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In vivo and *in vitro* inhibition of cholinesterase activity in *Colossoma macropomum* (tambaqui) fingerlings by the herbicide trifluralin

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Abstract

The Amazonian fish *Colossoma macropomum* (tambaqui) is farmed intensively in rice paddies around the São Francisco River delta in northeast Brazil, where the herbicide trifluralin is regularly used. The aims of this study were to evaluate the inhibitory effects of trifluralin on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) of brain and muscle from *C. macropomum* in order to assess the value of this species as a sentinel for herbicide contamination. Trifluralin was highly toxic to fingerlings *in vivo* (96 h-LC_{50} = 0.42 mg $L^{-1)}$. Cholinesterase activity in cell-free extracts of brain was associated with two isoforms, AChE and BChE, although the former predominated. The specific activity of brain AChE was reduced significantly ($p < 0.05$) following 96 h exposure of fingerlings to trifluralin at 0.5 and 0.75 mg L^{-1}, but increased by 30% after exposure to 1.0 mg L^{-1} of herbicide. Muscle AChE was not affected by exposure to trifluralin. K_m^{app} and V_{max}^{app} values of brain AChE were 0.043 ± 0.015 mmol L^{-1} and 0.301 ± 0.014 mmol min^{-1} mg^{-1}protein, respectively. Brain AChE was moderately sensitive to trifluralin (IC_{50} = 0.78 mg L^{-1}), but was very sensitive to the anticholinesterase agent eserine (IC_{50} = 0.043 mg L^{-1}). AChE inhibition in C. macropomum may be employed as a biomarker for biomonitoring trifluralin contamination in water bodies.

Keywords: Acetylcholinesterase, Biomarker, Brain, Muscle, Freshwater fish, Herbicides

INTRODUCTION

Knowledge of the sensitivity of native fish and other freshwater organisms to chemical pollutants is essential if aquatic systems are to be fully protected. Brazil has the highest biodiversity of freshwater fish in the world with more than 3,000 species having been identified (Martins & Bianchini, 2011). However, even though ecotoxicological research has expanded in Brazil over the last decade, the number of fish species that have been assessed remains low and most studies have focused on species from the southern and southeastern regions of the country.

The serrasalmid *Colossoma macropomum* (Cuvier, 1816) [Characiformes: Characidae: Serrasalmidae], commonly known as tambaqui, is a freshwater fish that is native to the Amazon and Orinoco river basins. The species was introduced successfully into the São Francisco River in northeastern Brazil (10°27'S, 36°25'E) in 1984 (Woynarovich, 1993) and has become highly adapted to many other river systems in the country. Farming of *C. macropomum* in the shallow waters of the rice paddies that abound in the São Francisco River delta has been encouraged by the federal agency Companhia do Desenvolvimento dos Vales do São Francisco e do Parnaiba (CODEVASF, 2006). In this context, the association between irrigated rice crops and fish farming offers great advantages, since rice improves the phytoplankton and dissolved oxygen in the water body while fish augment the amount of organic matter present. Thus, fish, especially tambaqui, and rice have become two important commodities around the São Francisco River delta and represent the current driving force of the economy of the area (Holanda *et al*., 2009).

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Herbicides such as trifluralin, clomazone, quinclorac, bentazon, 2,4-dichlorophenoxy acetic acid, propanil, alachlor, paraquat and atrazine are used widely in the rice fields of the São Francisco River (Costa *et al.*, 2008; Cattaneo *et al.*, 2012). Trifluralin, a component of several brands of herbicide marketed in Brazil, is a selective dinitroaniline herbicide that acts by disrupting cell division (Lima *et al.*, 1999). It is used to control annual grasses and broadleaf weeds around fruit and nut trees, and amongst vegetable and grain crops such as soybean, sunflower, cotton and alfalfa. The herbicide is highly toxic to fish and other aquatic organisms as demonstrated in previous various studies involving rainbow trout, bluegill sunfish and catfish (U.S. Environmental Protection Agency, 1996; Könen & Çavas, 2008; Hartless *et al*., 2009).

Animal cholinesterases, which are members of the class of serine esterases, are widespread in cholinergic and non-cholinergic tissues as well as in plasma and other body fluids (Massoulié *et al*., 2008). According to their tissue-specific distribution, substrate specificity and susceptibility to inhibitors, cholinesterases may be classified as acetylcholinesterases (AChE; acetylcholine acetylhydrolases; E.C. 3.1.1.7) or butyrylcholinesterases (BChE; acylcholine acylhydrolases; E.C. 3.1.1.8). AChE breaks down the neurotransmitter acetylcholine at the cholinergic synapses and, for this reason, AChE inhibitors are important in medicine to correct the effects of acetylcholine insufficiency in some neurological disorders.

AChE inhibition has been used in occupational and environmental medicine as a marker of the effects of pesticides on the nervous system (Houghton *et. al*., 2006; Lionetto *et. al*., 2013). Moreover, by virtue of their high specificity, cholinesterases from fish have been employed as biomarkers of contamination by these organophosphate and carbamate pesticides (Alpuche-Gual & Gold-Bouchot, 2008). However, sensitivity to such agents varies among fish species and even among different tissues of the same species (Varò *et al.*, 2003). For example, clomazone inhibited AChE in brain tissue of the teleost fish *Leporinus obtusidens*, but induced a significant increase in AChE activity in muscle tissue (Moraes *et al.* 2007).

The aims of this study were to characterize the cholinesterases of the freshwater fish *C. macropomum* and to determine the inhibitory effects *in vivo* and *in vitro* of trifluralin on AChE and BChE of brain and muscle tissue from this species. *Colossoma macropomum* was chosen for this study not only because it is omnivorous and well adapted to the floodplains of the São Francisco River delta, but also because it is relatively easy to maintain and manipulate in the laboratory and is, therefore, a good candidate as a sentinel species in immunotoxicity assays (Salazar-Lugo *et. al.,* 2009). The value of *C. macropomum* as an indicator species for trifluralin contamination in the rice paddies of the São Francisco River delta, as well other water bodies, was assessed from the anti-cholinesterase effects of the herbicide on brain and muscle tissue from the fish.

MATERIALS AND METHODS

Details of the study were submitted to and approved by the Ethics Commission on the Use of Animals of the Universidade Federal de Alagoas (protocol no. 024854/2010-11).

Reagents

All chemicals, including 5,5-dithio-bis-nitrobenzoic acid (DTNB; 98%), dithiothreitol (DTT; ≥ 98%), acetylthiocholine iodide (ASCh; 97%), butyrylthiocholine iodide (BSCh; 97%), bovine serum albumin (BSA; 98%), triton X-100, sodium phosphate, sodium dodecyl sulfate (SDS; 99%), dimethyl sulfoxide (DMSO; 99.9 %), tetraisopropyl pyrophosphamide (iso-OMPA), eserine free base (physostigmine; 99%) and trifluralin (99 %), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received.

Origin of C. macropomum and fish acclimatization

Fingerlings of *C. macropomum* were produced in an agrochemical-free environment and supplied by CODEVASF. Fish (mean weight 47.8 ± 2.2 g; mean length 14.2 ± 0.8 cm) were acclimatized to laboratory conditions in 250 L tanks containing herbicide-free dechlorinated tap water for seven days at room temperature (26.0 ± 1.0 °C) under a 12 h photoperiod (Fernandes *et al*., 2004). Tank water was continuously aerated using a static pump system according to the specifications of Associação Brasileira de Normas Técnicas (2011), and the fish were fed once a day with commercial fish pellets containing 38% crude protein.

Trifluralin toxicity assay

Laboratory-acclimatized fish were maintained in 70 L glass aquariums and exposed to trifluralin for 96 h following the guidelines of the Associação Brasileira de Normas Técnicas (2011). Fingerlings were distributed into five groups of eight animals each according to the concentration of trifluralin (i.e. 0, 0.25, 0.5, 0.75 and 1.0 mg L^{-1}) employed in the assay. The range of concentrations tested was chosen on the basis of the 96 h lethal concentration 50 (LC_{50}) values determined for other freshwater fish species exposed to this herbicide (U.S. Environmental Protection Agency, 1996). Since trifluralin is only sparsely soluble in water (0.3 mg L^{-1} at 25 °C, CAS#1582-09-08), stock solutions of the herbicide were prepared in DMSO and diluted appropriately so that the concentration of the solvent in the aquarium was 0.001 % (v/v). According to Assis *et al.* (2007), DMSO has no effect on the activity of AChE in brain tissue from *C. macropomum*. Water quality was monitored throughout the assays and conditions were recorded every 24 h (Associação Brasileira de Normas Técnicas, 2011) as follows: water temperature was determined using an Instrutherm (São Paulo, Brazil) model TE-300 digital portable thermometer, pH was measured using an AZ Instrument (Taichung City, Taiwan) model 8651 portable pH-meter, and dissolved oxygen was assessed using

a Lutron Electronic Enterprise (Taipei, Taiwan) model DO-5510 digital oxygen meter. Each assay was replicated four times, and fish remained unfed during the assays.

After 96 h of exposure to the herbicide, the numbers of dead fish in each group were recorded and LC^{50} values (with 95% confidence intervals; 95% CI) for trifluralin against *C. macropomum* were calculated by Probit analysis using SAEG software version 9.0 (Fundação Arthur Bernardes, Viçosa, Brazil). Fish were subsequently removed from the aquarium, placed in an ice bath and sacrificed by decapitation. Brains were removed through incisions made in the skulls, weighed, wrapped in Parafilm™, immersed in liquid nitrogen and stored in the freezer at -20 °C until required for enzyme assay. Dorsal muscle tissue was obtained using analogous procedures.

Preparation of cell-free extracts

Cell-free extracts were prepared from brain and muscle tissues removed from control and trifluralin-exposed fish according to the method of Golombieski *et al.* (2008) with minor modifications. After thawing in ice, tissues were cut into small pieces and samples (80 mg) were suspended in 1 mL of cold 0.9% sodium chloride solution and homogenized in an all-glass Potter homogenizer cooled in an ice bath. Homogenates were submitted to sonication (three cycles of 15 s each) using a ThermoFisher Scientific (Waltham, MA USA) model D100 sonic dismembrator and subsequently centrifuged (Sigma model 3K30, Osterode, Germany) at 3000 x *g* for 15 min at 4 °C. In the case of muscle tissue, a second centrifugation was carried at 5000 x *g* for 10 min at 4 °C.

Cholinesterase activity assay

Cholinesterase activities in cell-free extracts of brain and muscle tissue from control and trifluralin-exposed fish were determined at room temperature (range 25 - 28 °C) according to the method described by Ellman *et al.* (1961). The substrates ASCh and BSCh were employed in AChE and BChE assays, respectively, and reactions were monitored at 420 nm using a Perkin Elmer (São Paulo, Brazil) model Lambda 2 UV/VIS spectrophotometer. The reaction mixture (3 mL) contained 0.1 M potassium phosphate buffer (pH 8.0), 0.34 mM of DTNB, 0.5 mM of substrate and 100 µL of diluted cell-free extract. Substrates were replaced with 20 μL of demineralized water in control assays. The reaction time was 5 min and all assays were carried out in triplicate. Total protein contents of the extracts were determined in triplicate according to the method of Bradford (1976) and estimated using a calibration curve constructed with BSA standard. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydroxylation of 1 µmol of substrate per min (U = μmol min^{-1}) while specific activity was expressed as activity per mg of total protein (μmol min^{-1} mg^{-1}).

Polyacrylamide gel electrophoresis (PAGE)

Cell-free extracts of brain from control fish were subjected to PAGE in order to verify the presence of bands corresponding to the proteins of interest (AChE and BChE). PAGE under denaturing conditions (SDS-PAGE) was performed using 8.0% acrylamide gel containing 5 mM DTT, to prevent oxidation of thiol groups, as described by Laemmli (1970). PAGE under non-denaturing conditions (native PAGE) was performed using 8.0% acrylamide gel, as described by Sagane *et al.* (2005). Gels were stained with Coomassie Brilliant Blue G-250 solution. Native PAGE gels were also stained for AChE activity by incubation with 6 mM ASCh and 3 mM DTNB in 100 mM sodium phosphate buffer (pH 7.0) at room temperature with gentle shaking until colored protein bands developed. Molecular weights of proteins were estimated by comparison with bands produced by co-electrophoresed Mark12™ unstained protein standard (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; Cat. #LC 5677).

Enzyme kinetics

Kinetic studies on acetylcholinesterase were performed using crude cell free extracts of brain from control fish with ASCh substrate in the range 0.00624 - 4 mM. All determinations were carried out in triplicate. Apparent Michaelis-Menten constants (K_m^{app}; mmol L^{-1}) and maximum velocities of hydrolysis (V_{max}^{app}; µmol min^{-1} mg^{-1} protein; De La Torre *et al.*, 2002) were determined by nonlinear regression analysis of Michaelis-Menten functions using GraphPad Prism software version 5 for Windows (GraphPad Software, San Diego, USA).

Cholinesterase inhibition assay

The AChE inhibition assay was performed according to the method of Tortelli *et al.* (2006) using trifluralin and the anticholinesterase agent eserine. Stock solutions of each inhibitor were prepared in 0.001 % DMSO. Aliquots of cell-free extracts of brain from control fish were incubated for 20 min with solutions containing trifluralin in the range 0.075 to 9 µmol L^{-1} or eserine in the range 0.05 to 10 µmol L^{-1}, following which residual AChE activity was evaluated as described above. The effect of the solvent DMSO on enzyme activity was assessed and found to be negligible even at the highest concentration tested (0.05 %). The concentration of inhibitor that reduced AChE activity by half (IC_{50}) was calculated from the dose-response curve (log [inhibitor] *vs.* response) with the aid of GraphPad Prism software version 5 for Windows.

Statistical analyses

The Lilliefors test was employed to determine the normality of data and the Cochran C test was employed to verify homogeneity of variances. Data were expressed as mean ± standard deviation, and mean values were compared using one-way analysis of variance (ANOVA) and the Dunnett test. Differences were considered statistically significant at the 5 % probability level ($p \leq 0.05$).

RESULTS

Water quality, expressed in terms of temperature, pH and dissolved oxygen, exhibited no significant differences (Dunnett test) between the 96 h toxicity tests with trifluralin, and the mean values of the parameters were 26.48 ± 0.17 °C, pH 7.10 ± 0.35 and 7.00 ± 0.29 mg L^{-1} of dissolved oxygen, respectively. The 96 h-LC_{50} value of trifluralin against *C. macropomum* fingerlings was determined to be 0.42 mg L^{-1}.

The specific activities of AChE (with ASCh as substrate) and BChE (with BSCh as substrate) in brain and muscle from *C. macropomum* after 96 h exposure to trifluralin are shown in Table 1. In unexposed fish (control), AChE activity in brain was higher than both BChE activity in the same tissue and AChE activity in muscle. In contrast, BChE activity in control fish was lower in brain than muscle.

Some significant ($p < 0.05$) variations were observed in the specific activities of AChE and BChE in brain tissue from fish exposed to trifluralin in comparison with the control. Exposure of fish to increasing concentrations of herbicide (up to 0.75 mg L^{-1}) led to a decline in brain AChE activity, but exposure to 1.0 mg L^{-1} gave rise to an increase in activity of around 30% compared with unexposed fish. In contrast, increasing concentrations of the herbicide had no significant effect on the specific activity of AChE in muscle. Regarding brain BChE activity, samples from trifluralin-treated fish exhibited some significant ($p < 0.05$), but inconsistent, oscillations compared with the control, although exposure to 1.0 mg L^{-1} of herbicide increased the activity by 19%. On the other hand, BChE activity in muscle was not influenced by exposure to trifluralin at concentrations up to 0.75 mg L^{-1}, but exposure to 1.0 mg L^{-1} of herbicide gave rise to a significant ($p < 0.05$) increase in activity of around 63%.

The cell-free extracts of brain and muscle from fish exposed to trifluralin contained significantly less protein in comparison with those obtained from control fish (Fig. 1). Thus, exposure to 1.0 mg L^{-1} trifluralin led to a decrease in protein content of brain and muscle samples by 73 and 72%, respectively, in comparison with the control. This pronounced reduction in protein content was most likely responsible for the high specific activities of cholinesterase recorded in such tissues since specific activity is expressed as µmol.min^{-1} per mg of protein present.

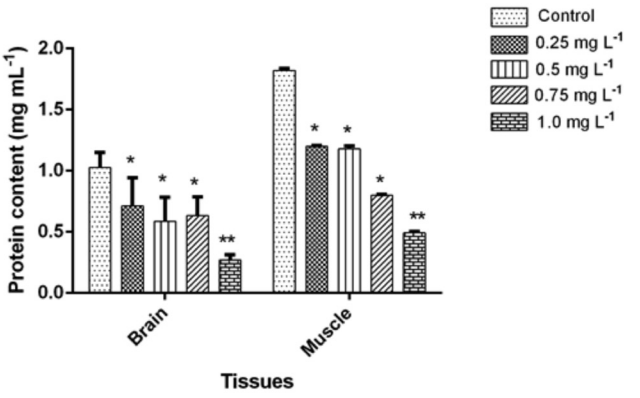

Figure 1 - *In vivo* effect of different concentrations of trifluralin on the protein content of cell-free extracts of brain and muscle from *Colossoma macropomum* fingerlings

The SDS-PAGE fingerprints obtained from crude cell-free extracts of brain from *C. macropomum* (Fig 2a) were typical of those from fish brain tissue and showed a band at around 62

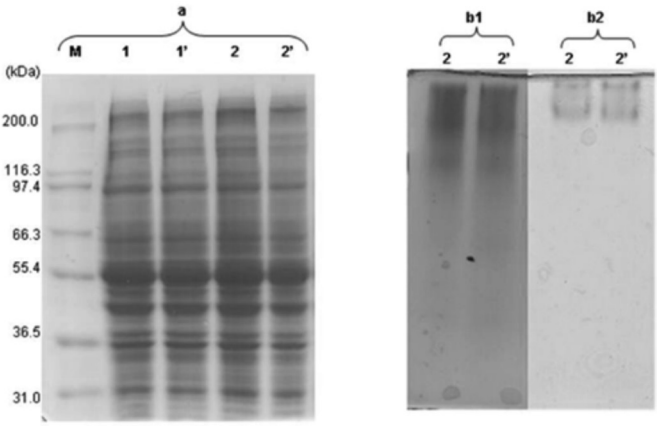

Figure 2 – Polyacrylamide gel electrophoresis (PAGE) of cell-free extracts of brain from *Colossoma macropomum* fingerlings: **a**) SDS-PAGE (8.0% acrylamide with 5 mM DTT) gel stained with Coomassie Brilliant Blue. Lane M - Mark12™ unstained protein standard. Proteins present in cell-free extracts are shown in lanes 1 and 1' (50 µg protein) and lanes 2 and 2' (90 µg protein); **b1**) Native PAGE (8.0% acrylamide) gel of cell-free extracts (90 µg protein) stained with Coomassie Brilliant Blue; **b2**) Native PAGE (8.0% acrylamide) gel of cell-free extracts (90 µg protein) stained for acetylcholinesterase activity (6 mM ASCh and 3 mM DTNB in 100 mM sodium phosphate buffer, pH 7.0).

Table 1 – Specific acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities of cell-free extracts of brain and muscle from *Colossoma macropomum* exposed to different trifluralin concentrations for 96 h.

Treatments mg L^{-1}	Brain		**Muscle**	
	AChE µmol **min^{-1} mg^{-1} protein**	**BChE** µmol **min^{-1} mg^{-1} protein**	AChE µmol **min^{-1} mg^{-1} protein**	**BChE** µmol **min^{-1} mg^{-1} protein**
0 (control)	2.089 ± 0.040^{a}	0.068 ± 0.009^{a}	0.339 ±0.135^{a}	0.132 ± 0.013^{a}
0.25	1.982 ± 0.320^{a}	0.088 ± 0.042^{b}	0.352 ± 0.121^{a}	0.176 ± 0.084^{a}
0.5	1.668 ± 0.070^{b}	0.036 ± 0.016^{b}	0.182 ± 0.068^{a}	0.162 ± 0.038^{a}
0.75	1.218 ± 0.060^{b}	0.044± 0.045^{b}	0.247 ± 0.114^{a}	0.176 ± 0.020^{a}
1.0	2.706 ± 0.160^{c}	0.081 ± 0.012^{b}	0.278 ± 0.064^{a}	0.215 ± 0.034^{b}

Parameters expressed as mean ± standard deviation.
Within each column, mean values followed by dissimilar superscript letters are significantly different according to Dunnett test ($p < 0.05$)

kDa corresponding to fish AChEs. Native PAGE gels stained with Coomassie Brilliant Blue (Fig 2b1) exhibited two diffuse bands in the same areas as those of the colored bands revealed by AChE activity staining (Fig 2b2).

Figure 3 presets a typical Michaelis-Menten kinetic plot showing AChE activity in a cell-free extract of brain from *C. macropomum* as a function of concentration of substrate ASCh. Values of the Michaelis-Menten constants K_m^{app} and V_{max}^{app} for AChE from *C. macropomum* brain were determined to be 0.043 ± 0.015 mmol L^{-1} and 0.301 ± 0.014 mmol min^{-1} mg^{-1} protein, respectively (Table 2).

Curves showing AChE activity remaining in cell-free extracts of brain from *C. macropomum* following exposure to trifluralin or eserine are presented in Figure 4. The IC_{50} values derived from the dose response curves were 0.78 mg L^{-1} (95% CI = 0.3276 - 2.520 mg L^{-1}) for trifluralin and 0.043 mg L^{-1} (95% CI = 0.0804 - 41.69 mg L^{-1}) for eserine. Hence, brain AChE from *C. macropomum* was approximately 18.1-fold more sensitive to eserine than to trifluralin.

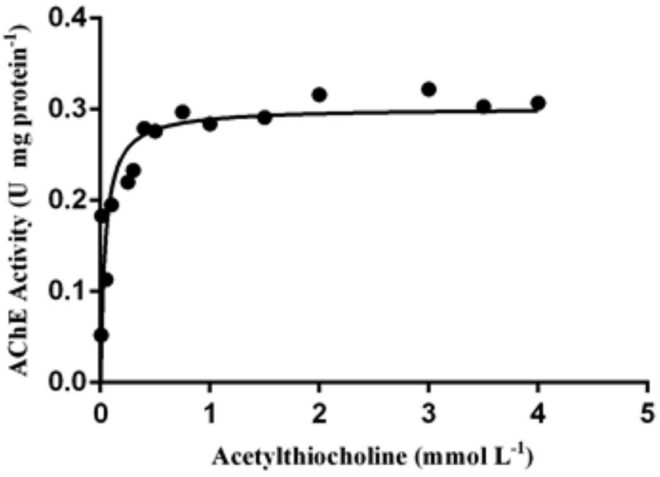

Figure 3 – Michaelis-Menten plot of acetylcholinesterase (AChE) activity in a cell-free extract of brain from *Colossoma macropomum* fingerlings as a function of concentration of the substrate acetylthiocholine iodide. Data reported represent mean ± standard deviation of three replicates.

DISCUSSION

The toxicity of trifluralin to different species of freshwater fish varies from high to very high according to the U.S. Environmental Protection Agency (1996, 2015). For example, Poleksić & Karan (1999) reported that trifluralin was very highly toxic to *Cyprinus carpio* (96 h-LC_{50} = 0.045 mg L^{-1}), while Gangolli (1999) considered that the herbicide was only highly toxic to *Oncorhynchus mykiss* (96 h-LC_{50} = 0.21 mg L^{-1}). The 96 h-LC_{50} value of 0.42 mg L^{-1} obtained in the present study indicates that the toxicity of trifluralin to *C. macropomum* fingerlings must also be considered high.

The specific activity of AChE in brain and muscle from *C. macropomum* was higher than that of BChE in the corresponding tissue. Because AChE preferentially catalyses the hydrolysis of acetyl esters, the predominant enzyme activity observed in brain and muscle from *C. macropomum* in the presence of ASCh originated from AChE (Gagnaire *et al*., 2007).

The results shown herein demonstrate that the specific activity of brain AChE was influenced significantly ($p < 0.05$) by increasing concentrations of trifluralin, whereas that of muscle AChE remained relatively unaffected. This finding is in accordance with the study of Halappa & David (2009), who reported that the highest inhibitory effects of chlorpyrifos on AChE activity could be observed in brain followed by muscle, gill and liver of the freshwater fish *Cyprinus carpio*.

In contrast to the effect on specific activities, increasing concentrations of trifluralin led to reductions in protein content of both brain and muscle tissues. According to Argese *et al*. (2002), the reduction in protein content could be associated with alterations in the hydrogen-bonding capacity of the substituted amino group of trifluralin in relation to the polar groups at the membrane/water interface, leading to disruption of the structure

Table 2 - Comparison of apparent Michaelis–Menten constants (K_m^{app}), maximum velocities of hydrolysis (V_{max}^{app}) and IC_{50} values reported for acetylcholinesterase of brain tissues from different freshwater fish using acetylthiocholine as substrate and eserine as inhibitor.

Fish species	K_m^{app} mmol L^{-1}	V_{max}^{app} mmol min^{-1} mg^{-1}protein	IC_{50} µmol L^{-1} (mg L^{-1})	References
Colossoma macropomum	0.043 ± 0.015	0.301 ± 0.014	0.158 (0.043)	Present work
Odontesthes bonaeriensis	0.040 ± 0.01	0.260 x 10^{-3} ± 0.01	0.0743 (0.020)	Monserrat & Biachini, 2001
Cyprinus carpio	0.230	0.482	0.5 (0.137)	De la Torre *et al.*, 2002
Cnesterodon decemmaculatus	0.170	0.464	1.43 (0.393)	De la Torre *et al*, 2002
Cathorops spixii	0.196 ± 0.078	0.021 ± 5.0	0.0770 (0.021)	Tortelli *et al.*, 2006
Micropogonias furnieri	0.201 ± 0.076	0.018 ± 1.2	4.47 (1.230)	Tortelli *et al.,* 2006

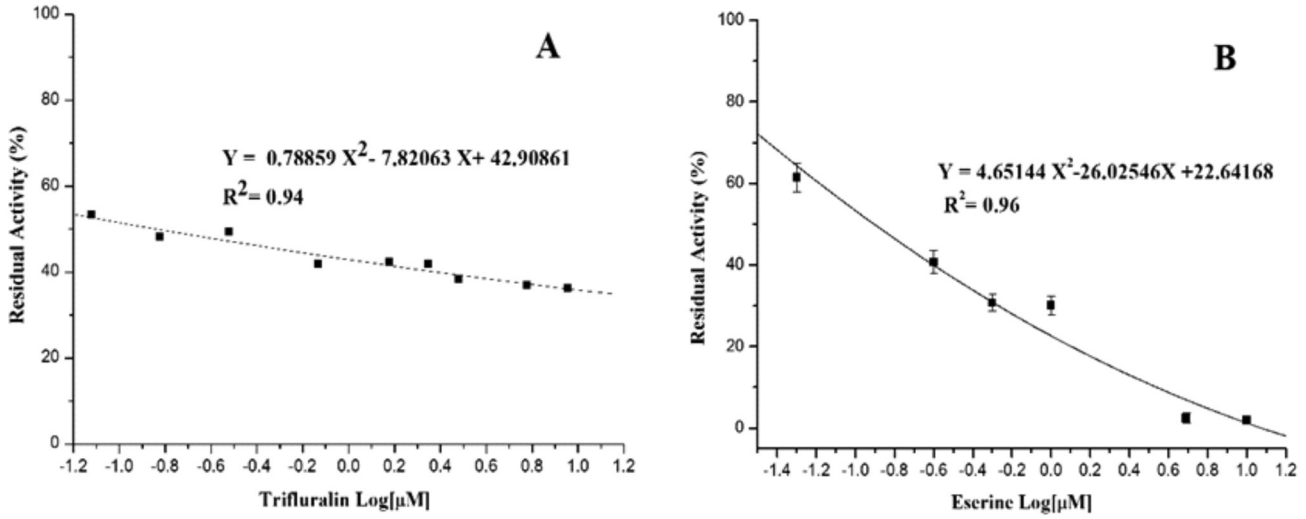

Figure 4 - Effects of different concentrations of (**A**) trifluralin and (**B**) eserine on acetylcholinesterase (AChE) activity present in the cell-free extracts of brain from *Colossoma macropomum* fingerlings. Data reported represent mean ± standard deviation of three replicates.

and function of the inner mitochondrial membrane. These authors also reported that the toxicity of aniline derivatives intensified with increasing hydrogen-bonding capacity. In the present study, the reduction in protein content of brain extracts from fish that had been exposed to 1.0 mg L^{-1} trifluralin was so large that the specific activity of AChE became significantly higher than that of the control, a phenomenon that was not observed when fish were exposed to lower concentrations of the herbicide. In contrast, the specific activities of muscle AChE remained relatively constant, independent of the concentrations of trifluralin employed. Such divergent behavior could be explained by differential transcription and multiple molecular forms of AChE present in vertebrates. It is known, for example, that AChE variants differ with respect to their post-translational modifications, oligomerization properties and interactions with cell membranes (Massoulié *et. al*., 2008).

With respect to BChE, the significant variations, in comparison with the control, observed in the specific activities of the enzyme in brain tissue from trifluralin-treated fish were somewhat inconsistent, and no plausible justification for such behavior could be established. On the other hand, the specific activity of BChE in muscle treated with 1.0 mg L^{-1} trifluralin was significantly higher than that of the control, a finding that may be explained in terms of the observed reduction in protein content of muscle tissue in fish that had been exposed to high concentrations of the herbicide.

Two cholinesterase-active bands were observed when native PAGE gels were stained with DTNB in the presence of substrate ASCh. Cell free extracts of brain from control fish showed both AChE and BChE activities and, since the latter is also able to hydrolyze ASCh, it likely that the active bands were associated with the two cholinesterases.

AChE of brain from control *C. macropomum* exhibited typical Michaelis-Menten kinetics in the presence of ASCh in the concentration range 0.00624 to 4 mM with no inhibition pattern. The kinetic constants of brain AChE from *C. macropomum* obtained in this study were compared with the values reported in the literature for other freshwater fish species. Thus, the K_m^{app} value of AChE from *C. macropomum* was analogous to those reported for the freshwater fish *Odontesthes bonaeriensis* (Monserrat & Biachini, 2001; Table 2). In contrast, the V_{max}^{app} values of brain AChEs from *C. macropomum* and *O. bonaeriensis* were very different, indicating dissimilar turnover numbers with respect to the acetylcholine substrate.

Eserine is a specific AChE inhibitor, and IC_{50} values for this compound have been used as indicators of cholinesterase sensitivity to pesticides and of limits of exposure of aquatic and terrestrial organisms to pesticides (Monserrat & Biachini, 2001). According to published IC_{50} values (Table 2), AChE from *C. macropomum* showed sensitivity to eserine that was higher than the cholinesterases from *Cyprinus carpio*, *Cnesterodon decemmaculatus* and *Micropogonias furnieri* but lower than those from *O. bonaeriensis* and *Cathorops spixii*.

IC_{50} values for various other inhibitors of fish brain cholinesterases have been reported, including 0.081 mg L^{-1} for dichlorvos against *C. macropomum* (Assis *et al.* 2007), 7.4 mg L^{-1} for dichlorvos against *Dicentrarchus labrax* (Varò *et al.*, 2003), 1.425 g L^{-1} for glyphosate against *Jenynsia multidentata* (Sandrini *et al*., 2013) and 5.6 – 10.4 and 7.9 – 14.8 mg L^{-1}, respectively, for atrazine and molinate against *Melanotenia fluviatilis* (Phyu *et al*., 2006).

Based on comparisons with published IC_{50} values, AChE from *C. macropomum* brain should be considered moderately sensitive to trifluralin (IC_{50} = 0.78 mg L^{-1}), but very highly sensitive to the anticholinesterase agent eserine (IC_{50} = 0.043 mg L^{-1}).

CONCLUSIONS

Our results show that the toxicity of trifluralin to *C. macropomum* fingerlings was high. Cholinesterase

activities present in cell-free extracts of brain and muscle from *C. macropomum* were associated with the presence of two isoforms, AChE and BChE, with the former being predominant. *In vivo* exposure of fish to trifluralin influenced significantly the specific activity of brain AChE, but not of muscle AChE. Since *C. macropomum* is well adapted to Brazilian rivers and brain AChE showed moderate sensitivity to trifluralin, this species may be employed as a sentinel for biomonitoring contamination by the herbicide in the São Francisco River delta as well as in other water bodies. Further studies under field-simulated conditions are required in order to verify these preliminary findings because river water contains sediment and zooplankton that could influence the absorption of pollutants by the fish.

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