Effects of chlorpyrifos on acetylcholinesterase activity in two freshwater fish species (*Cnesterodon decemmaculatus and Gambusia affinis*)

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Abstract: Chlorpyrifos (CPF) is a widely used organophosphate insecticide. The effect of chlorpyrifos exposure on acetylcholinesterase activity (AChE, biomarker of neurotoxicity) was evaluated in two freshwater fish species (*Cnesterodon decemmaculatus* and *Gambusia affinis*). The animals were exposed to sublethal concentrations (1 and 5 μg CPF × L^{-1}) or remained in control water for 96 h. AChE activity was inhibited 48% and 69% in

C. decemmaculatus and 27% and 36% in *G. affinis* for 1 and 5 µg CPF × L^{-1}, respectively. *In vitro* assays showed no differential sensitivity of the enzyme to CPF between species. However, the IC_{50} for *in vitro* exposure to chlorpyrifos-oxon (CPF-oxon, a product of the natural oxidative desulfuration of CPF) were 10.2 nM and 54.2 nM for *C. decemmaculatus* and *G. affinis*, respectively. Therefore, a different sensitivity of the enzyme between both species could explain the dissimilar behaviour of AChE after *in vivo* exposure to CPF.

Keywords: chlorpyrifos; pesticides; biomarkers; acetylcholinesterase; AChE; *Gambusia affinis*; *Cnesterodon decemmaculatus*; freshwater fish; teleost.

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1 Introduction

Water pollution by agrochemicals is currently one of the most serious problems that may affect aquatic environments, since they serve as the final sink of chemically complex

mixtures of natural contaminants and xenobiotics. In Argentina, the area involved in row crop agriculture increased from approximately 9 million ha at the end of the 1990s up to 20 million ha in 2012/2013 (MAGyP, 2014). This led to a parallel increase in the pesticide application rate (herbicides plus insecticides, fungicides, acaricides, among others) from 127,500 tons in 1999 to more than 314,000 tons in 2012 (CASAFE, 2013).

Once applied, part of the pesticides can be mobilised by the influence of a number of factors such as wind, rain or irrigation that increase infiltration and surface run-off. Thus, the organic compound can reach nearby aquatic environments and adversely affect the biota. These contaminants may cause environmental stress, operating on different levels of biological organisation, ranging from subcellular levels, to individual and up to the population. Consequently, it may result in alterations of the ecosystem structure, functionality and integrity (De Coen et al., 2000; van der Oost et al., 2003).

In particular, chlorpyrifos (*O*,*O*-diethyl *O*−3,5,6-trichloro-2-pyridinyl phosphorothioate, CPF) is a broad-spectrum organophosphate insecticide widely used in the region. Approximately, 9,100 tons out of 36,400 tons of insecticides used in the country correspond to CPF (CASAFE, 2011). It is a slightly volatile, non-systemic insecticide of low water-solubility, classified as fat-soluble. Although hydrolysis and photolysis in solution is slow, CPF is generally considered of low persistence in the environment (WHO, 2009). However, Marino and Ronco (2005) studied the CPF levels in surface water bodies of the Rolling Pampa Region (Argentina) and reported CPF concentrations of up to 10.8 $\mu g \times L^{-1}$. Moreover, the accumulation of the organophosphorus pesticide CPF in fish species and its transfer throughout the food chain have been studied (Varo et al., 2002; Venkateswara Rao et al., 2005). Therefore, it is necessary to characterise the fate and unforeseen toxicity of these pesticides in non-target species to assess the risk associated with their use.

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 (Conti, 2008; Schlenk et al., 2008). 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 specific biomarkers.

Acetylcholinesterase (AChE) (EC 3.1.1.7) belongs to the esterase family of enzymes and its physiological role is terminating neurotransmission by hydrolysis of acetylcholine, a neurotransmitter present in the synapses of vertebrates and invertebrates. The determination of AChE activity has been used in many ecotoxicological studies (Bradbury et al., 2008) as a sensitive biomarker for carbamates and organophosphates (OP) pesticides (Soreq and Seidman, 2001; Thompson, 1999; van Dyk and Pletschke, 2011). Carbamates cause the reversible inhibition of AChE, whereas OP induces irreversible inhibition. AChE activity inhibition leads to an accumulation of acetylcholine in the synaptic cleft and thereby to overstimulation of the postsynaptic membrane. These pollutants are known as disruptors of cholinergic nerve transmission, binding at the catalytic site of the enzyme and thus preventing the inactivation of acetylcholine. The resulting overstimulation of the central and peripheral cholinergic receptors culminates eventually with the development of cardiorespiratory collapse that can lead to death.

In fish, the inhibition of AChE has been shown to disrupt many physiological functions such as locomotor capacity, predator evasion, prey location, orientation towards

food and feeding, spatial distribution pattern and social interactions (Bradbury et al., 2008). Furthermore, Behra et al. (2002) have reported a non-classical function: the enzyme was required for the neuronal and muscular development in zebrafish embryos (*Danio rerio*). This finding suggests that exposure of fish to toxicants that alter the functionality of AChE during early development could induce alterations in the structure of the fish population.

Cnesterodon decemmaculatus (Jenyns, 1842) is a freshwater non-migratory and viviparous teleost (Cyprinodontiformes, Poeciliidae), endemic in Neotropical America, which has a wide distribution in a variety of water bodies of the Rio de la Plata basin (Ringuelet et al., 1967; Ringuelet, 1975). This species has a high tolerance to physicochemical variations, being resistant to polluted environments (Teixeira de Mello, 2007). *C. decemmaculatus* is one of the native species proposed as suitable for use in ecotoxicity studies because it is easily handled and acclimatised to laboratory conditions and has been used for *in situ* biomonitoring of aquatic ecosystems (de la Torre et al., 2002, 2005; Ossana et al., 2016; Salibián, 2006; Vera-Candioti et al., 2014). Furthermore, AChE inhibition in *C. decemmaculatus* after exposure to environmental contaminants, such as heavy metals and herbicides, has also been reported (de la Torre et al., 2002; Menendez-Helman et al., 2012).

Gambusia affinis (Baird and Girard, 1853) (Cyprinodontiformes, Poeciliidae), also commonly known as mosquitofish, was introduced into water bodies of Argentina to reduce the proliferation of mosquito larvae (Haro and Bistoni, 1996). This teleost, like *C. decemmaculatus*, is a species with external sexual dimorphism and the ability to establish in a wide range of habitats that has also been used as a test species for toxicity testing and biomonitoring of aquatic environments (Carr et al., 1997; Cengiz and Ünlü, 2002; Venkateswara Rao et al., 2005).

The aim of this study was to evaluate the differential susceptibility of *C. decemmaculatus* and *G. affinis* to CPF exposure by studying CPF-induced AChE inhibition.

2 Materials and methods

2.1 Chemicals

All reagents were of analytical grade and solutions were prepared using milli-Q water. Bovine serum albumin (BSA), DTNB and acetylthiocholine iodide were purchased from Sigma (St. Louis, MO, USA). CPF and CPF-oxon were purchased from Chem Services (USA).

2.2 Animals

Fish without previous exposure to pollutants were provided by a commercial dealer. The AChE activity in both species was characterised before carrying out the bioassays. For this purpose, a fish subsample from the stock was randomly chosen during summer season.

2.3 Semi-static bioassay

Semi-static toxicity bioassays were performed following the protocol for freshwater fish of the Argentine Normalization and Certification Institute (IRAM, 2008). All animals of each species were of a homogeneous size and were acclimatised to glass aquarium conditions (22 ± 2ºC, 12L:12D photoperiod) for 15 days, in dechlorinated tap water, aerated and fed daily *ad libitum* with commercial dried fish food. After this first acclimatisation period, fish were newly acclimatised 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} $CaCO_3$; alkalinity equivalent to 60–70 mg × L^{-1} $CaCO_3$) (USEPA, 1993) under similar previous aquarium temperature and photoperiod conditions. All exposures were performed at the end of the summer to avoid any bias. Fish were exposed to 0 (control), 1 and 5 µg CPF × L^{-1} for 96 h (5 fish per container, 10 fish per treatment). The CPF solutions were prepared by dilution of the stock solution (1000 mg CPF × L^{-1} in ethanol) with MHW. The final test solutions contained ethanol $1 \times 10^{-4} - 5 \times 10^{-4}$% (v/v). All test solutions were completely renewed after 48 h, and the ratio of the organisms' weight/MHW volume was kept constant (1 g × L^{-1}). At the end of the exposure period, animals were anaesthetised 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 head was immediately processed. This methodology was adopted considering the small size of the animals as was previously reported by other authors (Nunes et al., 2005; Varó et al., 2008). Throughout this study, fish pain or discomfort was avoided following the established instructions of animal handling protocols mentioned before (Baumans, 2005).

Homogenisation of tissue was performed using a glass-Teflon electrically operated tissue homogeniser at 3500–4000 rpm. Homogenisation of the anterior section was done in 0.1 M K_2HPO_4 buffer pH 8 using a ratio of 1/25 tissue weight/buffer volume. The homogenates were centrifuged (Hermle Z 216 MK microcentrifuge) at 10,000 g for 15 min at 4ºC; the supernatants were stored at –20ºC and used before 10 days for AChE activity and total tissue protein biochemical determinations.

2.4 Determination of AChE activity and tissue protein content

The absorbance measurements were carried out in triplicate using a 1-cm path length cuvette and a double beam UV/Vis Pharmasec 1700 Shimadzu Spectrophotometer with UV-Probe Software.

The activity of AChE (E.C. 3.1.1.7) was determined by the method of Ellman et al. (1961). Each reaction mixture was made at room temperature and contained: 3 mL of 0.1 M K_2HPO_4 buffer (pH 8), a homogenate aliquot (10 µL), 100 µL of 5,5-dithiobis (2-nitrobenzoic acid) solution (10 mM DTNB) and 20 µL of substrate (0.075 M acetylthiocholine iodide). The hydrolysis reaction rate of substrate mediated by AChE was followed spectrophotometrically by absorbance measurements at 412 nm for 2 min at 8 s intervals. In each case, the absorbance was corrected by subtracting the background for homogenate sample with reagents (without substrate). AChE activities were calculated using an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$.

Total protein content was quantified by Lowry's method. The absorbance was measured at 650 nm, using BSA as standard in the range 0–30 µg. The total protein content was expressed as mg × (g wet tissue) $^{-1}$.

2.5 *In vitro assay*

Preliminary studies were performed by analysing different CPF concentrations and co-incubation times (30 min to 3 h). The range of concentrations was determined considering previous reports of *in vitro* effects of CPF on fish AChE activity (Kavitha and Venkateswara Rao, 2008; Venkateswara Rao and Kavitha, 2010). The longest co-incubation time (for the highest percentages of inhibition), which does not affect the activity of the enzyme in the control (less than 10% decrease), was selected. Co-incubations of AChE with different concentrations of CPF (final concentrations: 0, 25, 50, 100 and 200 mg CPF × L^{-1}) were carried out. For this purpose, different volumes of stock solution of CPF (1000 mg CPF × L^{-1} ethanol 10% in phosphate buffer) were added to 60 µL aliquots of anterior section homogenates from unexposed animals ($n = 4$); a final volume of 75 µL was reached by adding phosphate buffer to obtain the concentrations previously specified. Two different controls were performed by adding 15 µL of phosphate buffer or 15 µL ethanol 10% in phosphate buffer (the highest ethanol concentration in final volume was 2%) to 60 µL homogenate. AChE activity was determined as previously indicated after 3 h of co-incubation.

Similar procedure was conducted for the CPF-oxon co-incubations. In this case, the final concentrations of CPF-oxon were 0, 10, 50, 100 and 200 nM, and the co-incubation time was 15 min according to the procedure described by other authors (Boone and Chambers, 1997; Carr et al., 1997).

2.6 *Expression of results and statistical analysis*

AChE activity was expressed as specific activity units (1 = nmol of substrate hydrolysed × min^{-1} × mg $protein^{-1}$). To obtain a single value of AChE activity for each fish sample, the activity values were averaged from 3 replicates. For a comparative analysis, AChE activities were also calculated per mg of wet tissue.

Fulton's condition factor index (K), a morphometric parameter, was calculated for each fish as $K = [(W \times 100)/L^3]$, 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) for the characterisation of AChE activity in both species. Differences in the AChE activity between *C. decemmaculatus* and *G. affinis* were assessed by *t*-test.

The AChE activities determined in fish from the bioassays are presented as mean ± SEM, as relative percentage, where mean controls are referred as 100%, for each treatment and both species. Two-way ANOVA followed by Bonferroni post-test was performed to assess differences between CPF-exposed groups and the control, and also between both species.

One-way ANOVA followed by Dunnett's or Dunn's multiple comparison test were performed to assess differences in each morphometric parameter between CPF-exposed groups and the control.

The half maximal (50%) inhibitory concentration (IC_{50}) was obtained for *in vitro* assays by using the dose–response curves–inhibition function and choosing the 'log(inhibitor) vs. normalised response – variable slope' equation.

The value of $p < 0.05$ was considered to indicate a significant difference in all statistical analyses. All statistical analyses were performed using GraphPad Prism 5.0 Software.

3 Results and discussion

The basal levels of physiological biomarkers are a key tool to be used in toxicity tests, as well as in biomonitoring protocols for fish species as sentinel organisms (Schlenk et al., 2008). In this sense, AChE activity in *C. decemmaculatus* and *G. affinis* was evaluated. The AChE activity mean values from the anterior section, the body weight, length and *K* index (mean ± SEM) of both species corresponding to unexposed animals are detailed in Table 1. *C. decemmaculatus* showed greater AChE activity than *G. affinis* ($p < 0.0001$) in the same season.

Table 1 Acetylcholinesterase activity (expressed as specific activity units, *U*) in homogenates of the anterior section of unexposed fish *Cnesterodon decemmaculatus* or *Gambusia affinis*; total length (*L*), weight (*W*) and Fulton´s condition factor index (*K*)

	L (cm)	*W* (g)	*K*	*AChE* (U/mg)	*N*
C. decemmaculatus	2.27 ± 0.05	0.083 ± 0.004	0.71 ± 0.03	275 ± 10	19
G. affinis	2.64 ± 0.10	0.15 ± 0.02	0.73 ± 0.04	199 ± 10	26

Data as means ± SEM (*N* = number of biological replicates).

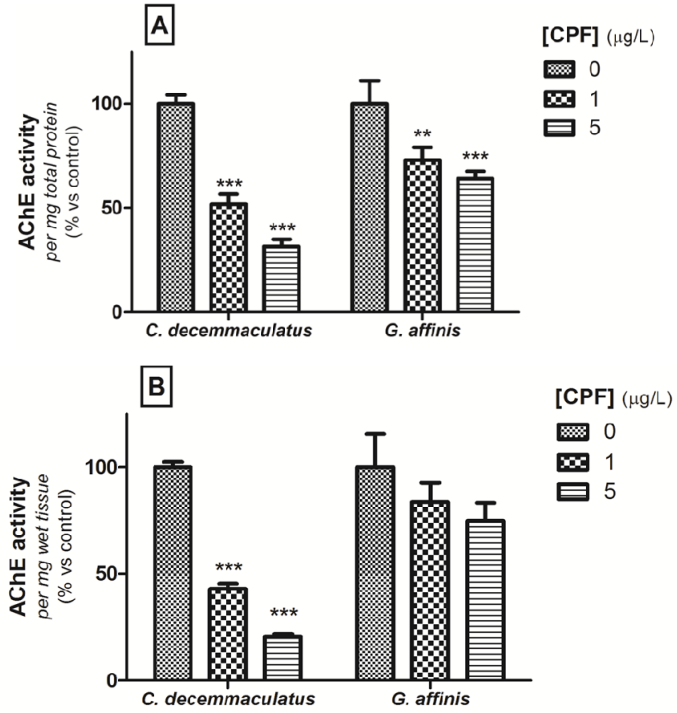
The use of enzymatic activity as a tool to assess responses of organisms exposed to pollutants in toxicity bioassays, as well as in biomonitoring protocols, requires the understanding of the toxic-biological response relationship, the natural fluctuation of a particular biomarker and the validation in the system under study. Previously, Menendez-Helman et al. (2015) determined a sinusoidal function $f(x) = 192.2 + 82.6 * \sin((2\pi * x/11.2) + 6.3)$ to describe the temporal behaviour of AChE in the anterior section of *C. decemmaculatus*. When the length as well as the season are considered, a quadratic surface function explains the anterior section enzyme activity.

AChE activity for the current fish sample was estimated using these two functions. Using the sinusoidal function, 274 U of AChE activity was estimated for specimens analysed in March, whereas the AChE activity estimated, using a quadratic function, was 261 U for the summer and the average length of 2.3 cm. The AChE activity values (Table 1) determined for *C. decemmaculatus* in this study are very similar to the estimations of these functions. Similarly, de la Torre et al. (2005) reported a brain AChE activity of 296 ± 7 U for specimens of *C. decemmaculatus* collected in summer from a permanent artificial pond that was used as reference site. Otherwise, Carr et al. (1997) reported a brain AChE activity of 233 ± 19 U for *G. affinis* similar to the values obtained in this work.

The CPF-induced AChE inhibition was evaluated in both species after an acute exposure to CPF. Figure 1 shows the activities of AChE calculated as relative percentages of the controls for the anterior section of the animals exposed to different

concentrations of CPF or maintained in control conditions for 96 h. The AChE activity values (as specific activity units, per mg of total protein) in the anterior section of the control fish were 264 ± 11 ($N = 9$) and 177 ± 19 ($N = 8$) (means ± SEM) for *C. decemmaculatus* and *G. affinis*, respectively. In all exposed groups (1 and 5 µg CPF × L^{-1}), the activity was significantly different from that observed in the controls. The inhibition ranged from 48% to 69% and 27% to 36% for *C. decemmaculatus* and *G. affinis*, respectively (Figure 1 (A)). The absence of solvent effect was verified (data not shown) for the highest ethanol concentration tested (0.0005%) by a previous trial.

Figure 1 Acetylcholinesterase activity in homogenates of the anterior section of *Cnesterodon decemmaculatus* or *Gambusia affinis* after 96 h of exposure to chlorpyrifos (1 or 5 µg CPF × L^{-1}) or maintained in MHW (control, 0): (A) AChE as specific activity units, per mg of total protein and (B) AChE activity expressed per mg of wet tissue. Data as means ± SEM ($N = 8$–10), as percentage relative to controls. Asterisks indicate significant differences from the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)



Moreover, there were statistically significant differences in the inhibition degree between the species for each treatment. *C. decemmaculatus* showed greater AChE inhibition than *G. affinis*.

The morphometric parameters and the total protein content are shown in Table 2. A significant decrease in the *K* index and total protein was determined for *C. decemmaculatus* exposure to 5 µg CPF × L^{-1}. In *G. affinis*, these parameters were not affected.

Furthermore, since the total protein content is affected by exposure to CPF, it should be noted that this is a parameter used to calculate the AChE specific activity. Therefore,

these results should be analysed carefully. Then, to determine the overall effect on AChE activity, without discarding the unspecific effect, AChE activities were recalculated per mg of wet tissue (rather than per mg of total protein) (Figure 1(B)). It is noteworthy that AChE inhibition was even greater for *C. decemmaculatus* (57% and 79% for 1 and 5 µg CPF × L^{-1}, respectively) using this expression. Besides, AChE inhibition differed even more between species when AChE relative activity was expressed per mg of wet tissue.

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* or *Gambusia affinis* after acute exposure to chlorpyrifos (1 or 5 µg CPF × L^{-1}) or maintained in MHW (control, 0)

	[*CPF*] (µg × L^{-1})	*L* (cm)	*W* (g)	*K*	*Total protein* (mg × g wet $tissue^{-1}$)
C. decemmaculatus	0	2.44 ± 0.03	0.091 ± 0.006	0.63 ± 0.03	61 ± 2
	1	2.43 ± 0.06	0.082 ± 0.009	0.55 ± 0.04	53 ± 4
	5	2.47 ± 0.05	0.074 ± 0.006	0.49 ± 0.04*	42 ± 2**
G. affinis	0	2.46 ± 0.02	0.117 ± 0.010	0.79 ± 0.07	49 ± 5
	1	2.45 ± 0.09	0.114 ± 0.012	0.77 ± 0.05	*58 ± 4*
	5	2.45 ± 0.04	0.102 ± 0.008	0.71 ± 0.06	58 ± 5

Data as means ± SEM. Asterisks indicate significant differences from the control (*$p < 0.05$, **$p < 0.01$).

The CPF concentrations tested correspond to sublethal concentrations for both species. The LC_{50} reported for *G. affinis* is 297 µg CPF × L^{-1} (Venkateswara Rao et al., 2005), whereas for *C. decemmaculatus* it is 105.3 µg CPF × L^{-1} (Paracampo et al., 2014).

The data available for acute or chronic exposure of these species to CPF are sparse. Venkateswara Rao et al. (2005) reported the inhibition of AChE enzyme activity in brain, alterations of the locomotor behaviour and also bioaccumulation of the toxicant in different parts of fish body for *G. affinis* after exposure to 60 µg CPF × L^{-1} for 20 days. Kavitha and Venkateswara Rao (2008) also determined the decrease (82%) in brain AChE activity and the several impairments of the locomotor behaviour after acute exposure of *Gambusia affinis* to 297 µg × L^{-1} (LC_{50} for 96 h). Later, Bonifacio et al. (2016) found that the brain AChE activity of *C. decemmaculatus* increased significantly in those specimens exposed to Clorfox (containing CPF concentrations near 0.2 and 1 µg × L^{-1}) for 6 weeks. To explain this unexpected result, they suggest that fish brain may respond in a compensatory manner by increasing the AChE synthesis to cope with the inhibition caused by the CPF. The exposure to formulated Clorfox also affected the fish general condition (a decrease in the Fulton condition factor), altered behavioural locomotor activity parameters, modified the AST/ALT ratio, and activated antioxidant mechanisms.

Nevertheless, there are no previous reports about the effect on AChE activity after exposures of *G. affinis* to concentrations of CPF below 60 µg × L^{-1}. For *C. decemmaculatus*, no previous references were found in the literature concerning the effect of CPF (as active ingredient) in AChE activity. In this study, a significant inhibitory effect on both species for acute exposure (96 h) at concentrations of 1 and 5 µg CPF × L^{-1} was determined. The low concentrations tested are environmentally relevant,

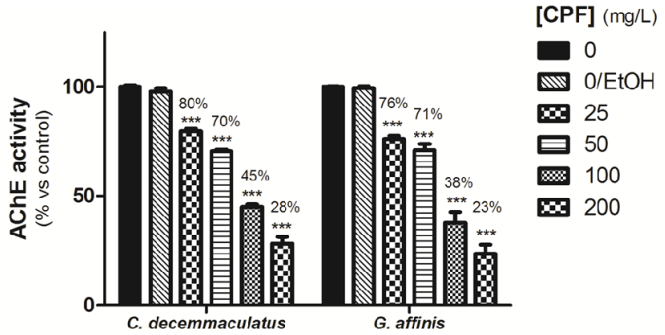
as CPF concentrations close to 10 μg × L^{-1} have been reported for water bodies in agricultural areas of the Rolling Pampa region (Marino and Ronco, 2005). Also, a decrease in the condition factor *K* in *C. decemmaculatus* was determined, showing that the more susceptible species have the general fish health condition altered. Similarly, the reduction in protein content, indicating that the contaminant also produces an unspecific effect, was observed for *C. decemmaculatus.*

Previously, Carr et al. (1997) had reported the inhibition of AChE activity in fish after an accidental spillage of CPF into an aquatic environment. Thus, the small pond was contaminated with CPF via run-off, but the CPF concentration was unknown. These authors indicated that several fish species whose members were either dead or near death were largemouth bass (*Micropterus salmoides*), bluegill sunfish (*Lepomis macrochirus*) and golden shiners (*Notemigonus crysoleucas*). However, no lethality was observed in the mosquitofish (*Gambusia affinis*) population of the pond. In this sense, although *C. decemmaculatus* and *G. affinis* belong to the same family, Poeciliidae, the LC_{50} of *C. decemmaculatus* was lower than the LC_{50} of *G. affinis.* This fact suggested that susceptibility to CPF for *C. decemmaculatus* is greater than for *G. affinis*. The higher AChE inhibition determined in this work for *C. decemmaculatus* when is compared with *G. affinis* is according to this assumption.

Furthermore, to analyse the cause of the difference between *C. decemmaculatus* and *G. affinis* in the AChE inhibition *in vivo*, the sensitivity of the enzyme to exposures to CPF in both species *in vitro* was studied.

The AChE activity showed an inhibition pattern when homogenates of unexposed animals were co-incubated for 3 h with CPF solutions *in vitro* (Figure 2). In this case, the inhibitory effect was observed for exposure to high concentrations of CPF (more than a thousand times greater than those used for *in vivo* bioassays). The AChE inhibition by CPF ranged from 20% to 72% and 24% to 77% for *C. decemmaculatus* and *G. affinis*, respectively, and the differences were statistically significant for all treatments compared with the control. The solvent effect was ruled out for the highest ethanol concentration tested.

Figure 2 Acetylcholinesterase activity in homogenates of the anterior section of unexposed fish *Cnesterodon decemmaculatus* or *Gambusia affinis* after *in vitro* exposure to chlorpyrifos (25, 50, 100, 200 mg CPF × L^{-1}) or after adding the same volume of buffer solution (control, 0) or buffer with the highest ethanol concentration tested (0/EtOH). Data as means ± SEM ($N = 4$), as percentage relative to controls. Asterisks indicate significant differences from the control (***$p < 0.001$)



On the other hand, there was no difference in the inhibition degree between species for each treatment.

The toxicological risk of CPF for both species expressed by their IC_{50} values was calculated from the experimental data. The IC_{50} values for *in vitro* exposure under these conditions were 89.3 ($R^2 = 0.9561$) and 76.5 ($R^2 = 0.8658$) mg CPF × L^{-1} for *C. decemmaculatus* and *G. affinis*, respectively.

Kavitha and Venkateswara Rao (2008) performed an *in vitro* kinetic study for brain AChE of *G. affinis.* The typical double reciprocal plots of initial rate vs. substrate concentration in the presence of various concentrations of CPF showed a common intersection of all slopes at the ordinate, and the slope increase corresponded to CPF increase. This result indicates that CPF alters the apparent kinetic rate constant values, resulting in a competitive type of inhibition.

From the experimental results of this study, the IC_{50} obtained (more than 200 μM) was in the order of 10,000 times greater than those reported for CPF-oxon according to the higher toxicity of the latter. It is noteworthy that CPF is a poor anticholinesterase and must undergo oxidative desulphuration to its active metabolite CPF-oxon to effectively inhibit AChE. The oxon metabolite is a potent irreversible inhibitor of the target enzyme, AChE.

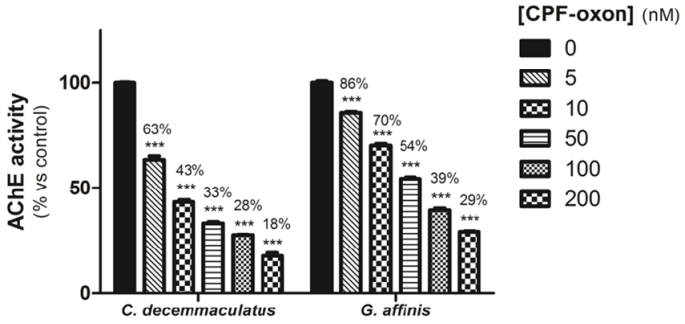
Moreover, no statistically significant differences in the sensitivity of the enzyme were determined between both species. As there was no difference in the sensitivity of the enzyme to CPF between *G. affinis* and *C. decemmaculatus,* this could not be the reason for the dissimilar behaviour of AChE after *in vivo* exposure to CPF. However, it cannot be ruled out that the difference *in vivo* toxicity between the species is due to differences in the enzyme sensitivity of each fish species to the active metabolite (CPF-oxon). Another possibility is the existence of differences in the metabolism between species affecting the levels of CPF-oxon that reach the brain. Different processes affect the uptake of chemicals from water, the biotransformation rate, excretion, factors that affect the distribution of the chemical throughout the organism and its half-life. These processes may explain changes in the pollutant concentrations in the tissues, and therefore, different effects among the species. For example, Carr et al. (1997) proposed a protective role of skeletal muscle of *G. affinis* considering that the amount of skeletal muscle present in fish is much larger than the amount of brain, and also the 20-fold more sensitivity AChE of this tissue. The authors hypothesised that it is possible that once the AChE inhibitor enters the body, it phosphorylates the skeletal muscle AChE destroying the inhibitor and thus reducing the amount available to inhibit the less sensitive brain AChE. Then, if the muscle AChE of *G. affinis* has a protective role that is not equally present in *C. decemmaculatus*, this could explain the different effect of the *in vivo* CPF exposure on brain AChE between these species observed in this work.

Finally, to analyse one of these hypotheses, the sensitivity of AChE to CPF-oxon was evaluated *in vitro* in both species.

The AChE inhibition by CPF-oxon ranged from 37% to 82% and 14% to 71% for *C. decemmaculatus* and *G. affinis*, respectively (Figure 3). The differences were statistically significant for all treatments compared with the control. In this case, the CPF-oxon concentrations used are in the nanomolar range, as well as the CPF concentrations of the *in vivo* bioassays. In addition, there were significant differences in response between the two species. The IC_{50} for *in vitro* exposure to CPF-oxon in these conditions

were 10.2 nM ($R^2 = 0.9170$) and 54.2 nM ($R^2 = 0.9746$) for *C. decemmaculatus* and *G. affinis*, respectively, which supports the existence of a different sensitivity of the enzyme between both species.

Figure 3 Acetylcholinesterase activity in homogenates of the anterior section of un-exposed fish *Cnesterodon decemmaculatus* or *Gambusia affinis* after *in vitro* exposure to chlorpyrifos-oxon (5, 10, 50, 100, 200 nM) or adding the same volume of buffer solution (control, 0). Data as means ± SEM ($N = 4$), as percentage relative to controls. Asterisks indicate significant differences from the control (***$p < 0.001$)



The IC_{50} for the brain cholinesterase (ChE) of *G. affinis* exposed to CPF-oxon was previously determined for other authors. Boone and Chambers (1997) estimated the $IC_{50} = 50 \pm 2$ nM and Carr et al. (1997) estimated the $IC_{50} = 64 \pm 2$ nM. These values are very similar to the IC_{50} determined in this study. Moreover, Carr et al. (1997) found that the *in vitro* sensitivity of *G. affinis* brain AChE to inhibition by CPF-oxon is lower than that of the AChE from other species (bass, bluegill and shiners) suggesting that the species difference in toxicity exhibited after an environmental exposure to CPF, resulted primarily from species differences in the sensitivity of brain AChE to inhibition by CPF-oxon. This result is consistent with the dissimilar inhibition of brain AChE after *in vivo* exposure and the absence of lethality of *G. affinis* after a pollution event, which caused high fish mortality. In this sense, despite *C. decemmaculatus* and *G. affinis* are closely related species, AChE of *G. affinis* showed a lower sensitivity to CPF-oxon than *C. decemmaculatus*. Therefore, the different response observed for *in vivo* exposure could be due to the higher sensitivity of AChE of *C. decemmaculatus* to CPF-oxon.

4 Conclusions

In this work, the differential susceptibility of *C. decemmaculatus* and *G. affinis* to CPF exposure was evaluated by studying CPF-induced AChE inhibition. Brain AChE activity appears to be a sensitivity biomarker for CPF exposure in both species, *Cnesterodon decemmaculatus* and *Gambusia affinis*, even at low CPF concentrations that are environmentally relevant (<10 μg CPF × L^{-1}). Furthermore, there are no previous reports about the effect on AChE activity after exposures of *C. decemmaculatus* and *G. affinis* to these low CPF concentrations. In addition, the AChE inhibition *in vivo* differs between *G. affinis* and *C. decemmaculatus*, the latter being the more sensitive species to CPF exposure. No differential sensitivity of the enzyme to CPF between *G. affinis* and *C. decemmaculatus* was determined by the *in vitro* assays. However, the sensitivity of the

enzyme to the active metabolite CPF-oxon differs between these species. Certainly, it is possible that the lower sensitivity of brain AChE from *G. affinis* than the AChE from *C. decemmaculatus* to the inhibition by CPF-oxon could explain the dissimilar behaviour of AChE after *in vivo* exposure to CPF.

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