *Bd*-infected tadpoles (McMahon et al. 2013).

Malformations of oral morphology were reported in other amphibians exposed to herbicides: *R. arenarum* exposed to atrazine (Svartz et al. 2012), *Scinax nasicus* exposed to glyphosate (Lajmanovich et al. 2003), and *Hypsiboas pulchellus* exposed to imazethapyr (Pérez-Iglesias et al. 2015). Anomalies in the mouth morphology due to the reduction of keratodonts can affect the diet of tadpoles and compromise the performance and survival of larvae (Christopher et al. 1996; Pérez-Iglesias et al. 2015).

Furthermore, regarding the food, changes in the intestine shape can hinder the food processing. This anomaly has already been reported in *Xenopus laevis*, when exposed to atrazine concentrations of 10, 25, and  $35 \text{ mg L}^{-1}$ . At  $35 \text{ mg L}^{-1}$ , 80% of the exposed larvae had a “straight” intestine instead of spiral (Lenkowski et al. 2008), very close to the maximum acceptable toxicant concentration of atrazine to intestinal format in *P. gracilis* ( $37.5 \text{ mg L}^{-1}$ ).

The presence of edema may be associated with alterations of ionic balance (Nieves-Puigdoller et al. 2007) and the endocrine system (Herkovits et al. 1980). Although we could not identify the physiological process of edema formation in consequence of atrazine exposure, in the present study, it is, in fact, an additional evidence of the effects caused by atrazine. The maximum acceptable toxicant concentration for edemas was  $37.5 \text{ mg L}^{-1}$ , a higher concentration than the observed for *R. arenarum* exposed to atrazine, where the significant emergence of edemas occurred at the concentration of  $15 \text{ mg L}^{-1}$  (Svartz et al. 2012).

Brazilian legislation stated  $0.002 \text{ mg L}^{-1}$  as the maximum allowed value of atrazine in water (for human consumption, Class 1 and Class 3 freshwater) for both surface and underground water (Brazil 2008, 2011). In Brazil, the maximum concentration detected in the water was  $0.075 \text{ mg L}^{-1}$  (Moreira et al. 2012). In other countries, as in the midwest United States, concentrations up to  $0.33 \text{ mg L}^{-1}$  have been found in surface waters (USEPA 2014; Belanger et al. 2015, 2016). However, published studies about atrazine concentration in water were performed mostly in rivers (Albuquerque et al. 2016), which are distinct environments from the reproduction sites of most Brazilian amphibians. There are no data on the concentrations of atrazine in water from lentic environments similar to those used by *P. gracilis*. In this study, we

showed that although with low acute toxicity, concentrations  $> 1 \text{ mg L}^{-1}$  of commercial atrazine formulation may cause changes in mobility and malformations in the early development stages of *P. gracilis*. Although the MATC data presented values of 1.35 and  $37.5 \text{ mg L}^{-1}$ , it is not possible to exclude the fact that lower concentrations affected the exposed larvae.

## Conclusions

This is the first study on the effects of the commercial formulation of atrazine on *P. gracilis*. Because it is a common and widely distributed species in southern South America, it is constantly exposed to pesticides, and therefore it is important to understand the effects of the atrazine herbicide on its life cycle. In the present study, the early stages of development of *P. gracilis* were tolerant to the commercial formulation of atrazine if the acute and chronic concentrations, which affected mobility and caused malformation, were compared with the levels recorded in surface waters and to the allowed by legislation. Such tolerance may be necessary for the survival of nontarget species that reproduce in sites contaminated by pesticides.

Atrazine is a banned herbicide in some countries due to its persistence in water (Rao et al. 2013), but it is widely used in agriculture in Brazil (IBAMA 2014) and frequently found in surface waters (Albuquerque et al. 2016). Therefore, it is a pesticide with high potential for contact with anuran amphibians that reproduce in agroecosystems, as is the case of *P. gracilis*. Only by understanding the effects of pesticides on nontarget species that naturally have contact with the contaminated environment, we can better understand the extent of tolerance or sensitivity of amphibian populations to environmental contamination. We suggest that more studies test concentrations close to those found in the environment to provide greater clarity about the toxicity of this pesticide.

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## Compliance with Ethical Standards

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

**Ethical approval** This study was licensed by IBAMA (50398-1) and authorized by the Ethics Committee for Animal Use of the Federal University of Fronteira Sul.

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