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Effects of the herbicide Roundup on the polychaeta *Laeonereis acuta*: Cholinesterases and oxidative stress

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ABSTRACT

Glyphosate based herbicides, including Roundup, are widely employed in agriculture and urban spaces. The objective of this study was to evaluate the toxicological effects of Roundup on the estuarine polychaeta *Laeonereis acuta*. Biomarkers of oxidative stress as well as acetylcholinesterase and propionilcholinesterase activities were analyzed. Firstly, the LC_{50} 96 h for *L. acuta* was established (8.19 mg/L). After, the animals were exposed to two Roundup concentrations: 3.25 mg/L (non-observed effect concentration – NOEC) and 5.35 mg/L (LC_{10}) for 24 h and 96 h. Oxygen consumption was determined and the animals were divided into three body regions (anterior, middle and posterior) for biochemical analysis. An inhibition of both cholinesterase isoforms were observed in animals exposed to both Roundup concentrations after 96 h. A significant reactive oxygen species (ROS) reduction was observed in the posterior region of animals in both periods, while antioxidant capacity against peroxyl radicals (ACAP) was reduced in the posterior region of animals exposed for 24 h. Considering the antioxidant defense system, both GSH levels and enzyme activities (catalase, superoxide dismutase, glutathione s-transferase, glutathione peroxidase and glutamate cysteine ligase) were not altered after exposure. Lipid peroxidation was reduced in all analyzed body regions in both Roundup concentrations after 24 h. Animals exposed to the highest concentration presented a reduction in lipid peroxidation in the anterior region after 96 h, while animals exposed to the lowest concentration presented a reduction in the middle region. Overall results indicate that Roundup exposure presents toxicity to *L. acuta*, causing a disruption in ROS and ACAP levels as well as affects the cholinergic system of this invertebrate species.

1. Introduction

Agrochemicals are one of the most widespread contaminants observed in the aquatic environment (Silva et al., 2003). Glyphosate based herbicides such as Roundup are the most employed in agriculture, comprising up to 40% of pesticides commercialized in Brazil (Carneiro et al., 2015). Besides its use in agriculture, Roundup can be used in silviculture, horticulture and in various domestic cases (USEPA, 2011). Its primary action is as a non-selective herbicide employed in weed control by the inhibition of the enzyme 5-enolpyrivilshikimate-3 phosphate synthase (EPSPS), which is a key enzyme in the shikimate pathway and responsible for the biosynthesis of aromatic amino acids in plants and microorganisms (Giesy et al., 2000).

Although animals do not possess the physiological targets for specific glyphosate toxicity, it has been demonstrated that both the active ingredient and its commercial formulations affect physiological and biochemical parameters in animals. These effects include: an inhibition of cell respiration (Peixoto, 2005), a reduction in acetylcholinesterase activity in mollusks and fish (Sandrini et al., 2013) and reproduction impairment in fish (Lopes et al., 2014). Besides these effects, it has been proposed that glyphosate exposure may cause alterations in enzymes of the antioxidant defense system in invertebrates such as annelids (Contardo-Jara et al., 2009) and insects (Aguiar et al., 2016). Such alterations to enzymes responsible for maintaining the cell redox state would lead to an imbalance between prooxidants and antioxidants in favor of the former. This then leads to a disturbance in cell redox signaling and oxidative damage, a situation called oxidative stress (Sies, 1991; Jones, 2006). Based on this, Roundup exposure may lead to a similar situation in aquatic organisms such as the fish species *Carassius auratus* and *Prochilodus lineatus*

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leading to the alteration of antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST) and glutathione reductase (GR) (Lushchak et al., 2009; Modesto e Martinez, 2010b).

Aquatic environments represent the final destination for a variety of anthropogenically based contaminants, including those introduced by industrial activity and agriculture (Saiz-Salinas and González-Oreja, 2000). Among these aquatic recipients, estuaries are considered highly stressful environments due to their great variability in salinity, dissolved oxygen, temperature and pH (D'Incao et al., 1992; Elliott and Quintino, 2007), which may be exacerbated by contaminants. Consequently, organisms that live in such environment are exposed to both natural and anthropogenic stimulus. These organisms consequently possess adaptations in order to neutralize or reduce damage caused by such variation in abiotic factors.

Polychaetes are among the organisms that inhabit estuarine environments, and *Laeonereis acuta* represents one of the primary Brazilian species. This benthonic species occurs in the Atlantic shores of South America (Omena and Amaral, 2001), living in burrows constructed within sediment. Such organisms are important elements in the food chain since they are non-selective filter-feeders and represent a food source for invertebrates, fish and birds (Botto et al., 1998; Palomo and Iribarne, 2000; Palomo et al., 2004). This polychaete species has been employed as an experimental model for assessing the effects of pro-oxidant agents of both natural and anthropogenic sources, such as hydrogen peroxide (Rosa et al., 2005), copper (Geracitano et al., 2002), cadmium (Sandrini et al., 2008) and arsenic (Ventura-Lima et al., 2011).

Since this polychaete species inhabits sediments from estuarine environments that represent a common recipient of pollutants, including glyphosate and glyphosate based herbicides, due to its use in urban and agricultural areas around the shore, the objective of the present study was to evaluate the toxic potential effects of Roundup in *L. acuta* with respect to mortality, oxidative balance and cholinesterase enzyme activity. To this, the biochemical parameters were analyzed in different body regions (anterior, medium and posterior) from *L. acuta* since previous studies related that antioxidant activity were differently expressed in body regions (Rosa et al., 2005).

2. Material and methods

2.1. Animal collection and maintenance

Laeonereis acuta were collected in the Patos Lagoon Estuary in Rio Grande, Southern Brazil and transferred in plastic bags containing cold water from the collection site. The license for collection was provided by the Chico Mendes Institute for Biodiversity Conservation – Brazil (ICM-Bio/license number 40868-1). This species were selected based in its presence and ecological relevance in the estuaries and due to its previous application on environmental monitoring programs in Southern Brazil (Monserrat et al., 2003) and laboratory studies concerning biochemical and physiological effects of pollutants (Geracitano et al., 2002; Rosa et al., 2005; Sandrini et al., 2008; Ventura-Lima et al., 2011). Animals were acclimated for 7 days under lab conditions in plastic boxes containing sediment and saline water (10 ppm), temperature 20 ± 2 °C, pH 8.0 and photoperiod 12L:12D. Animals received commercial fish food every 2 days, and the water was renewed every two days.

2.2. Experimental procedures

Since Roundup Original is a complex mixture composed of both glyphosate (360 g/L) and other ingredients, Roundup concentrations in the present study were based on the relative glyphosate equivalents. In order to define sub-lethal Roundup concentrations to *L. acuta* for further physiological and biochemical analyses, polychaetes were exposed to Roundup concentrations from 0.065 mg/L (representing the maximum commercial concentration with respect to the preservation of flora and fauna according to Brazilian legislation – CONAMA 357) to 65 mg/L. Ten (10) animals per treatment were exposed to herbicide for 96 h in individual flasks containing water (100 mL). Initially, five test concentrations (in a geometric series) and a control group were used. The test was then repeated employing three concentrations (in a geometric series) ranging from the lowest concentration that resulted in 100% mortality and the highest concentration that did not cause any mortality. In all sets of experiments, mortality was verified daily and the experimental water was renewed every 48 h (semi-static system). Animals were exposed without sediment and water conditions and were maintained during the acclimation period. Results were analyzed by the Probit method (EPA Probit Analysis Program – version 1.5); the CL_{50} and CL_{10} 96 h were determined as well as the non-observed effect concentration (NOEC). For the posterior tests, the animals were exposed to Roundup 3.25 and 5.35 mg/l, the NOEC and CL_{10} respectively.

2.3. Oxygen consumption

Animal oxygen consumption was determined according to the methodology of Nithart et al. (1999). Animals (n=10) were transferred to glass flasks containing water (10 mL) at the same characteristics of salinity, pH and temperature as the exposure period. The initial oxygen concentration was recorded with a digital oxymeter (DO-5519, Lutron Eletrônica – CO), after which the flasks were sealed for 20 min. This measurement time was chosen in order to not reduce pO_2 to hypoxic levels (Rosa et al., 2005). Then, the flasks were opened and the final oxygen concentration was determined. Blank flasks were maintained in the same conditions in order to take in account bacterial respiration. The animals were weighed and flask volumes were verified. The oxygen consumption was expressed as mg O_2/mL/mg wet weight/h.

2.4. Cholinesterase activity

In order to determine cholinesterase activity, animal tissue samples were prepared according to the methodology of Sandrini et al. (2013). Samples consisted of pools (n=6) from 4 anterior regions, homogenized in 200 μL of cold phosphate buffer (100 mM) mixed with glycerol 20%. Homogenates were centrifuged at 9000×*g* for 30 min at 30 °C; the resulting supernatant was employed as the soluble fraction (SF). Pellets were re-suspended in homogenization buffer mixed with Triton X-100 (0.5%) and incubated for 30 min at room temperature. Following incubation, re-suspended pellets were centrifuged with the same conditions described above. The supernatant from this centrifugation was employed as the enzyme source for the membrane fraction (MF).

Enzyme activity was determined via the method described by Elman et al. (1961). DTNB (5,5-dithiobis-2-nitrobenzoic acid – Sigma-Aldrich) and acetylthiocholine (7.5 mM) or propionylthiocholine (4 mM) were used. Enzyme activity was analyzed using a microplate reader (412 nm). Results were expressed as ηmoles/mg of protein/min. Protein concentrations from supernatants were measured by the biuret method as described before.

2.5. Oxidative stress analysis

After the exposure periods, each animal was divided into anterior region (20 initial segments), middle region (next 20 segments) and posterior region (final segments). Such division was based on previous studies that determined differences in the antioxidant responses in distinct regions of the animal body (Rosa et al., 2005). This division was employed for all the biochemical determinations, except for cholinesterase activity, in which the anterior region was the only one employed.