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LETTER TO EDITOR

Glyphosate as an Acetylcholinesterase Inhibitor in *Cnesterodon decemmaculatus*

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Abstract The toxic effect of sublethal concentrations (1, 17.5 and 35 mg L^{-1}) of pure glyphosate was evaluated on acetylcholinesterase (AChE) activity in the fish species, *Cnesterodon decemmaculatus*. Acute bioassays (96 h) under laboratory conditions were conducted and homogenates for each specimen corresponding to the anterior, middle and posterior body sections were performed. Fish survival was 100%, even at the highest concentration tested (35 mg L^{-1}), in accordance with the low lethal toxicity reported for glyphosate. However, a significant inhibitory effect on AChE activity was recorded even for the lowest herbicide concentration tested (1 mg L^{-1}), in the homogenates corresponding to the anterior body section. The inhibition ranged from 23 to 36%. The analytical determination of glyphosate in assay media by ion chromatography, was used to verify its stability. These results indicate that AChE—a neurotoxicity biomarker—in *C. decemmaculatus* may be affected by exposure to environmentally relevant concentrations of glyphosate.

Keywords Glyphosate · Toxicity bioassay · AChE · *Cnesterodon decemmaculatus*

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Water pollution by agrochemicals is presently one of the most critical problems for the conservation of aquatic ecosystems. In Argentina, transgenic soybean production has grown steadily over the last decade. The cropped area increased from approximately 9 million ha at the end of the 1990s up to 16 million ha in 2006/2007. This led to a parallel increase in the pesticide application rate (herbicides plus insecticides) from 127.5 million kg in 1999 to more than 250 million kg in 2006. In particular, the application of glyphosate formulations, which are the herbicides most commonly used in agriculture, increased from 70 million kg to more than 160 million kg between 1999 and 2006. Glyphosate [N-(phosphonomethyl)glycine); PMG] is the main broad-spectrum herbicide used for the control of a wide range of pests in soybean crops. The physical properties of PMG, in particular its high solubility in water and strong binding capacity to soil organic matter (WHO (World Health Organization) 1994), explain its fast and easy distribution in aquatic compartments of ecosystems.

Acetylcholinesterase (AChE) (EC 3.1.1.7) belongs to a family of enzymes designated as esterases, responsible for the degradation of acetylcholine, a neurotransmitter in both vertebrates and invertebrates. The presence of acetylcholine in non-cholinergic neurons is also well documented in sensory neurons of mammals and birds. The AChE activity depends on its amount and its tissue-specific modulation. Since AChE activity is extremely important for many physiological functions of fish, its determination may be useful in ecotoxicity studies. This enzyme is used as a classical biomarker in biomonitoring studies with regard to the exposure of a number of organophosphate and carbamate pesticides (Thompson 1999). Its inhibition causes an accumulation of acetylcholine in the synapse, with the consequent over-stimulation of the post-synaptic membrane, a process that may lead to death. It has been recently

reported that the inhibition of fish AChE can also be detected after their exposure to other contaminants, such as formulations containing glyphosate (Glusczak et al. 2007; Modesto and Martinez 2010). In addition, Behra et al. (2002) have shown a non-classical function of the enzyme: they reported that it is required for neuronal and muscular development in zebrafish embryos; suggesting that the exposure of fish to toxics that affect AChE functionality during the early stages of development could alter the population structure. The aim of this study was to investigate the effect of acute exposure of the teleost fish, *Cnesterodon decemmaculatus*, to pure glyphosate solutions on AChE activity in different portions of their body.

Materials and Methods

Cnesterodon decemmaculatus (Jenyns, 1842) (Teleostei, Cyprinidontiformes) is an ovoviviparous teleost, widely distributed in the Río de la Plata basin. It is a species that is widely used as a test species for toxicity testing and biomonitoring of aquatic environments (Ferrari et al. 1998). Fish without previous exposure to pollutants were provided by a commercial dealer. The stock was kept in glass aquaria at constant environmental conditions (22 ± 2°C; 12L:12D photoperiod), in dechlorinated tap water of Lujan City, partially renewed daily, constantly aerated and daily fed ad libitum commercial fish food. Fish were weighed and their total length determined. The instrumental errors were ±1 mg and ±0.005 cm respectively. The body weight and length (means ± SEM) were 96 ± 5 mg and 2.36 ± 0.04 cm, respectively (n = 39). These values allowed the calculation of Fulton's Condition Factor (K), using the following formula: $K = [(W \times 100)/L^3]$, where W and L are the weight and length of the fish respectively. All animals were of a homogeneous size.

Acute semi-static bioassays were conducted. Before testing, fish were acclimated for five days in reconstituted moderately hard water (MHW; pH 7.4–7.8; hardness: 80–100 mg $CaCO_3$ L^{-1}; alkalinity: 60–70 mg $CaCO_3$ L^{-1}) (USEPA 1993) under environmental conditions similar to those of the stock animals. Fish were exposed to four solutions: 0, 1, 17.5 and 35 mg PMG L^{-1} (95% purity) for 96 h. The solution pH was adjusted to 7.7 using 4 M NaOH. The tests were performed with two replicates per concentration. The ratio of organism weight/volume of test media was 960 mg L^{-1}. Animals were not fed during the tests. The test media were maintained without aeration and were completely renewed after 48 h of the experiment. To verify the stability of the herbicide, samples of the media were taken at the initial time and after 48 h (prior to the renewal). The samples were frozen at −20°C until analytical determination of the concentrations of PMG. Animals were anesthetized in ice-cold water. Three homogenates for each specimen were performed, each corresponding to different portions of the body. The anterior body section (A) corresponded to the whole head (avoiding muscle tissue); the body midsection (M) corresponded to muscle and visceral tissues; and the posterior body section (P) contained mainly muscle. This methodology was adopted considering the small size of the animals, and has been previously used by other authors (Nunes et al. 2005; Varó et al. 2008). Each body portion was homogenized using a glass-Teflon tissue disrupter at 3,500–4,000 rpm. The anterior body section was homogenized in 0.1 M K-phosphate buffer pH 8 and the middle and posterior body sections in 0.1 M Na-phosphate/KCl buffer pH 7.4. All homogenates were frozen at −20°C and stored up to 5 days.

The activity of acetylcholinesterase was determined at room temperature on the homogenates by the method of Ellman et al. (1961). Measurements were carried out at 412 nm using the substrate acetylthiocholine iodide and a molar absorption coefficient of 13.6 mM^{-1} cm^{-1}. Total protein content was quantified by the Lowry et al. (1951) method, with BSA as the standard. All measurements were performed in triplicate and a mean value was considered for the calculations. The results were calculated as specific activity units (1 U = nmol of substrate hydrolyzed min^{-1} mg $protein^{-1}$) and expressed as relative percentages of the control group. The measurements were carried out with a UV/Vis Pharmasec 1700 Shimadzu Spectrophotometer with UV-Probe software.

PMG concentrations in the exposure water were analized by ion chromatography (Zhu et al. 1999), using a Dionex DX-100 chromatograph with a conductivity detector and a 25 µL sample loop. Dionex AG-4 and AS-4 were used as analytical columns. A mixture of NaOH/CO_3^{2-} (4 mM/9 mM) was chosen as eluent with a flow rate of 2 mL min^{-1}. Data acquisition was performed using the Clarity Lite software. Samples with nominal concentrations of 0, 1, 17.5 and 35 mg PMG L^{-1} and five standards freshly prepared at the time of measurement were injected.

All reagents were of analytical grade and solutions were prepared using milli-Q water. The PMG (95%) used in the toxicity assays was given by Monsanto (Zarate, Argentina); the PMG used as standard, bovine serum albumin and acetylthiocholine iodide were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Comparisons between different experimental groups were performed using one-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test. The significance level was set at $H_o < 0.05$.

Results and Discussion

The survival of the fish exposed to the herbicide was 100%, and the Condition Factor showed no significant differences between treatments (Table 1). The average Condition Factor for all fish used in the study was 0.71 ± 0.02 (mean ± SEM; n = 39).

There are numerous reports on the toxicological effects of commercial glyphosate-based formulations on aquatic animals (Giesy et al. 2000), but few on the acute toxicity of pure glyphosate solutions. In preliminary studies carried out by Menéndez-Helman et al. (personal communication), *C. decemmaculatus* specimens exposed to 140 mg L^{-1} of PMG for 96 h showed a survival of 100%. These results are consistent with those described by Carriquiriborde (2010), who determined that the CL50-96 h for juveniles of *C. decemmaculatus* is >225 mg L^{-1}. This author also reported that this parameter for another native teleost (*Odontesthes bonariensis*) was >165 mg L^{-1}. These results suggest that pure PMG does not exhibit a significant acute lethal toxicity for freshwater fish (CL50-96 h >100 mg L^{-1}). However, several authors have noted that formulations containing PMG as an active ingredient together with POEA (polyethoxylene amine) as a nonionic surfactant exhibit a significant increase in lethal toxicity for various aquatic organisms (algae, zooplankton, amphibians and fish) (Pérez et al. 2011). There are also reports that indicate the existence of sublethal effects of these formulations, for example on biomarkers of neurotoxicity. It is interesting to mention that the variation in the activity of acetylcholinesterase, which provides information on the effects of a pesticide on the nervous system, may help to explain some behavioral changes in fish, such as loss of balance and changes in the pattern of locomotion (Tierney et al. 2007).

Figure 1 shows the activities of AChE calculated as relative percentages of the controls for each body portion of the animals (A, M, P) exposed to different concentrations of pure glyphosate. The activity in A, M and P of the control fish (n = 9) was 235.2 ± 24.1; 517.7 ± 114.0 and 346.2 ± 31.6 U (means ± SEM), respectively.

Table 1 Condition factor (K) of *C. decemmaculatus* exposed to pure PMG solutions for 96 h

Glyphosate (mg PMG L^{-1})	n	K
0	9	0.67 ± 0.05
1	10	0.75 ± 0.04
17.5	10	0.71 ± 0.05
35	10	0.73 ± 0.05

Data as means ± SEM

n number of fish

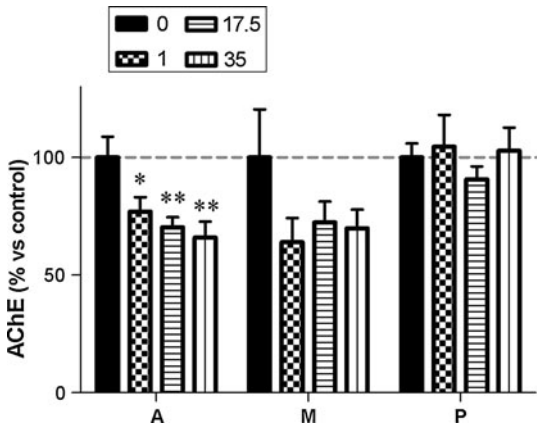

Fig. 1 AChE activity in the anterior (A), middle (M) and posterior (P) body sections of *C. decemmaculatus* after 96 h of exposure to 1, 17.5 and 35 mg PMG L^{-1}; controls in MHW. Data as means ± SEM (n = 9–10), as percentage relative to controls. Asterisks indicate significant differences from control (*$p < 0.05$, **$p < 0.01$)

Table 2 PMG concentration in assay media at initial time (t_0) and after 48 h (t_{48})

Concentration of glyphosate (mg PMG L^{-1})		
Nominal	Analytical	
	t_0	t_{48}
1	1.0 ± 0.1	1.0 ± 0.1
17.5	16.4 ± 0.7	17.0 ± 1.6
35	33.1 ± 2.1	33.9 ± 2.8

Data as means ± SEM (n = 4)

The anterior and middle body sections showed a decreasing trend in AChE activity. In A, this activity was significantly different from that observed in controls. In M, AChE activity showed a higher variability, which may explain that the differences observed were not statistically significant. The inhibition ranged from 23 to 36%. The herbicide did not affect AChE activity in the posterior body section. It could be inferred that AChE presented a different sensitivity to glyphosate depending on the enzyme location in the body.

This effect on AChE activity is comparable with that described for other fish species exposed to formulated glyphosate for a short time. In *Rhamdia quelen*, after 96 h exposure to 0.2 and 0.4 mg L^{-1} of Roundup, AChE activity decreased significantly in the brain, while no changes were found in the muscle with respect to the control (Glusczak et al. 2007). In *Prochilodus lineatus*, AChE activity in the brain decreased significantly when exposed to 1 and 5 mg L^{-1} of RoundupTransorb, whereas in muscle this decrease was significant only for the highest concentration of herbicide (Modesto and Martinez 2010).

Table 2 shows the analytical concentrations of PMG in the test media at initial time and after 48 h. The herbicide

concentrations were not significantly changed. This implies that there were no changes in herbicide concentrations due, for example, to abiotic degradation processes during the time of the trial. In another series of determinations, we found the same stability of the PMG solutions containing up to 140 mg L^{-1} of the herbicide (data not shown).

The lowest concentration tested is environmentally relevant, as there have been reports of background ambient concentrations of PMG close to 1 mg L^{-1} in water bodies in agricultural areas of the Pampas region, close to sites where formulated glyphosate was applied (Peruzzo et al. 2008). In the present work, that concentration led to changes in AChE activity in the anterior and middle body sections of fish. The present results are complementary to those provided by other authors for commercial glyphosate-based formulations.

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