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CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM THE BRAIN OF THE AMAZONIAN TAMBAQUI *(COLOSSOMA MACROPOMUM)* AND IN VITRO EFFECT OF ORGANOPHOSPHORUS AND CARBAMATE PESTICIDES

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Abstract—In the present study, acetylcholinesterase (AChE) from the brain of the Amazonian fish tambaqui (*Colossoma macropomum*) was partially characterized and its activity was assayed in the presence of five organophosphates (dichlorvos, diazinon, chlorpyrifos, and tetraethyl pyrophosphate [TEPP]) and two carbamates (carbaryl and carbofuran) insecticides. Optimal pH and temperature were 7.0 to 8.0 and 45°C, respectively. The enzyme retained approximately 70% of activity after incubation at 50°C for 30 min. The insecticide concentration capable of inhibiting half of the enzyme activity (IC50) for dichlorvos, chlorpyrifos, and TEPP were calculated as 0.04 μmol/L, 7.6 μmol/L, and 3.7 μmol/L, respectively. Diazinon and temephos did not inhibit the enzyme. The IC50 values for carbaryl and carbofuran were estimated as 33.8 μmol/L and 0.92 μmol/L, respectively. These results suggest that AChE from the juvenile *C. macropomum* brain could be used as an alternative biocomponent of organophosphorus and carbamate biosensors in routine pesticide screening in the environment. Environ. Toxicol. Chem. 2010;29:2243–2248. © 2010 SETAC

Keywords—Organophosphorus pesticide Carbamate pesticide Acetylcholinesterase Biomarkers *Colossoma macropomum*

INTRODUCTION

Organophosphorus and carbamate are major classes of pesticides in use throughout the world. Together, they share about 50% of the world market of insecticides/acaricides. Their relatively fast hydrolysis and low persistence in the environment have supported their increasing use. However, their toxicity to mammals and other nontarget organisms, together with the large amounts used, constitute a threat to human health and the environment. Both classes are cholinesterase inhibitors, and several methodologies have been developed using these enzymes from various species to monitor their environmental presence. These neurotoxic agents have been distributed throughout the world without control in recent decades and, due to misuse and a lack of specificity, have become a serious problem to both humans and the environment [1]. Therefore, methods for organophosphorus and carbamate detection using either organisms or their enzymes as bioindicators and biomarkers, respectively, have been evaluated [2,3]. The cholinesterase group stands out among such molecules [4–6].

Acetylcholinesterase (AChE; enzyme classification 3.1.1.7) is widely known as a specific biomarker of organophosphorus and carbamate pesticides due to the inhibition of its activity [7]. This enzyme is responsible for modulating neural communication in the synaptic cleft by hydrolyzing the ubiquitous neurotransmitter acetylcholine. A lack of AChE activity causes central and peripheral nervous system disorders and death [8].

Studies have confirmed cholinesterases as suitable for monitoring the occurrence of these pesticide classes in environmental compartments [6,9–11]. For example, biosensors have been proposed based on AChE from electric eel and both genetically engineered (B394) and wild-type strains of *Drosophila melanogaster* [12]. However, the high interspecific and intraspecific polymorphism of these enzymes cause varied responses to insecticide compounds, thereby hindering the evaluation and comparison of results from different studies [13]. Consequently, it is necessary to characterize AChE activity in each species and type of tissue.

In previous work, AChE from the brain of the juvenile Amazonian fish tambaqui (*Colossoma macropomum*) was shown to be sensitive to dichlorvos [14]. This enzyme source could be proposed as a feasible alternative for setting up biosensors once it is located in a discarded tissue (brain) of this fish, which is the third most farmed species in Brazil (30,598 tons in 2007, according to the Brazilian Ministry of Environment; http://www.ibama.gov.br/recursos-pesqueiros/documentos/estatistica-pesqueira/).

The aims of the present study were to partially characterize some kinetic and physicochemical parameters of this enzyme, and to evaluate the effect of seven relevant organophosphorus and carbamate pesticides on its activity, to propose it as the biocomponent of an in vitro biosensor.

MATERIALS AND METHODS

Acetylthiocholine iodide, bovine serum albumin, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), Tris (hydroxymethyl) aminomethane, and dimethyl sulfoxide were purchased from Sigma. Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), temephos (97.5%), tetraethyl pyrophosphate (97.4%), carbofuran (99.9%), and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal®. Disodium hydrogen

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phosphate and HCl were obtained from Merck. Trisodium citrate was acquired from Vetec. Glycine was acquired from Amersham Biosciences. The spectrophotometer used was Bio-Rad Smartspec™ 3000. The juvenile specimens of *C. macropomum* were supplied by Mar Doce Piscicultura e Projetos. Tambaqui specimens, 16.5 ± 3.7 cm in length and 93.8 ± 7.9 g in weight, were captured from a 750-m^3 pond.

Enzyme extraction

Twenty juvenile fish were acclimatized in 100-L aquaria (dissolved oxygen 8.04 ± 0.05 mg/L, temperature 26.04 ± 0.07°C, pH 6.93 ± 0.22, salinity 0.17 g/L) for one week and then sacrificed by immersion in an ice bath (0°C). The brains were immediately removed, joined in pairs, and homogenized in 0.5 mol/L Tris-HCl buffer, pH 8.0, maintaining a ratio of 20 mg of tissue per ml of buffer using a Potter–Elvehjem tissue disrupter. The homogenates were centrifuged for 10 min at 1,000 *g* (4°C) and the supernatants (crude extracts) were frozen at −20°C.

Enzyme activity and protein determination

The crude extract (30 μl) was added to 500 μl of 0.25 mmol/L DTNB dissolved in 0.5 mol/L Tris-HCl buffer, pH 7.4, and the reaction started by the addition of 0.125 mol/L acetylthiocholine iodide (30 μl) [14]. Enzyme activity (quadruplicate) was spectrophotometrically determined by following the absorbance at 405 nm for 180 s, in which the reaction exhibited a first-order kinetics pattern [14]. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 μmol of substrate per minute. A blank assay was similarly prepared except that 0.5 mol/L Tris-HCl buffer, pH 8.0, replaced the crude extract sample. Protein content was estimated according to a modified dye-binding method [15], using bovine serum albumin as the standard.

Optimal pH and temperature

Assays were performed using DTNB solutions in a pH range from 2.5 to 9.5 by using citrate-HCl (2.5 to 4.5), citrate-phosphate (4.0 to 7.5), Tris-HCl (7.2 to 9.0) buffers. Substrate nonenzymatic hydrolysis (in basic pH) was corrected by subtracting their values from the activities. Optimum temperature was established by assaying the enzyme activity at temperatures ranging from 5 to 70°C for 180 s.

Thermal stability

Thermal stability of juvenile *C. macropomum* AChE was evaluated by exposing crude extract samples for 30 min at temperatures ranging from 25 to 80°C and assaying the activity retained after 5 min of equilibration at 25°C (room temperature).

Inhibition assay

Acetylcholinesterase inhibition was assayed using five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos, and tetraethyl pyrophosphate [TEPP]) and two carbamates (carbaryl and carbofuran). The insecticides were first dissolved in dimethyl sulfoxide and then diluted in distilled water to five final concentrations ranging from 0.001 to 10 ppm, with each subsequent concentration 10-fold higher than the previous concentration. These concentrations correspond respectively: 0.0045 to 45.2 μmol/L (dichlorvos); 0.0032 to 32.8 μmol/L (diazinon); 0.0028 to 28.5 μmol/L (chlorpyrifos); 0.0021 to 21.4 μmol/L (temephos); 0.0034 to 34.5 μmol/L (TEPP); 0.0061 to 61.3 μmol/L (carbaryl); and 0.0045 to 45.2 μmol/L (carbofuran). The insecticide solutions (10 μl) were incubated with crude extract (20 μl) for 1 h [14] and the residual activity (%) was determined as previously described, using the absence of pesticide as 100% activity. All enzymatic and inhibition assays were carried out at room temperature (25°C). Five crude extracts from 10 fish brains were analyzed in triplicate for each insecticide concentration and data were expressed as mean ± standard deviation. These data were statistically analyzed by nonlinear regression fitted to polynomial or exponential decay ($p > 0.05$) modeling using the software MicroCal® Origin Version 8.0. The concentration capable of inhibiting half of the enzyme activity (IC50) was estimated for each pesticide.

RESULTS AND DISCUSSION

Optimum pH for juvenile *C. macropomum* AChE was found to be in the range 7.0 to 8.0 (Fig. 1A) similar to those described in the literature for other fishes (Table 1): *Solea solea* (7.0), *Scomber scomber* (8.0), and *Pleuronectes platessa* (8.5) [9]; *Cymatogaster aggregate*[16] and *Hypostomus punctatus* [17] (between 7.0 and 7.2). Optimum temperature was estimated as 45°C (Fig. 1B). Bocquené et al. [9] found temperatures in the range 32 to 34°C for *Pleuronectes platessa*; Beauvais et al. [4] at 25°C for *Lepomis macrochirus*, and Hazel [18] at 35°C for *Carassius auratus*. In the present study, AChE from juvenile

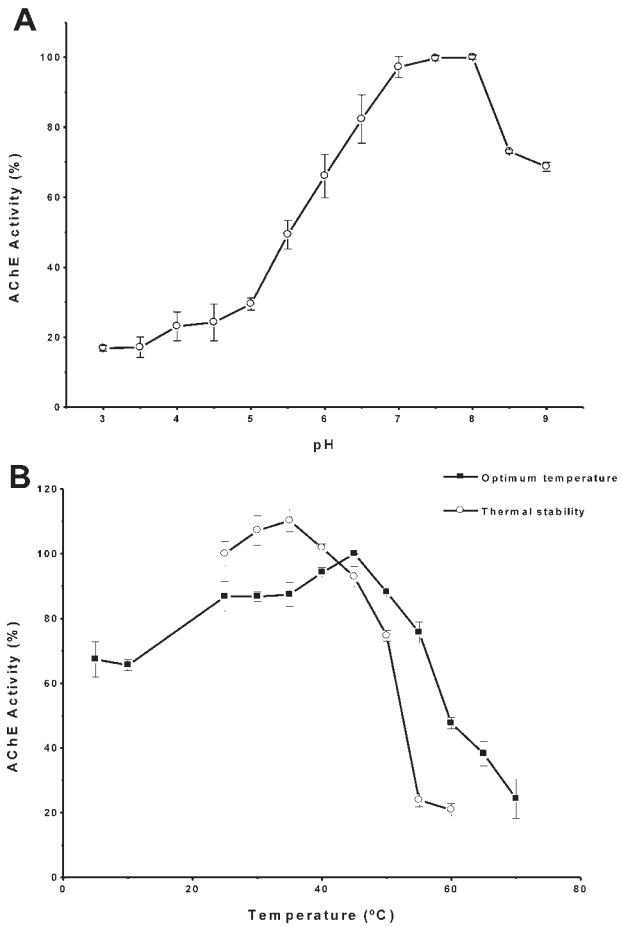

Fig. 1. Effect of pH (**A**) and temperature (**B**) on the AChE from brain of juvenile *C. macropomum*. The pH range was attained by using citrate-HCL, citrate-phosphate, and tris-HCl buffers, whereas the temperature effect was investigated either on the enzyme activity (optimum temperature, ■) or on the enzyme preparation (thermal stability, ○) for 30 min; after 5 min (25°C equilibrium), its activity was estimated. AChE = acetylcholinesterase.

Table 1. Kinetics and physicochemical parameters of AChE from some freshwater and marine species[a]

Species [reference]	K_m (mmol/L)	V_{max} (U/mg of protein)	Optimum pH	Optimum temperature (°C)	Source	Life stage
Oreochromis niloticus[5]	0.10 ± 0.03	0.229 ± 0.014	ND	ND	Brain	Juvenile 48.2 ± 3.9 g
Pleuronectes vetulus[6]	1.69 ± 0.26	0.482 ± 0.034	ND	ND	Muscle	Juvenile 13.5–29.5 cm
Pleuronychtis verticalis [6]	0.30 ± 0.07[b] 0.23 ± 0.06[c]	0.524 ± 0.032[b] 0.120 ± 0.08[c]	ND	ND	Muscle	Juvenile
Solea solea [9]	ND	ND	7.5	ND	Brain	ND
Pleuronectes platessa [9]	ND	ND	8.5	33	Brain	ND
Scomber scomber [9]	ND	ND	8.0	ND	Brain	ND
Colossoma macropomum [present work]	0.43 ± 0.02	0.13 ± 0.05	7.5	45	Brain	Juvenile 16.6 ± 3.7 cm

[a] AChE = acetylcholinesterase; K_m = Michaelis–Menten constant; V_{max} = maximum velocity of enzyme activity; ND = not determined.
[b] Female specimens.
[c] Male specimens.

C. macropomum after being incubated for 30 min at 50°C retained about 70% of its activity at 35°C (Fig. 1B). Zinkl et al. [19] reported absence of cholinesterase activity in the brain of *Oncorhyncus mykiss* (formerly known as *Salmo gairdneri*) subjected to temperatures higher than 45°C.

The Michaelis–Menten kinetics is displayed in Figure 2, from which the maximal velocity (V_{max}) and apparent bimolecular constant (K_m) were 0.128 ± 0.005 U/mg protein and 0.434 ± 0.025 mmol/L, respectively, using acetylthiocholine iodide as substrate. The Lineweaver–Burk plot is also presented. Varó et al. [20] reported acetylthiocholine iodide inhibition at concentrations greater than 5.12 mmol/L in brain tissue from *Sparus aurata*, in contrast to muscle tissue, for which inhibition occurred at 20.48 mmol/L. Rodríguez-Fuentes and Gold-Bouchot [5] found acetylthiocholine inhibition at 4.89 mmol/L in AChE from the brain of *Oreochromis niloticus*. However, in the present study, no substrate inhibition was observed even at the 15 mmol/L acetylthiocholine iodide. According to Table 1, the apparent Michaelis–Menten constant of the juvenile *C. macropomum* AChE was lower than that estimated for *Pleuronectes vetulus* muscle and higher than *Pleuronychtis verticalis* muscle and *Oreochromis niloticus* brain, whereas the maximum velocity was smaller than those reported for these mentioned tissues.

Among the anticholinesterasic agents, organophosphates and its analogues play a different role in the metabolic paths before reaching sites of neuronal transmission. Some of them are produced in a less toxic form (thion form, P = S) which is more stable in the environment. When absorbed by an organism, this form of pesticide undergoes bioactivation to a more toxic form (oxon form, P = O) by monooxigenases from the cytochrome P450 complex present in some organs/tissues including liver, kidneys, lungs, and brain. Therefore, this phenomenon and the diverse effect of the resulting products on the AChE can determine differences in the behavior of the enzyme.

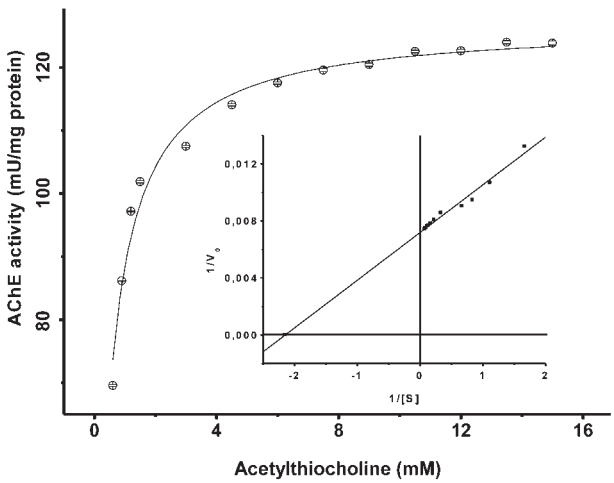

Fig. 2. Michaelis–Menten plot of the AChE from brain of juvenile *C. macropomum* acting on acetylthiocholine. Data are expressed as the mean ± standard deviation of three replicates from four homogenates. The inset shows the Lineweaver–Burk plot. AChE = acetylcholinesterase.

The Food and Agriculture Organization [21] recommends that 20% inhibition is the relevant end-point to determine acceptable daily intakes of an anticholinesterasic compound. In the present study, some of the compounds analyzed were highly toxic to tambaqui AChE, and the inhibition they caused could rapidly reach the above-mentioned levels.

Results from inhibition assays are displayed in Figure 3 and Table 2 and summarize the IC50 values estimated from these data for the five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos, and TEPP) and two carbamate insecticides (carbaryl and carbofuran). Dichlorvos as previously demonstrated [14] was shown to strongly inhibit the juvenile *C. macropomum* AChE. Among the investigated pesticides in the present study, this insecticide presented the lowest IC50 value (0.04 μmol/L; 0.01 ppm) and the lowest value compared with those reported in the literature for other fish species. Chuiko [22] estimated the IC50 value of 0.31 μmol/L for *Leuciscus idus* and *Esox lucius*, and 0.63 μmol/L for *Alburnus alburnus*. Dichlorvos is a direct inhibitor of AChE. It is an oxon organophosphate compound [23] and does not require bioactivation for enzyme inhibition in contrast with thion compounds, for which only a fraction of the total amount is activated in the tissues [24,25]. Chlorpyrifos also displayed lower IC50 value (7.6 μmol/L) than that reported for *Cyprinus carpio* [26]. Diazinon and temephos did not show inhibition effect on the juvenile *C. macropomum* AChE under the experimental conditions used in the present study. According to a number of studies, acute toxicity from phosphorothionate pesticides such as diazinon and chlorpyrifos is strongly influenced by differences in the activity of cytochrome P450-mixed oxidase systems, which bioactivate these compounds [27,28]. Nevertheless, these influences only determine toxic effects through the balance between activation and detoxification pathways: P450 dearylation, carboxylesterase and butyrylcholinesterase phosphorylation, and oxonase-mediated hydrolysis [29]. Thus, the contrast between high sensitivity to oxons and apparent lower oxidation activity possibly could be a *C. macropomum* enantiostatic mechanism when facing xenobiotic threats [30].

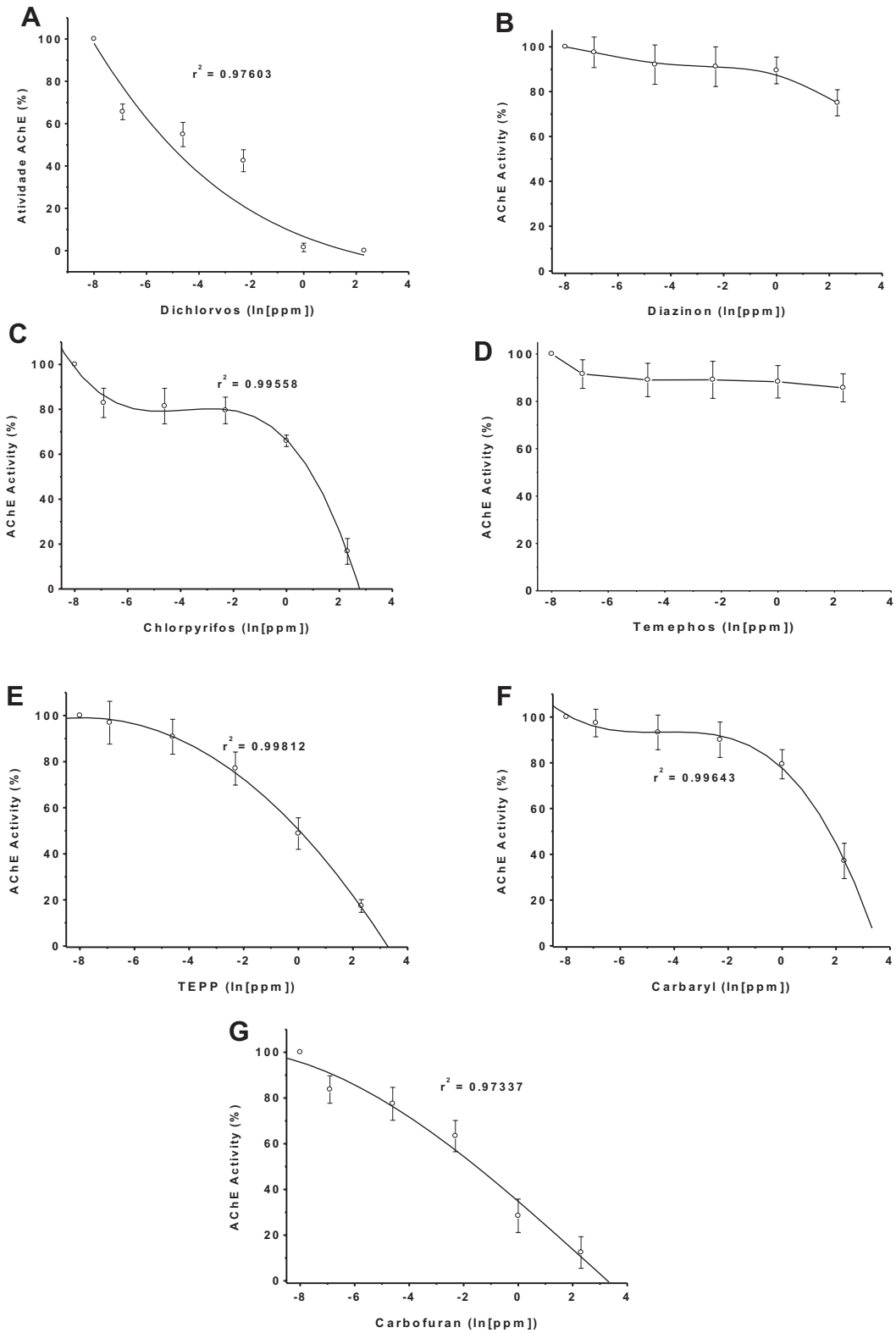

Fig. 3. Effect of organophosphates and carbamates on the activity of AChE from brain of juvenile *C. macropomum*. Dichlorvos (**A**), diazinon (**B**), chlorpyrifos (**C**), temephos (**D**), TEPP (**E**), carbaryl (**F**), and carbofuran (**G**) concentrations ranged from 0.001 to 10 ppm. All the assays were performed at 25°C, and the experimental points are the mean ± standard deviation of triplicate of four crude extracts. AChE = acetylcholinesterase; TEPP = tetraethyl pyrophosphate.

Another condition that may cause discrepancies, particularly in case of chlorpyrifos, is that this compound accumulates in tissues, which likely affects other results. Antwi [31] also found no statistical differences in four fish species (*Oreochromis niloticus*, *Sarotherodon galilaea*, *Alestes nurse*, and *Schilbe mystus*) between controls and individuals living in areas treated weekly with temephos over a six-year period. Temephos is also a thion compound, but the reasons for such results are not only caused by the circumstances mentioned for diazinon and chlorpyrifos. This pesticide is known to exhibit moderate to low

Table 2. Pesticide IC50 values for in vitro freshwater fish[a,b]

Species [reference]	IC50 (μmol/L)
Dichlorvos	
Alburnus alburnus [23]	0.63
Leuciscus idus [23]	0.31
Esox lucius [23]	0.31
Colossoma macropomum [14][c]	0.36
Colossoma macropomum [present study]	0.04
Chlorpyrifos	
Cyprinus carpio [27]	810
Colossoma macropomum [present study]	7.6
Diazinon	
Oncorhynchus mykiss [26]	2.5
Danio rerio [26]	20.0
Poecilia reticulata [26]	7.5
Cyprinus carpio [26]	0.2
Colossoma macropomum [present study][d]	No effect
*T*emephos	
Oreochromis niloticus, Sarotherodon galilaea, Alestes nurse, and *Schilbe mystus* [32]	No effect
Colossoma macropomum [present study]	No effect
TEPP	
Colossoma macropomum [present study]	3.7
Carbaryl	
Colossoma macropomum [present study]	33.8
Carbofuran	
Cyprinus carpio [27]	0.45
Colossoma macropomum [present study]	0.92

[a] IC50 = insecticide concentration capable of inhibiting 50% of enzyme activity; TEPP = tetraethyl pyrophosphate.
[b] Pesticide purity degree varied from 97.4 to 99.9%.
[c] Commercial formulation.
[d] Up to 1.0 ppm.

toxicity to mammals and other nontarget organisms, and is commonly used in potable water treatment against mosquito larva vectors of diseases in public health campaigns [31]. Tetraethyl pyrophosphate displayed an IC50 value of 3.7 μmol/L. This is an organophosphorus known to be highly toxic to mammals; it is the biotransformation product of another pesticide, which is classified as extremely hazardous by the World Health Organization [32]. Table 3 displays its in vivo LC50 for other fish species provided by the U.S. Environmental Protection Agency Ecotoxicology Database (http://cfpub.epa.gov/ECOTOX/), which reflects the high toxicity of this compound (6.8 h at 25°C) [33]. Tetraethyl pyrophosphate is currently classified as an obsolete pesticide [32], but in fact is responsible for part of the toxic action in some organophosphate products, such as diazinon, chlorpyrifos, parathion, and demeton, where it appears as an impurity or breakdown product due to the manufacturing process or unsuitable storage conditions [33]. The two analyzed carbamate insecticides, carbaryl and carbofuran, presented IC50 values of 33.8 μmol/L and 0.99 μmol/L, respectively. The latter IC50 value is similar to that reported by Dembélé et al. for in vitro, *Cyprinus carpio* [26], namely, 0.45 μmol/L (0.1 ppm).

Table 3. TEPP LC50 in several fish species[a,b]

Species	TEPP (%)	LC50 (mg/L)
Carassius auratus	40.0	21.00
Gambusia affinis	40.0	2.84
Ictalurus punctatus	40.0	1.60
Lepomis macrochirus	40.0	0.79
Pimephales promelas	40.0	1.00
Poecilia reticulata	40.0	1.80
Oncorhynchus tshawytscha	40.0	0.31

[a] TEPP = tetraethyl pyrophosphate; LC50 = concentration resulted in death for half of the animals.
[b] Source: U.S. Environmental Protection Agency ECOTOX Database.

The monitoring of pesticides such as organophosphates and carbamates can be evaluated by using organisms in aquatic environments (in vivo assays). In these cases, tanks, animal manipulation, feeding demands, and specially trained personnel are required. Otherwise, animals can be collected from their environment and these toxic components analyzed in their tissues. The use of enzymes, namely, cholinesterases, allows in vitro procedures that are less costly, less time-consuming, less laborious, and more sensitive. The analysis of reactions can take place without interfering compounds present in tissues or animal sensors that could interact with anticholinesterasic agents, thereby causing false positives or negatives. Moreover, biosensors based on these enzymes can be built and used in environmental monitoring. The findings described here confirm previous findings [14] related to the sensitivity of AChE from the brain of the juvenile Amazonian tambaqui towards dichlorvos, and its possible use as the biocomponent of in vitro sensor for this pesticide, and also for chlorpyrifos, carbaryl, and carbofuran.

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