Ecotoxicology and Environmental Safety 136 (2017) 173–179

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae)

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ARTICLE INFO

Keywords:
Amphibians
Pesticides
Fipronil
Toxicity
Oxidative stress

ABSTRACT

Amphibians are very sensitive to environmental change and pollution because they have both aquatic and terrestrial life cycle stages and high skin permeability. Particularly during the larval stages, when these animals are restricted to small, transient ponds, exposure to high concentrations of pesticides is inevitable in agricultural areas. Given that pesticide application increases during the summer, which coincides with the reproductive season and the occurrence of most neotropical tadpoles in their natural environment, strong indications exist that tadpoles are developing in contaminated ponds. Fipronil is one of the primary insecticides used in sugarcane cultivation in Brazil, and little is known about its toxic effects on non-target organisms such as tadpoles. The purpose of this study was to evaluate the effects of fipronil and its metabolites on oxidative stress in *Eupemphix nattereri* tadpoles after exposure in water and sediment at concentrations of 35, 120 and 180 μg kg^{-1}. We assessed the activities of the antioxidant enzymes glutathione S-transferase (GST), glucose 6-phosphate dehydrogenase (G6PDH) and catalase (CAT) and lipid peroxidation (malondialdehyde, MDA). The results showed that fipronil has an inherent capacity to cause oxidative stress in tadpoles, as evidenced by a decrease in CAT activity and an increase in lipid peroxidation levels at all concentrations tested. Fipronil sulfone also produced elevated MDA levels at two of the tested concentrations and increased G6PDH activity in tadpoles exposed to the highest concentration of this metabolite but did not affect MDA levels. Our data showed that fipronil and its degradation products promoted oxidative stress in *Eupemphix nattereri* tadpoles exposed to environmentally relevant concentrations and could lead to a decrease in the long-term physiological performance of these animals, leading to detrimental effects at the population level.

1. Introduction

The intensification of agricultural activity in Brazil has caused extensive environmental impacts on many ecosystems, especially due to intensive pesticide use. Brazil is the largest consumer of pesticides in the world, with São Paulo being responsible for approximately 20% of the national consumption. In northwest São Paulo, the principal crop is sugarcane, and the cultivated area has increased in recent years (UNICA, 2015). Sugarcane cultivation requires a large amount of pesticides, especially the herbicides diuron, tebuthiuron and glyphosate and the insecticides carbofuran and fipronil (Bicalho et al., 2010; Peret et al., 2010). In Brazil and other tropical regions of the world, application of these pesticides is generally intensified during the rainy season, which coincides with the reproductive period of most amphibian species. Because many amphibians are distributed in areas common to agriculture practice, there is a great concern that many species are being affected by exposure to local pesticides.

The phenylpyrazole fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfonyl)pyrazole-3-carbonitrile) is among the most commonly used insecticides, which acts directly on the γ-aminobutyric acid (GABA) chloride channels in insects to disrupt neuronal signalling (Gunasekara and Troung, 2007). GABA antagonists such as fipronil are known to cause hyperactivity, convulsions and death in fish (Beggel et al., 2012). In addition, recent evidence suggests

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http://dx.doi.org/10.1016/j.ecoenv.2016.10.027
Received 7 July 2016; Received in revised form 20 October 2016; Accepted 22 October 2016
Available online 18 November 2016
0147-6513/

that fipronil also induces reactive oxygen species (ROS) production in cells, which can lead to increased lipid peroxidation and oxidative stress (Ki et al., 2012; Margarido et al., 2013; Möhler et al., 2004).

Under the aerobic conditions and sandy soils that characterize regions of sugarcane cultivation, fipronil has a half-life of 122–128 days. According to the Groundwater Ubiquity Score (GUS) index, fipronil and its degradation products have a low affinity for water, moderate mobility in soil and a sorption coefficient (Koc) of 803. Fipronil sulfone and fipronil sulfide also have low mobility and Koc values of 2511 and 3981, respectively. Fipronil has high affinity for natural organic matter present in water and sediments and is more susceptible to photodegradation than hydrolysis, except under alkaline conditions (Gunasekara and Troung, 2007; Gustafson, 1989).

In general, pesticides may be transported by drift, precipitation and runoff and may be found in different environmental matrices such as soil, surface water, and sediment, thus affecting various non-target organisms (Edwards, 1993; Hoerger et al., 2014; LeNoir et al., 1999; Moreira et al., 2010; Peret et al., 2010; Rand et al., 1995). Once in the environment, pesticides can be degraded, generating by-products that can be more or less toxic than the original compound (Cheyns et al., 2010; Gunasekara and Troung, 2007; Katagi, 2004; Muneer et al., 1999). Fipronil degradation gives rise to several products, especially fipronil sulfide and fipronil sulfone, which are generated by reduction and oxidation reactions, respectively. The concentrations of fipronil, fipronil sulfone and fipronil sulfide in the soil of São José do Rio Preto, São Paulo, Brazil were recently shown to range between 35 and 180 μg kg^{-1} (de Toffoli et al., 2015). These soils undergo flooding during the rainy season, forming temporary ponds that are used by anurans for reproduction. Due to their proximity to the cultivation areas, fipronil concentrations in these ponds can be higher than those commonly found in most permanent aquatic environments, such as rivers and lakes, thereby posing a substantial risk for local anuran species. However, to our knowledge, data regarding fipronil concentrations in small temporary ponds formed close to agriculture areas are not described in the scientific literature.

Amphibians are known to be more sensitive to pesticide contamination during the larval stage than as adults because tadpoles are strictly aquatic and have higher skin permeability (Yan et al., 2008). Once in water, fipronil rapidly partitions between the water column and the sediment (Maul et al., 2008; Tingle et al., 2003), posing a risk to benthic organisms such as *Eupemphix nattereri* tadpoles, which use the sediment and organic matter as a food source. However, studies on how fipronil affects tadpoles are still limited. Many studies have found that exposure of tadpoles to pesticides such as atrazine, glyphosate, quinclorac and fipronil in laboratory assays may alter the antioxidant response and may trigger oxidative stress (e.g., Dornelles and Oliveira, 2016; Margarido et al., 2013). Margarido and collaborators showed that fipronil impairs the antioxidant defense system in *Scinax fuscovarius* tadpoles, increasing their susceptibility to oxidative stress. However, no studies have investigated the effects of fipronil metabolites. Additionally, the study by Margarido et al. (2013) considered only fipronil dissolved directly in water and used a commercial formulation (Regent® 800WG). Due to the low water solubility of fipronil, it is possible that its association with aquatic sediment contributes more to its toxicity, especially towards benthic species that feed by foraging through the sediment. Therefore, the present study aimed to evaluate the effect of fipronil and its metabolites, fipronil sulfone and fipronil sulfide, at different environmental concentrations found in soils near sugarcane crops. To monitor oxidative stress, we measured malondialdehyde (MDA) levels to indicate lipid peroxidation and the activities of the antioxidant enzymes catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH) and glutathione S-transferase (GST, also a phase II biotransformation enzyme) in *Eupemphix nattereri* tadpoles, one of the most common benthic tadpole species found in the northwest region of São Paulo State, Brazil.

2. Material and methods

2.1. Test organisms

Spawn of the anuran *Eupemphix nattereri* (Leiuperidae) were collected from temporary ponds in the São José do Rio Preto region in northwest São Paulo State, Brazil (20° 47' 07.05"S, 49° 02' 42.09" W), during the rainy season (November–February). The larvae were kept in the laboratory under ideal temperature (28 °C), pH (7.5–8.0) and oxygen (~5 mg/L) conditions until they reached stages 29–33 (0.081 ± 0.018 g) just before the development of legs (Gosner, 1960). The animals were collected under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), and this work was approved by the Ethics Committee on Animal Use in Research of the São Paulo State University (CEUA-IBILCE/UNESP nº 086/2013).

2.2. Preparation of fipronil and its metabolites in the water and the sediment

Prior to exposure, the concentrations of fipronil and its metabolites in the water and the sediment were measured in aquariums in the absence of tadpoles to better understand the dispersion and availability of the compounds in the sediment and water. For this experiment, fipronil, fipronil sulfide and fipronil sulfone were tested at three concentrations: 35, 120 and 180 μg kg^{-1}. For the preparation of the experimental concentrations (35, 120 and 180 μg kg^{-1}), 7, 24 and 36 μg of each compound (fipronil, fipronil sulfide or fipronil sulfone), from stock solutions (72 mg L^{-1}**)** were diluted in acetone and then separately added to 200 g of soil. Water (1 L) was added to aquariums only after the acetone had evaporated. The soil was collected from non-agricultural areas of Sao Jose do Rio Preto, SP-Brazil, cleaned and washed twice with water and acetone, and used in the experiments only after the solvents had totally evaporated. No traces of fipronil or any of its metabolites were detected in the soil samples after the clean-up procedure. The concentrations of fipronil and the metabolites that were added to the soil were selected based on a recent study that found similar concentrations in soils close to agricultural areas in the region of São José do Rio Preto, Brazil (de Toffoli et al., 2015). The aquariums were agitated to disperse the compounds into the aqueous phase and placed on a horizontal surface to allow the soil to settle. Control aquariums with the solvent alone were also prepared using the same procedure for comparison. After 24 h, when the soil fraction had completely settled, water and soil were collected for chemical analysis. Chemical analysis of soil and water were also performed after 7 days after experimental exposure to the compounds in all aquariums containing tadpoles.

2.3. Chemical analysis

Fipronil and its metabolites were extracted from the water and sediment samples according to the method described by de Toffoli et al. (2015), which involves solid-phase extraction (SPE) on C18 cartridges (Supelco Analytical, Bellefonte, Pennsylvania, USA) for the water samples and liquid-liquid extraction (acetone/dichloromethane) for the sediment. To monitor the recovery rate, all water and sediment samples were spiked with the surrogate diazinon d-10 (40.0 μg L^{-1}). Deuterated d-10 phenanthrene (1.0 mg/L) was also used as an internal standard, before separation on C18 SPE cartridges. The cartridges were conditioned with 3.0 mL of a 3: 1 solution of hexane/isopropanol (Sigma-Aldrich, Germany) followed by 0.5 mL of methanol (Merck - SupraSolv®, Germany) and finally 1.0 mL of ultrapure water. With the aid of a manifold system (Agilent Technologies, USA), 400.0 mL of sample that had been pre-filtered through 0.7-μm glass fibre (Sartorius Stedim Biotech, Germany) was percolated through the cartridge and then extracted, and analytes were eluted with 3.0 mL of ethyl acetate