Seasonal variations in the dose-response relationship of acetylcholinesterase activity in freshwater fish exposed to chlorpyrifos and glyphosate

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ABSTRACT

The herbicide glyphosate [N-(phosphonomethyl) glycine; PMG] and the insecticide chlorpyrifos [O, O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, CPF] are widely used in agricultural practices around the world and can reach aquatic environments. Therefore, it is necessary to characterize the toxicity of these pesticides on non-target species. The use of biomarkers as a tool to assess responses of organisms exposed to pollutants requires the understanding of their natural fluctuation and the dose-response relationship. In the present work, the effect of the exposure to PMG and CPF on the acetylcholinesterase activity (AChE, biomarker of neurotoxicity) in Cnesterodon decemmaculatus, a native teleost, was evaluated in different environmental conditions. Circannual rhythms in the basal levels of AChE activity in homogenates of the anterior section were confirmed. Statistically significant average inhibition of AChE activity (47.1 ± 0.7% for 1 μg CPF × L−1; 69.7 ± 2.5% for 5 μg CPF × L−1; 23.1 ± 1.1% for 1 mg PMG × L−1 and 32.9 ± 3.3% for 10 mg PMG × L−1) was determined during summer, winter and spring weather seasons. Interestingly, animals exhibit an increased susceptibility to exposure during the autumn season (inhibition of 55.4 ± 0.6% for 1 μg CPF × L−1; 81.9 ± 3.3% for 5 μg CPF × L−1; 41.4 ± 1.7% for 1 mg PMG × L−1 and 61.1 ± 0.3% for 10 mg PMG × L−1). A different sensitivity of the enzyme between seasons was evaluated by in vitro tests. The inhibition pattern for chlorpyrifos-oxon (CPF-oxon, the active metabolite of CPF) was not affected when test was performed using homogenates of unexposed specimens of summer or autumn. Otherwise, PMG in vitro inhibitory effect was not observed in a wide range of concentrations.

The results confirm that AChE activity is a sensitive biomarker for exposure to CPF and PMG, even at environmentally relevant concentrations. Finally, this work highlights the existence of seasonal variations in the dose-response relationship, which could be due to variations in the metabolism of the pollutants.

1. Introduction

The pollution of freshwater ecosystems is one of the most critical problems that may affect aquatic environments, since they serve as final receivers of chemically complex mixtures of natural pollutants and xenobiotics. In Argentina, the continuous expansion of the agricultural frontier led to a parallel increase in the pesticide application rate (herbicides plus insecticides, fungicides, acaricides, among others) from 62,800 tons of active ingredients in 1999 to more than 200,000 tons in 2016 (FAO, 2018). At present, organophosphorus (OP) pesticides are one of the most widely used and have largely replaced organochlorine pesticides. Glyphosate [N-(phosphonomethyl) glycine; PMG] is the most used herbicide for agricultural purposes, while chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate; CPF] is the most used insecticide for genetically modified soybean crops in the region (Aparicio et al., 2015). Once applied, part of the pesticides can reach nearby aquatic environments and adversely affect the biota. These contaminants may cause environmental stress, which often produces abnormal regulatory responses in aquatic organisms.

Changes beyond the normal homeostatic limits of a particular parameter can be monitored by selected biomarker measures. The term biomarker (WHO, 1993) broadly includes parameters whose alteration
reflects the interaction between a particular biological system and the environmental stressors. Ecotoxicological biomarkers are frequently used in toxicity bioassays, as well as in environmental quality and risk assessment protocols, as early-warning signals reflecting the adverse sublethal responses to pollutants. Moreover, it is possible to quantify the degree of stress from the magnitude of changes in selected biomarkers.

Acetylcholinesterase (AChE) belongs to a family of enzymes designated as esterases and its physiological role is to terminate neurotransmission by hydrolysis of acetylcholine. AChE is a biomarker of the alterations in the neuromuscular cholinergic and sympathetic synaptic junctions within the Central Nervous System (CNS). The determination of AChE activity has been used in many ecotoxicological studies, it is a sensitive biomarker for organophosphates and carbamates pesticides exposure (Soreq and Seidman, 2001; Thompson, 1999). AChE inhibition due to organophosphate exposure is caused by irreversible binding of the phosphate group of the OP to the enzyme active site (Thompson, 1999). Instead for organophosphorothioates, which are less toxic, a biotransformation involving an oxidative desulphurization must occur before enzyme inhibition (Assis et al., 2012). These OP pollutants are generally known as disruptors of cholinergic nerve transmission, preventing the inactivation of acetylcholine. AChE inhibition causes an accumulation of acetylcholine in the synaptic cleft and thereby to overstimulation of the postsynaptic membrane, which can lead to death. Several studies have indicated that besides organophosphates and carbamates, AChE from brain and muscle was also sensitive to other pollutants such as heavy metals and glycosylase-based herbicides (Modesto and Martinez, 2016; Richetti et al., 2011).

In the case of fish, inhibition of AChE activity has been shown to be extremely important for many physiological functions such as locomotor capacity, predator evasion, prey location, orientation towards food and feeding, spatial distribution pattern and social interactions (Dutta and Arends, 2003).

Cnesterodon decemmaculatus (Jenyns, 1842) (Teleostei, Cyprinodontiformes) is a freshwater non-migratory and live-bearer teleost (Pisces, Poeciliidae), endemic in Neotropical America, which has a wide distribution in a variety of water bodies of the Rio de la Plata basin. C. decemmaculatus is one of the native species proposed as suitable species for use in ecotoxicity studies because it is easily handled and acclimatized to laboratory conditions and has been used for in situ biomonitoring of aquatic ecosystems (Sallabí, 2006). Furthermore, AChE inhibition in C. decemmaculatus after the exposure to environmental contaminants, such as heavy metals and pesticides, has also been reported (Bernal-Rey et al., 2017; de la Torre et al., 2002; Menéndez-Helman et al., 2012).

In environmental context, important physiological biomarkers may fluctuate temporally in response to changing periods of organism development (Wendelaar Bonga, 1997). The use of enzymatic activity as a tool to assess responses of organisms exposed to pollutants in toxicity bioassays, as well as in biomonitoring protocols, requires the understanding of the toxic-biological response relationship, the natural fluctuation of a particular biomarker and the validation in the system under study. Previous reports indicated that basal levels of AChE activity can also be affected by fish length and seasonal variability (Beauvais et al., 2002; Chuiko et al., 1997; Menéndez-Helman et al., 2015). In this regard, the response to a stress factor cannot be assumed to be always the same. Thus, the aim of this study was to analyse the effect of seasonal variation on the dose-response relation of the acetylcholinesterase activity in the teleost fish Cnesterodon decemmaculatus exposed to the OP pesticides chlorpyrifos and glyphosate.

2. Material and methods

2.1. Chemicals

All reagents were of analytical grade and solutions were prepared using Milli-Q water. PMG, Bovine serum albumin (BSA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and acetylcholine iodide were purchased from Sigma (St. Louis, MO, USA). CPF and chlorpyrifos-oxon [diethyl (3,5,6-trichloro-2-pyridinyl) phosphate, CPF-oxon] were purchased from Chem Services (USA).

2.2. Animals

Fish were provided by a local commercial supplier who collected them from streams of Buenos Aires Province during summer, autumn, winter and spring. The body weight and length (mean ± SEM) of the animals used for the bioassays were 93 ± 2 mg and 2.53 ± 0.01 cm, respectively (N = 227) at the final time.

2.3. Semi-static bioassays

Semi-static toxicity bioassays were performed following the protocol for freshwater fish of the Argentine Normalization and Certification Institute (IRAM, 2008). Animals captured in each weather season were acclimatized to glass aquarium conditions (22 ± 2 °C, 12L:12D photoperiod) for 15 days, in dechlorinated tap water, aerated and fed daily ad libitum with commercial dried fish food. For each bioassay, 50 adult fish of a homogeneous size (around 2.5 cm length) were selected from the sample after the first acclimatization period. Then, fish were newly acclimatized for 48 h in a chamber in polypropylene containers with reconstituted moderately hard water (MHW; pH 7.4–7.8, hardness equivalent to 80–100 mg × L⁻¹ CaCO₃; alkalinity equivalent to 60–70 mg × L⁻¹ CaCO₃) under similar previous aquarium temperature and photoperiod conditions.

Fish were exposed to 0 (control), 1 and 5 μg CPF × L⁻¹ or 1 and 10 mg PMG × L⁻¹ for 96 h. Each exposure treatment was performed by duplicate. All test media concentrations used for the bioassays, were below those reported for surface water, 10.8 μg CPF × L⁻¹ and 10.9 mg PMG × L⁻¹, in the Pampenan Region (Marino and Ronco, 2005; Ronco, 2011). Furthermore, the CPF concentrations used in these assays were much lower than the LC₅₀ of 105.3 μg CPF × L⁻¹ (Paracampo et al., 2015) or 75 μg CPF × L⁻¹ (Carriquiriborde, 2010) previously reported for C. decemmaculatus. Similarly, the PMG concentrations used were much lower than the LC₅₀ > 225 mg PMG × L⁻¹ (Carriquiriborde, 2010) reported for the same fish species.

The CPF solutions were prepared by dilution of the stock solution (1000 mg CPF × L⁻¹ in ethanol) with MHW. The final test solutions of CPF contained ethanol 1 × 10⁻⁴, 5 × 10⁻⁴ and 10⁻³ (v/v). The PMG solutions were prepared in MHW and the pH was adjusted to that of the control group (MHW, pH 7.4–7.8) by addition of NaOH 4 M. All test solutions were completely renewed after 48 h. To verify the concentration and stability of pesticides, samples of the media were taken at the beginning, after 48 h (prior and post to the renewal) and at final exposure time and preserved at -20 °C for further analysis.

Mortality was recorded after 24, 48, 72 and 96 h of exposure and dead specimens were removed from the containers. The ratio of the organisms’ weight/MHW volume was kept constant (1 g × L⁻¹). At the end of the exposure period, animals were anesthetized in ice-cold chilled water, and the spinal cord was carefully cut. Fish were weighed (W) with an analytical balance, and the total length (L) was measured with a digital caliper. Each specimen was dissected, and the anterior section corresponded to the whole head was immediately processed. This methodology was adopted considering the small size of the animals, as was previously reported by different authors (Menéndez-Helman et al., 2012, 2015; Varó et al., 2008; among others). Throughout this study, fish pain or discomfort was avoided following the established instructions of animal handling protocols mentioned before.

Homogenization of tissue of the anterior section was performed using a glass-Teflon electrically operated tissue homogenizer at 3500–4000 rpm. The ratio was 1/25 tissue weight/buffer volume, using...
0.1 M K$_2$HPO$_4$ buffer pH 8. The homogenates were centrifuged (Hermle D.L. Bernal-Rey, et al.
nebulizer pressure 60 psig; drying gas 13 L × min$^{-1}$; gas temperature
sensitive mode. The operation parameters were: capillary voltage 3500 V; 6530 detector, equipped with electrospray ionization (ESI) in the po-
terms were not exposed to any inhibitory agent.
In both cases (CPF and PMG), samples of the bioassays media were
analyses were performed using MassHunter Software and with an accurate
mass database and spectral library. Besides, samples concentrations
omaximum injection enrichment factor 20:1 (Dionex AS-22 and AS-22 were used as analytical columns. A mixture of NaOH/
Na$_2$CO$_3$ (4 mM/9 mM) was chosen as eluent with a flow rate of
250 nM) were carried out. For this purpose, different volumes of stock
concentrations of CPF-oxon (final concentrations: 0, 10, 50, 100 and
200 nM) were run. For this purpose, different volumes of stock
concentrations of CPF-oxon (final concentrations: 0, 2.5, 5, 7.5, 10 and 20 mM). After 3 h of co-incubation AChE activity for each fish sample, the activity mean values were cal-
calculated for each fish as K = [(W × 100)/L$^3$], where W and L are
weight and length of the fish, respectively.
The results of AChE activity, L, W and K index are presented as
mean ± SEM (N = number of fish). The AChE activities determined in
fish from the bioassays are expressed as relative percentage, where
mean controls are referred as 100%, for each treatment and season.
Kruskal–Wallis non-parametric test followed by Dunn’s post-test or
ANOVA followed by Bonferroni post-test were used for multiple com-
parison analysis.
The values grouped by sex during different weather seasons were
analysed by unpaired Student’s t-test.
For in vitro assays the concentration of 50% inhibition (IC$_{50}$) was
calculated from the dose-response curve applying the equation “log
(inhibitor) vs. normalized response - variable slope” (GraphPad Prism
6.01 software).
The level of significance for all statistical tests was set at α = 0.05. All statistical analyses were performed using GraphPad Prism 6.01 and SigmaPlot 12.0 softwares.

3. Results and discussion
The appropriate use of an enzyme activity as a biomarker requires
good knowledge of its basal level, its natural variability related to
intrinsic biotic and environmental abiotic factors, and the susceptibility
of the response to pollutants. In this sense, based in bioassays results,
basal levels of soluble AChE activity in C. decemmaculatus control group
at different weather seasons were first analysed. Subsequently, the
existence of seasonal variations in the dose-response relation was eval-
uated.

3.1. Circannual rhythms in the basal levels of AChE activity
The unexposed specimens were seasonally collected in autumn,
winter, spring and summer throughout one year. The higher AChE ac-
activity was observed in summer (264 ± 11), whereas intermediate val-
ues were determined in autumn and spring, and the lowest levels were
found in winter (116 ± 8). It is noteworthy that AChE activity in
summer season is more than twice the winter level (p < 0.001) and
one and half times the spring one (p < 0.05), even though the spec-
imens were not exposed to any inhibitory agent.
The influence of both season and fish size on AChE activity was
3.2. Seasonal variations. Effect of the exposure to pesticides on AChE activity in *C. decemmaculatus*

The effects of pesticide exposure on AChE activity were evaluated throughout the year considering the existence of circannual rhythms in the baseline levels. *C. decemmaculatus* specimens were exposed to different concentrations of CPF and PMG during the four seasons of the year (autumn, winter, spring and summer).

### 3.2.1. Seasonal variations of AChE activity in *C. decemmaculatus* exposed to CPF

The average mortality between the bioassays was 12 ± 4%; 14 ± 5% and 12 ± 4% for control and groups exposed to 1 and 5 μg CPF × L−1, respectively.

CPF concentrations of the different bioassays are shown in Table 1. The analytical concentrations were similar to the nominal ones. No significant decrease in concentration was determined for any of the treatments during the entire bioassay for all seasons (on average, it decreased less than 5%). It should be noted that, as previously mentioned, the low concentrations tested are environmentally relevant, as CPF concentrations close to 10 μg × L−1 have been reported for water bodies in agricultural areas of the Rolling Pampa region (Marino and Ronco, 2005).

The results of all bioassays of CPF exposure are presented in Fig. 2. CPF exposure showed a concentration-dependent inhibition effect on AChE activity of *C. decemmaculatus*, with greater inhibition as the CPF concentration increases, for all seasons. The absence of solvent effect was verified for the highest ethanol concentration tested (data not shown).

These results are consistent with previous reports that describes AChE inhibition after OP pesticides exposure in fish species (Fulton and Key, 2001). Several impairments of the locomotor behaviour after acute

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**Table 1** Analytical concentration of CPF (μg × L−1) and PMG (mg × L−1) determined in aliquots of the bioassays media, collected at the initial time, after 48 h (pre and post replacement) and at the end of the bioassays (96 h).

<table>
<thead>
<tr>
<th>Weather Season</th>
<th>Nominal Initial exposure</th>
<th>48 h (before replacement)</th>
<th>48 h (after replacement)</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>1.11 ± 0.01</td>
<td>1.06 ± 0.01</td>
<td>1.09 ± 0.01</td>
<td>1.06 ± 0.01</td>
</tr>
<tr>
<td>Winter</td>
<td>4.98 ± 0.01</td>
<td>4.87 ± 0.01</td>
<td>4.94 ± 0.01</td>
<td>4.80 ± 0.01</td>
</tr>
<tr>
<td>Spring</td>
<td>0.99 ± 0.01</td>
<td>0.94 ± 0.01</td>
<td>1.05 ± 0.01</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>Summer</td>
<td>5.01 ± 0.02</td>
<td>4.72 ± 0.01</td>
<td>4.92 ± 0.01</td>
<td>4.74 ± 0.01</td>
</tr>
<tr>
<td>Second autumn</td>
<td>1.08 ± 0.01</td>
<td>1.04 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td>0.99 ± 0.02</td>
</tr>
<tr>
<td>Winter</td>
<td>4.93 ± 0.01</td>
<td>4.61 ± 0.01</td>
<td>4.91 ± 0.01</td>
<td>4.71 ± 0.01</td>
</tr>
<tr>
<td>Summer</td>
<td>1.08 ± 0.01</td>
<td>1.07 ± 0.01</td>
<td>1.11 ± 0.01</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>Second autumn</td>
<td>4.92 ± 0.01</td>
<td>4.88 ± 0.02</td>
<td>4.99 ± 0.01</td>
<td>4.80 ± 0.01</td>
</tr>
<tr>
<td>PMG concentrations in the media of the bioassays (mg × L−1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>1.1</td>
<td>0.8</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Winter</td>
<td>10</td>
<td>8.0</td>
<td>9.8</td>
<td>8.2</td>
</tr>
<tr>
<td>Spring</td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Summer</td>
<td>10</td>
<td>10.0</td>
<td>10.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Second autumn</td>
<td>1.9</td>
<td>0.8</td>
<td>9.9</td>
<td>8.2</td>
</tr>
<tr>
<td>PMG concentrations in the media of the bioassays (mg × L−1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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![Fig. 1. AChE activity in homogenates of the anterior section of *C. decemmaculatus* specimens collected during different seasons of the year.](image-url)

C. decemmaculatus brain AChE activity of (Bernal Rey et al., 2017).

The average inhibition in autumn was 55.4 ± 0.6% and 81.9 ± 3.3%, for 1 and 5 μg CPF × L−1, respectively. No statistically significant differences were observed for the exposure to 1 μg CPF × L−1 between winter and first autumn (p < 0.05), winter and second autumn (p < 0.001), and between summer and second autumn (p < 0.001).

The possible correlation between AChE inhibition and protein content variation (as percentage of respective control) was also analysed, since the total protein content is affected by some treatments exposure and it is a parameter used to calculate the AChE specific activity. No correlation between these parameters was observed (data not shown), discarding that the variations in susceptibility were determined by changes in protein content.

It is also interesting to note that the peak insecticide application for soybean production in the Argentine Pampas, usually occurs in late December to early February, and chlorpyrifos was consistently detected in streams of this region from December to April (Hunt et al., 2016; Marino and Ronco, 2005). In this context, it is more probably that fish species are exposed during the spring and summer months when spec- imens have higher values of absolute AChE activity and consequently remain with greater residual activity that allows them to meet their physiological requirements. At the same time, it is worth mentioning that AChE decrease is not necessarily fatal. In some species, fish survival has been observed even in brain AChE activity inhibition > 90% (Fulton and Key, 2001).

3.2.2. Seasonal variations of AChE activity in C. decemmaculatus exposed to PMG

The average mortality between the bioassays was 12 ± 4%; 10 ± 3% and 14 ± 4% for control and groups exposed to 1 and 10 mg PMG × L−1, respectively.

PMG concentrations of the different bioassays are shown in Table 1. The analytical concentrations were similar to the nominal ones. A slight decrease in the PMG concentrations (on average, around 15% during 48 h) was determined for the treatments for all seasons.

PMG exposure also showed a concentration-dependent inhibition effect on AChE activity of C. decemmaculatus for all seasons (Fig. 3).

Different sublethal effects of the glyphosate-based herbicides have been reported in fish. The AChE activity has been widely used as a neurotoxicity biomarker of exposures to OP (of the phosphonothioate and phosphate subclass) and carbamates since these pollutants exert a direct inhibition of the enzyme. Glyphosate was not considered as an anti-acetylcholinesterase inhibitor, due to its chemical structure. However, in the last years some studies have reported the inhibition of the enzyme after in vivo exposure to glyphosate-based herbicides (Glusczak et al., 2007; Modesto and Martinez, 2010; among others). Moreover, although there are very few works on the toxicity of PMG in fish, the inhibition of AChE activity in the anterior and middle sections of C. decemmaculatus was reported due to the exposure to PMG at concentrations of 1; 17.5 and 35 mg × L−1 (Menéndez-Helman et al., 2012). The results of the present work are consistent and complementary to those previously reported.

Regarding the morphometric parameters, there were no statistically...
significant differences in size between groups, also verifying the homogeneity of the specimens used in the bioassays (Table 2). A decrease profile in weight and condition factor confirm the difference of susceptibility of the animals in autumn (p < 0.01) and between summer and second autumn (p < 0.001), winter and second autumn (p < 0.01), summer and first autumn (p < 0.001), hand, for the highest concentration tested, statistically significant differences were obtained between winter and first autumn (p < 0.01), summer and second autumn (p < 0.01), and between summer and second autumn (p < 0.001), confirming the difference of susceptibility of the animals in autumn season.

Glyphosate based-herbicides are products widely applied in the regional agriculture practice. About five applications of glyphosate per year are made in the soybean crops in the Argentine Pampas. Consequently, its concentration in surface water nearby to agricultural areas were determined, even before and after the cultivation cycle (at the end of winter and the beginning of autumn) (Aparicio et al., 2013; Peruzzo et al., 2008). In this sense, specimens could be exposed during long periods and, although no lethal effects are observed, it is important to note that the reproductive period of the species (Lorier and Berois, 1995) overlap with the months of greatest application. Furthermore, the presence of this herbicide in surface waters has also been found during the period of the greatest susceptibility of the specimens.

### Table 2

Morphometric parameters (total length, L; weight, W; Fulton's condition factor index, K) and total protein content in the anterior section of *Cnesterodon decemmaculatus* specimens after acute exposure to chlorpyrifos (1 or 5 μg CPF × L⁻¹), glyphosate (1 or 10 mg PMG × L⁻¹) or maintained in MHW (control, 0) during different weather seasons.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>L (cm)</th>
<th>W (mg)</th>
<th>K</th>
<th>Total Protein (mg × g wet Tissue⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.56 ± 0.09</td>
<td>97 ± 14</td>
<td>0.56 ± 0.02</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>1 CPF</td>
<td>2.62 ± 0.04</td>
<td>123 ± 6</td>
<td>0.68 ± 0.02*</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>5 CPF</td>
<td>2.57 ± 0.04</td>
<td>101 ± 9</td>
<td>0.58 ± 0.03</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>10 PMG</td>
<td>2.62 ± 0.07</td>
<td>128 ± 11</td>
<td>0.72 ± 0.05</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.50 ± 0.04</td>
<td>95 ± 4</td>
<td>0.61 ± 0.02</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>1 CPF</td>
<td>2.53 ± 0.04</td>
<td>88 ± 4</td>
<td>0.54 ± 0.02</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>5 CPF</td>
<td>2.52 ± 0.03</td>
<td>79 ± 4*</td>
<td>0.49 ± 0.02**</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>10 PMG</td>
<td>2.51 ± 0.04</td>
<td>79 ± 3**</td>
<td>0.50 ± 0.02**</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>Spring</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.45 ± 0.02</td>
<td>78 ± 3</td>
<td>0.54 ± 0.02</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>1 CPF</td>
<td>2.45 ± 0.03</td>
<td>73 ± 3</td>
<td>0.50 ± 0.01</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>5 CPF</td>
<td>2.43 ± 0.05</td>
<td>69 ± 4</td>
<td>0.49 ± 0.03</td>
<td>63 ± 5*</td>
</tr>
<tr>
<td>10 PMG</td>
<td>2.39 ± 0.05</td>
<td>67 ± 7</td>
<td>0.49 ± 0.02</td>
<td>82 ± 5</td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.39 ± 0.04</td>
<td>91 ± 6</td>
<td>0.67 ± 0.04</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>1 CPF</td>
<td>2.43 ± 0.06</td>
<td>82 ± 9</td>
<td>0.55 ± 0.04</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>5 CPF</td>
<td>2.47 ± 0.05</td>
<td>74 ± 6</td>
<td>0.49 ± 0.04**</td>
<td>42 ± 2***</td>
</tr>
<tr>
<td>10 PMG</td>
<td>2.49 ± 0.02</td>
<td>84 ± 4</td>
<td>0.54 ± 0.03*</td>
<td>65 ± 3</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (N = 7–10). Asterisks indicate significant differences from the respective control (*p < 0.05, **p < 0.01, ***p < 0.001) (ANOVA, Bonferroni test or Kruskal–Wallis, Dunn’s test).
is important to highlight that in vivo contaminant levels that reach the brain to exert its neurotoxic effect. It elimination (Monserrat et al., 2003). These factors could affect the body and its half-life such as assimilation, metabolization and processes that influence the distribution of the compound throughout expression of different isoforms. On the other hand, there are different processes that influence the distribution of the compound throughout the body and its half-life such as assimilation, metabolism and elimination (Monserrat et al., 2003). These factors could affect the contaminant levels that reach the brain to exert its neurotoxic effect. It is important to highlight that in vivo studies using AChE extracts from different fish species present much more varied responses to the same compounds than in vitro studies. The environmental conditions, the balance between the polymorphism of the enzyme itself and the detoxification and bioactivation processes in the whole organism, result in a range of global susceptibilities inter- and intra-species (Chandrasekara and Pathiratne, 2007). As a first approach in the present work the sensitivity of the anterior section AChE of C. decemmaculatus to CPF and PMG and the existence of possible seasonal variations were analysed.

The primary mechanism of action of the phosphorothioate insecticides is known to be the inhibition of AChE. In this regard, many authors reported that the same effect is also produced on non-target species (Fulton and Key, 2001). The inhibition of AChE has been determined for C. decemmaculatus by in vivo exposure to CPF. In addition, an inhibition pattern was determined by in vitro tests obtaining a IC_{50} = 10.2 nM for CPF-oxon, its active metabolite, confirming that it presents a mechanism of direct inhibition on the enzyme (Bernal Rey et al., 2017). Moreover, Carr et al. (1997) found that in vitro sensitivity of brain AChE of G. affinis to inhibition by CPF-oxon was lower than the AChE sensitivity from other species (bass, bluegill, and shiners). This result suggests that the difference in toxicity among species, after an exposure to chlorpyrifos-oxon (0, 5, 10, 50, 100, 200 nM). Data are expressed as mean ± SEM (N = 4), AChE activity was calculated in percentage relative to controls.

According to the obtained results, no seasonal differences in the sensitivity of AChE to CPF that could explain the changes observed after in vivo exposure were determined. Some reports point out changes in the AChE inhibition after OP pesticides exposure according to fish size in different species, showing the existence of susceptibility variations during the life cycle of an organism (Chandrasekara and Pathiratne, 2007; Rath and Misra, 1981). Taking into account that homogeneous animals were used in the present work, this parameter would not be the cause of the differences observed between seasons after in vivo exposure. On the other hand, these differences should be explained by variations in the metabolism of the contaminant, such as bioactivation or detoxification, throughout the year affecting the levels of CPF-oxon that reach the brain.

Regarding PMG, an organophosphorus herbicide of the phosphonates subclass, different authors have reported the inhibition of AChE after glyphosate-based herbicide in vivo exposure. However, this was not predictable from its chemical structure, and the mechanism of inhibition remains unclear. In this context, the enzyme sensitivity to PMG was studied.

No inhibitory effect on AChE activity was observed when the in vitro assays were performed over a wide range of PMG concentrations (Fig. 5). In the present work we have carried out procedures to regulate the pH in such a way that an effect of PMG can be distinguished beyond its acid characteristic.

These results suggest that the inhibition observed for PMG in vivo exposure would not involve a direct mechanism of inhibition.

Conversely, a recent work by other authors has reported the 50% inhibition of AChE activity in Jenynsia multidentata and Danio rerio by in vitro exposure to 0.62 and 6.67 mM PMG, respectively (Sandrini et al., 2013).

The current results suggested that seasonal differences observed after in vivo exposure to PMG, as well as CPF, could be due to variations in the metabolism of the contaminant.

Different authors have suggested that oxidative stress may be a nonspecific way present in different toxicity scenarios of fish exposed to pesticides (Slaninova et al., 2009). Particularly, in relation to

**Fig. 3.** AChE activity in homogenates of the anterior section of *Cnesterodon decemmaculatus* fish after acute in vivo exposure to 0, 1 and 10 mg PMG × L\(^{-1}\) during different weather seasons. Data are expressed as mean ± SEM (N = 7–10). Panel A: AChE activity expressed as nmol of substrate hydrolysed × min\(^{-1}\) × mg protein\(^{-1}\) and asterisks indicate statistically significant differences vs the respective control group (\(*p < 0.05; **p < 0.01; ***p < 0.001\) (ANOVA, Bonferroni test or Kruskal–Wallis, Dunn’s test). Panel B: AChE activity in percentage relative to controls.

**Fig. 4.** Acetylcholinesterase activity in homogenates of the anterior section of un-exposed fish *Cnesterodon decemmaculatus*, corresponding to control group of bioassay carried out during different weather seasons (summer or autumn), and after in vitro exposure to chlorpyrifos-oxon (0, 5, 10, 50, 100, 200 nM). Data are expressed as mean ± SEM (N = 4), AChE activity was calculated in percentage relative to controls.

In this context, the difference in enzyme sensitivity to CPF-oxon by in vitro assays using homogenates of non-exposed animals from summer or autumn bioassays was evaluated.

The inhibition curves have no significant differences as can be seen in Fig. 4. The IC\(_{50}\) for in vitro exposure to CPF-oxon in these conditions were 10.2 ± 1.3 nM (R\(^2\) = 0.9321) and 10.8 ± 1.3 nM (R\(^2\) = 0.9342) for specimens of summer and autumn, respectively. There were no differences between control treatments with and without ethanol, discounting the effect of solvent for the highest ethanol concentration ([ethanol] = 0.0125%) used to dissolve CPF-oxon (data not shown).

According to the obtained results, no seasonal differences in the sensitivity of AChE to CPF that could explain the changes observed after in vivo exposure were determined. Some reports point out changes in the AChE inhibition after OP pesticides exposure according to fish size in different species, showing the existence of susceptibility variations during the life cycle of an organism (Chandrasekara and Pathiratne, 2007; Rath and Misra, 1981). Taking into account that homogeneous animals were used in the present work, this parameter would not be the cause of the differences observed between seasons after in vivo exposure. On the other hand, these differences should be explained by variations in the metabolism of the contaminant, such as bioactivation or detoxification, throughout the year affecting the levels of CPF-oxon that reach the brain.
4. Conclusions

In the present study, the seasonal changes in susceptibility of *C. decemmaculatus* to chlorpyrifos and glyphosate exposure were evaluated by analysing the inhibition of soluble AChE in different weather seasons. Brain acetylcholinesterase activity appears to be a sensitivity biomarker for chlorpyrifos and glyphosate exposure in *C. decemmaculatus*, even at concentrations that are environmentally relevant (near 10 µg CPF X L⁻¹ and 10 mg PMG X L⁻¹). The existence of circannual rhythms in the basal levels of AChE activity was confirmed. However, to date there were no reports of seasonal variation in the dose-response relationship of AChE in fish exposed to OP pesticides. According to the results obtained in the present work, the AChE inhibition after in vivo exposure differs between autumn and the other weather seasons. Fish specimens present increased susceptibility to CPF and PMG exposure in autumn season. No differential sensitivity of the enzyme to CPF-oxon was determined between summer and autumn by the in vitro assays. At the same time, the results of the in vivo assays suggest that the inhibition observed for PMG in vivo exposure would not involve a direct mechanism of inhibition. Certainly, it is possible that the dissimilar behaviour of AChE after in vivo exposure and changes among seasons in the dose-response relationship could be due to seasonal variations in the metabolism of these pollutants.

The main contribution of this work is to highlight the importance of considering both circannual rhythms in the baseline levels and the existence of seasonal variation in the dose-response relationship, in toxicity bioassays and biomonitoring programs, to reach valid conclusions when using AChE activity as a biomarker of neurotoxicity.

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Appendix A. Supplementary data

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References


Fig. 5. Acetylcholinesterase activity in homogenates of the anterior section of un-exposed fish *Cnesterodon decemmaculatus* after in vitro exposure to PMG (0, 2.5, 5, 7.5, 10, 20 mM). Data are expressed as mean ± SEM (N = 4). AChE activity was calculated in percentage relative to controls. glyphosate, different studies have shown that glyphosate-based herbicides exposure alters the activity of the antioxidant and detoxificant enzymes in the fish tissues (Menéndez-Helman et al., 2013; and references cited therein). These pathways could be present in the indirect mechanisms involved in AChE inhibition after exposure to PMG; and the existence of seasonal variations in these processes could explain the changes observed between seasons in the dose-response relationship.

Although there are not many reports in relation to the life cycle and seasonal variations in the physiological status of this species, it is noteworthy that *C. decemmaculatus* has a strongly seasonal reproductive strategy. It is a matrotrophic viviparous teleost. Its reproductive period, related to the fact that they had just passed throughout the reproductive requirements of this period. On the other hand, it is possible that the greater susceptibility of the specimens in autumn could be related to the fact that they had just passed throughout the reproductive period with the associated energy demand, thus they are not adequately prepared to face the stressors.
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