ECOTOX - BRASIL

Ecotoxicol. Environ. Contam., v. 8, n. 1, 2013, 55-58
doi: 10.5132/eec.2013.01.008

EEC

Toxicity of Glyphosate on *Physalaemus albonotatus* (Steindachner, 1864) from Western Brazil

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(Received April 12, 2012; Accept April 05, 2013)

Abstract

Amphibian declines have been reported worldwide and pesticides can negatively impact this taxonomic group. Brazil is the world's largest consumer of pesticides, and Mato Grosso is the leader in pesticide consumption among Brazilian states. However, the effects of these chemicals on the biota are still poorly explored. The main goals of this study were to determine the acute toxicity (CL_{50}) of the herbicide glyphosate on *Physalaemus albonotatus*, and to assess survivorship rates when tadpoles are kept under sub-lethal concentrations. Three egg masses of *P. albonotatus* were collected in Cuiabá, Mato Grosso, Brazil. Tadpoles were exposed for 96 h to varying concentrations of glyphosate to determine the CL_{50} and survivorship. The CL_{50} was 5.38 mg L^{-1} and there were statistically significant differences in mortality rates and the number of days that *P. albonotatus* tadpoles survived when exposed in different sub-lethal concentrations of glyphosate. Different sensibilities among amphibian species may be related with their historical contact with pesticides and/or specific tolerances. Further studies are required to determine the degree to which this taxonomic group is threatened by chemical contaminants.

Key-words: Amphibia, $CL_{50,}$ Herbicide, Mato Grosso, survival, tadpole.

Toxicidade do glifosato em *Physalaemus albonotatus* (Steindachner,1864) do Oeste brasileiro

Resumo

O declínio populacional de anfíbios tem sido observado mundialmente e agrotóxicos podem impactar negativamente este grupo taxonômico. O Brasil é o maior consumidor mundial de agrotóxicos, e Mato Grosso, o estado brasileiro que lidera este consumo. Entretanto, efeitos dos agrotóxicos sobre a biota ainda foram pouco investigados. Os principais objetivos deste estudo foram determinar o efeito agudo (CL_{50}) do herbicida glifosato em *Physalaemus albonotatus* e avaliar a taxa de sobrevivência quando girinos são mantidos em concentrações sub-letais. Três desovas de *P. albonotatus* foram coletadas em Cuiabá, Mato Grosso, Brasil. Girinos foram expostos por 96 horas em concentrações variadas de glifosato para a determinação de CL_{50} e sobrevivência. O CL_{50} foi 5.38 mg L^{-1} e houve diferenças significativas nas taxas de mortalidade e no número de dias que os girinos de *P. albonotatus* sobreviveram quando expostos as diferentes concentrações sub-letais de glifosato. Sensibilidades diferentes entre espécies de anfíbios aos agrotóxicos podem estar relacionadas com o contato histórico e/ou tolerâncias específicas. Outros estudos são necessários para determinar o quanto este grupo taxonômico está sendo ameaçado por estes contaminantes químicos.

Palavras-chave: Amphibia, $CL_{50,}$ Herbicida, Mato Grosso, sobrevivência, girino.

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INTRODUCTION

Amphibian declines have been reported and discussed worldwide (Stuart *et al.*, 2004; Beebee & Griffiths, 2005; Wake & Vredenburg, 2008) with much of the recent interest focused on the role of pesticides (Houlahan *et al.*, 2001; Davidson *et al.*, 2001, 2002; Davidson, 2004; Hayes *et al.*, 2006, 2010; May, 2010; Lawrence & Isioma, 2010). Brazil has both the greatest biodiversity worldwide and is simultaneously the world's largest consumer of pesticides (Pacheco, 2009), mainly due to its industrial scale agriculture that has rapidly expanded into the Cerrado and Amazonian biomes (Schiesari & Grillitsch, 2011). However, the impacts of agricultural pesticide runoff on Brazilian amphibians have not been well explored. This is particularly alarming given that declining amphibian populations in United States have been correlated with greater amounts of up wind agriculture, where pesticide use is common (Davidson *et al.*, 2001, 2002).

Up to 90% of pesticides never reach their intended targets (Sparling *et al.*, 2001) and amphibians are one of the non-target biological groups mostly affected (Fulton & Chambers, 1985; Berrill *et al.*, 1994; Sparling *et al.*, 2001). Glyphosate is a post-emergence, non-selective aminophosphonate-type herbicide and is currently the most popular single crop protection chemical product on the market (Woodburn, 2000; Júnior & Santos, 2002), probably due to its broad-spectrum. Glyphosate is generally regarded as an environmentally friendly herbicide because of its biodegradability and strong adsorption by soil (Barja & Santos, 2005; Vereecken, 2005). However, some studies have indicated that glyphosate can cause negative impacts on the biota (Folmar *et al.*, 1979; Langiano & Martinez, 2008; Romano *et al.*, 2008), including amphibians (Releya, 2005; Releya & Jones, 2009). Here we evaluate the toxic effects of glyphosate on *Physalaemus albonotatus* (Steindachner, 1864), a widely distributed anuran in Brazil (Mato Grosso and Mato Grosso do Sul States), Argentina, Bolivia and Paraguay (Frost, 2011).

MATERIAL AND METHODS

Three egg masses of *Physalaemus albonotatus* were collected in Cuiabá municipality, Mato Grosso State, Brazil in March 2011. Egg masses of *Physalaemus* genus (Leiuperidae) are easily visible, forming a foam nest floating on the water surface (Haddad & Prado, 2005). Each egg mass was at least 1 km away from the nearest other sample to control for the effect of genetic interference on survivorship. Each egg mass was individually maintained in a white plastic container of 30x20x10cm of size partially filled with water. Experiments with tadpoles began after hatching.

Glyphosate [Isopropylammonium N-(phosphonomethyl)-glycinate] was purchased from Dow AgroSciences Industrial Ltda (Gliz 480 SL). Mineral water (pH= 4.92) was used in the preparation of all solutions.

Acute toxicity tests were conducted using four replicates of five tadpoles, each kept in 1 liter of four selected concentrations of glyphosate solutions for 96 hours. The control consisted of tadpoles maintained only in mineral water. Concentrations of glyphosate were 0.8, 1.6, 3.2 and 6.4 mg L^{-1}. Feeding was not provided during the experiment. Observations were made on a daily basis and mortality was recorded. Mean temperature during this experiment was 31.2˚C. A Trimmed Sperman-Karber (TSK) was used to determine the CL_{50} value and its confidence interval.

Survivorship rates of *P. albonotatus* were calculated for sub-lethal concentrations of glyphosate: 25, 50 and 75% of CL_{50} values. Each replicate consisted of ten tadpoles kept in two liters plastic receptacles under experimental concentrations. Water was partially renewed every day. Tadpoles were fed *ad libitum* with fish meals. The mean temperature during this experiment was 31ºC. Dead individuals were enumerated three, five, seven, ten and eleven days after the beginning of the experiment. The last living tadpole died on the eleventh day. A survivorship graph was generated using an analysis of variance for repeated measures (ANOVA) performed in SYSTAT 12.

RESULTS

The 96-h CL_{50} of Glys 480 S for tadpoles of *Physalaemus albonotatus* from Cuiabá was 5.38 mg L^{-1}.

The percentage of *P. albonotatus* tadpole mortality increased with an increase in glyphosate concentrations. Nevertheless, tadpoles maintained under sub-lethal concentrations of glyphosate were characterized by differential survivorship rates (p= 0.00004551) (Figure 1). There were statistical differences ($p < 0.001$) between different concentrations

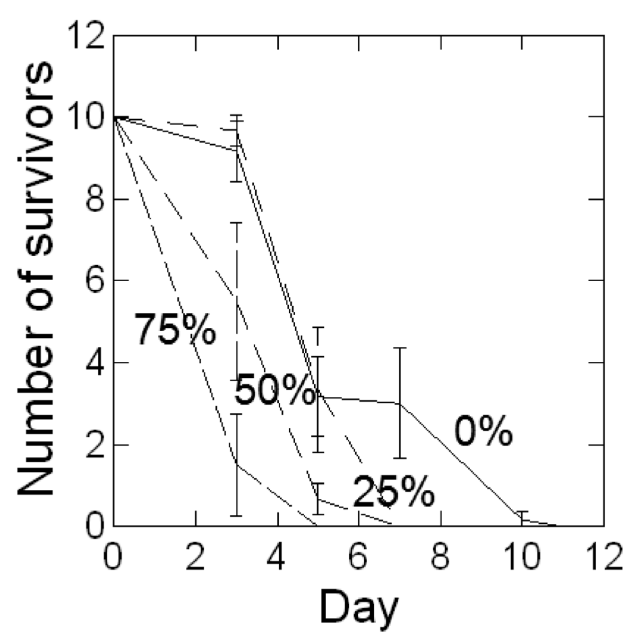

Figure 1 - The number of survivors of *Physalaemus albonotatus* tadpoles from Cuiabá, Mato Grosso, Brazil under control, 25, 50 and 75% of CL_{50} value of glyphosate by days of exposure in these concentrations. Overlap of vertical bars indicates no statistical difference and non-overlap bars shows statistical differences on survivorship.

of glyphosate for tadpole survivorship and number of days survived. After four days there were no survivors in the 75% of CL_{50} treatment, and there was only one survivor after the second day. For 25% and 50% of CL_{50} treatments there were no tadpoles which survived longer than five days. After seven days, only tadpoles in the control (mineral water) treatment were still alive.

DISCUSSION

The CL_{50} value for *Physalaemus albonotatus* was four times smaller than the value found for a congeneric species (*P. centralis*, CL_{50} 19.7 mg L^{-1}) using the same protocol (Figueiredo, 2010). Figueiredo (2010) found even higher values of CL_{50} for two other Brazilian amphibian species, the bufonid *Rhinella marina* (CL_{50} = 32 mg L^{-1}) and the microhylid *Elachistocleis* sp. (CL_{50} = 29.2 mg L^{-1}). The discrepancy in values of CL_{50} for Brazilian anuran species may reflect a differential resistance of anurans species to herbicides. Indeed, Figueiredo (2010) collected egg masses in puddles 10-50 meters away from a soybean plantation in Northern Mato Grosso where glyphosate has been used for at least a decade. These puddles were temporary (present only during the rainy season from December to March) and used for spawning by several anuran species. Anuran species with different historical contact with pesticides may thus be developing tolerance and/or may be in the midst of a population decline process.

The research protocols adopted for evaluating CL_{50} values of other anuran species differ somewhat from the present study making direct comparisons problematic. For example, Lajmanovich *et al*. (2003) tested Glys toxicity in tadpoles of *Scinax nasicus* (Hylidae) collected in Argentina, reporting a CL_{50} value of 3.62 mg L^{-1} (similar to that reported here). However, their experiment only lasted 48 hours rather than 96 hours. Mann & Bidwell (1998) using the pesticide Roundup to determine the CL_{50} for four Australian anuran species belonging to three families: Hylidae (*Litoria moorei*), Myobatrachidae (*Crinia insignifera*, *Heleioporus eyrei* and Limnodynastidae (*Limnodynastes dorsalis*). This experiment lasted 48 hours and CL_{50} values ranged from 8.1 to 32.2 mg L^{-1}. Also using roundup, Relyea (2005) determined the CL_{50} for six North American anuran species in three families: Ranidae (*Rana sylvatica*, *R. pipiens*, *R. clamitans*, *R. catesbeiana*), Bufonidae (Bufo *americanus*) and Hylidae (*Hyla versicolor*). This experiment lasted 16 days and values of CL_{50} ranged from 0.55 to 2.52 mg L^{-1}. Relyea & Jones (2009) also used a 96 hours protocol to determine the CL_{50} of Roundup in nine anuran species (three of them was not included in previous study). *Rana pipiens*, *R. clamitans*, *R. sylvatica*, *R. catesbeiana*, *R. cascadae*, *Bufo americanus*, *B. boreas*, *Hyla versicolor*, *Pseudacris crucifer* values of CL_{50} were 1.5, 1.4, 1.9, 0.8, 1.7, 1.6, 2.0, 1.7 and 0.8 mg L^{-1} respectively. Reyes *et al*. (2003) tested Glifosan for an Cuban anuran species (*Osteopilus septentrionali*, Hylidae) and the CL_{50} value in 96 hours was 20.81 mg L^{-1}. Wojtaszek & Staznik (2004) tested the pesticide Vision in *Rana clamitans* and *R. pipiens using a* 96 hours protocol, generating CL_{50} values of 2.70 and 11.50 mg L^{-1} respectively.

Methodological differences in experiments (time of exposure, commercial formulae tested) make direct comparisons among studies difficult. Nevertheless, species specific tolerances of glyphosate are apparent. These differences may be due to phylogenetic factors (diverse families of anurans analyzed) as well as environmental conditions (differential exposure to glyphosate) and merit further investigation.

The positive relationship between mortality of tadpoles and increased glyphosate concentrations was anticipated. Cauble & Wagner (2005) found the same pattern when they tested the effects of sub-lethal glyphosate concentrations (0ppm, 1ppm and 2ppm) during 43 days in the metamorphosis and development of *Rana cascadae* (Ranidae). All tadpoles including the control died before they metamorphosed. At a concentration of 0.96ppm the authors recorded a mortality of 8.6%, increasing to 51.4% when tadpoles were kept in a concentration of 1.94 ppm. Releya & Jones (2009) obtained a similar pattern of increasing mortality with increasing pesticide concentration.

There was considerable variation in the response of Brazilian anuran species to sub-lethal concentrations of glyphosate. For example, *Physalaemus centralis* had higher mortality rates when kept in higher sub-lethal concentrations of glyphosate (25, 50 and 75% da CL_{50}). For *Rhinella marina*, 50% and 75% of CL_{50} also affected survivorship, although for *Elachistocleis sp.* differences in mortality among different sub-lethal concentrations were not statistically significant (Figueiredo, 2010).

In conclusion, glyphosate exposure poses a different level of threat to different anuran species. However, further research is needed to assess whether resistance is influenced by phylogenetic signature, tolerance and/or historical contact with pesticides. Standardized protocols and additional studies on Brazilian anuran species are necessary to gain a better understanding of the impact of pesticides on Brazilian amphibians.

ACKNOWLEDGMENTS

We thank Fundação Oswaldo Cruz (FIOCRUZ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/**CT-SAÚDE** number **18/2006**) for financial support. We thank ICMBio/SISBio for collecting permits (number 25209-1) and Ethical commitee for animal research (number 23108.044717/10-4) for experimental permits. Thanks to R.J. Ladle for proof-reading the manuscript.

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