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Inhibition, recovery and field responses of *Astyanax fasciatus* (Cuvier, 1819) brain cholinesterases upon exposure to azinphos-methyl

G. Pistone[1,2]; G. Eguren[2] & D. Rodriguez-Ithurralde[1]

[1] Laboratory of Molecular Neuroscience and Developmental Toxicology Instituto de Investigaciones Biológicas Clemente Estable. Av. Italia 3318, 11600 Montevideo, Uruguay.

[2] Environmental Sciences and Ecology Institute Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, Uruguay.

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Abstract

Pesticides used in agriculture are among the most important environmental pollutants. Organophosphate and carbamate insecticides, intensely used in deciduous fruit-trees, may be transported to aquatic ecosystems by runoff. Northwest rural Montevideo possesses zones of fruit-tree farms, where azinphos-methyl is currently the most used pesticide. Despite the well-known neurotoxic properties of this agrochemical, studies of its effects on aquatic organisms are scarce in Uruguay. The main goal of this study was to evaluate effects on brain cholinesterases and erythrocyte micronuclei in *Astyanax fasciatus* exposed to azinphos-methyl in laboratory and field conditions. Dose-response curves showed concentration-dependent brain cholinesterase inhibitions and a LC_{50} (48-hour) of 2.31 mg L^{-1} for azinphos-methyl. Fishes exposed for 48 hours to toxicant and then transferred to clear water recovered 80% of brain cholinesterase activity in 10 days. Field study indicated that *A. fasciatus* from a watershed with low contamination showed a brain cholinesterase specific activity of 62.2±5.1 Units mg^{-1} protein (22ºC) 97.7 % of which was acetylcholinesterase. Specimens from a basin with intense fruticulture exhibited a spatial gradient, those collected downstream to the farms showed brain acetylcholinesterase activities 32% lower than fishes captured upstream. In conclusion, our data suggest that *A. fasciatus* is a suitable species for ecotoxicological biomonitoring.

Keywords: Acetylcholinesterase; *Astyanax fasciatus;* Aquatic Biomonitoring; *A*zinphos-methyl; Bioindicator; Brain cholinesterases; Fish; Organophosphate pesticide

Inhibição, recuperação, e respostas de campo das colinesterases cerebrais de *Astyanax fasciatus* (Cuvier, 1819) após exposição ao azinfos-metil.

Resumo

Os pesticidas utilizados na agricultura estão entre os poluentes ambientais mais importantes. Os inseticidas organofosforados e carbamates, intensamente utilizados em árvores caducifólias de fruto, podem ser transportados para os ecossistemas aquáticos. Montevidéu rural possui zonas de fruticultura intensiva onde atualmente o metil-azinfos é, de longe, o pesticida mais utilizado. Apesar de suas conhecidas propriedades neurotóxicas, os estudos sobre seus efeitos sobre organismos aquáticos são escassos no Uruguai. O principal objetivo deste estudo foi avaliar os efeitos da exposição ao metil-azinfos sobre colinesterases cerebrais e micronúcleos de eritrócitos de *Astyanax fasciatus*, em condições de laboratório e de campo. Curvas dose-resposta mostraram inibição de colinesterases cerebrais dependente da concentração e uma CL_{50} (metil-azinfos, 48 horas) de 2.31 mg L^{-1}. Peixes expostos por 48 horas ao tóxico e, em seguida, transferidos para a água clara, recuperaram 80% da atividade colinesterásica cerebral em 10 dias. O estudo de campo revelou que *A. fasciatus* selvagens coletadas de um curso de água com baixa contaminação mostraram uma atividade específica colinesterásica cerebral de 62.2 ± 5.1 Units mg^{-1} proteína (22 º C) da quais 97.7% foi acetilcolinesterase. Os espécimes coletados em uma bacia hidrográfica dedicada à fruticultura exibiram um gradiente espacial: a atividade da acetilcolinesterase cerebral dos peixes coletados rio abaixo da zona de fruticultura foi 32% menor que aquela dos peixes capturados rio arriba dessa zona. Em conclusão, nossos dados sugerem que *A. fasciatus* é uma espécie adequada para biomonitoramento eco-toxicológico.

Palavras chave: Acetilcolinesterase; *Astyanax fasciatus;* Bioindicador; Colinesterases cerebrais; Metil-azinfos; Monitoramento aquático; Peixe; Pesticida organofosforado.

*Corresponding author: Daniel Rodríguez-Ithurralde; e-mail: drit@iibce.edu.uy / drithurralde@gmail.com

INTRODUCTION

Considerable efforts are being made to improve the eco-toxicological assessment of South American water bodies (Gavilán *et al*., 2001; Barra *et al*., 2005; Oliveira *et al*., 2007). However, data gaps on biological and ecological characteristics of aquatic Neotropical species have been important obstacles for South American eco-toxicologists (Lizama & Ambrósio, 2002; Carrasco-Letelier *et al*., 2006). Thus, there is an urgent need to develop biomarkers using resident species that might be included in wide bio-surveillance programs. The sentinel species should be abundant, widely distributed, non-migratory and, on the other hand, it must respond as linearly and predictably as possible, and in the widest possible range of concentrations, to the agent to be monitored (Valdez-Domingos *et al*., 2009). *Astyanax fasciatus* is an abundant teleost which appears to combine most of these features, and that has been already proposed as a possible bioindicator of anthropogenic pollution (Schulz & Martins-Junior, 2000; Carrasco-Letelier *et al*., 2006; Moreira *et al*., 2010). Moreover, it is widely distributed from Southern Central America to the Río de la Plata (River Plate) basin (Ringuelet *et al*., 1967; Castro & Casatti, 1997) and, importantly, is small, non-migratory, and pelagic and inhabits streams and rivers devoid of strong currents and lentic systems (Castro & Casatti, 1997). Since in Uruguay toxicity data on native freshwater species are very scarce, we decided to investigate - in both laboratory and field conditions- responses of A. *fasciatus* brain cholinesterases to the organophosphate (OP) insecticide azinphos-methyl.

The area selected for the field study was the "Cañada del Dragón", a small stream belonging to the lower Santa Lucía River basin, Southern Uruguay (Fig. 1). Approximately 700 ha of its drainage area are occupied by deciduous fruit-tree farms (Carrasco-Letelier *et al*., 2006). Insecticides currently in use are OP insecticides, carbamates, and, in a lesser extent, synthetic pyrethroids. According to systematic interviews to landowners, azinphos-methyl is, by far, the most used pesticide in the area. OP and carbamates produce acute toxicity by quasi-irreversibly inactivating cholinesterase enzymes in blood, muscle and nervous system (Karlsson *et al*., 1984; Fulton and Key, 2001). In addition to acute toxicity and death caused by sudden cholinesterase inhibition, long-term exposure to low OP concentrations has been implicated in the genesis of several developmental and degenerative disorders of the nervous system (Rodríguez-Ithurralde *et al*., 1997; Olivera *et al*., 2003; Hayden *et al*., 2010).

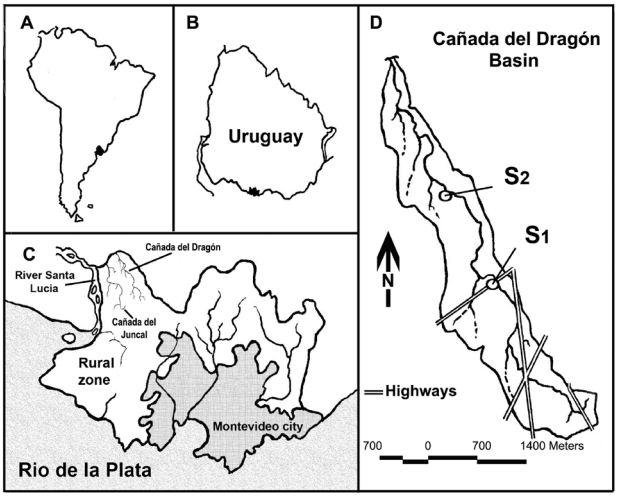

Figure 1 - Location of the study area. Highlighted areas (in black) indicate positions of Uruguay in South America (A) and of Montevideo Department in Uruguay (B). The left-lower panel (C) shows both *Cañada del Dragón* and *Cañada del Juncal* streams in Northwest Montevideo rural zone. The right panel (D) illustrates *Dragon* stream basin with location of sampling stations S1 and S2.

Cholinesterases comprise acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7) which split acetylcholine faster than other choline esters, and butyrylcholinesterase (BChE, acylcholine acylhydrolase, pseudocholinesterase; EC 3.1.1.8), which hydrolyzes butyrylcholine faster than propionyl or acetylcholine (Karlsson *et al*., 1984; Oliveira Ribeiro & Silva de Assis, 2005). In fishes, AChE is abundant in erythrocytes, nervous tissue and muscle, and its main function is to regulate cholinergic neurotransmission by catalyzing the hydrolysis of acetylcholine at both the neuromuscular junction and many central synapses (Karlsson *et al*., 1984; Rodrigues *et al*., 2011). AChE also subserves roles in neural development and synapse maturation (Rodríguez-Ithurralde *et al*., 1997; Olivera *et al*., 2003). In most teleosts, AChE accounts for more than 97% of total brain cholinesterases (Chuiko *et al*., 1997; Ferrari *et al*., 2007). BChE, the main plasmatic cholinesterase of most vertebrates, also occurs in fish brain, nerve and muscle, although in much smaller amounts than AChE. The sum of AChE and BChE enzymatic activities, usually termed "cholinesterase activity" (Karlsson *et al*., 1984) is frequently used in aquatic surveillance programs (Oliveira Ribeiro & Silva de Assis, 2005). Inhibition of cholinesterase activity in fish blood, brain (de la Torre *et al*., 2002, 2007; Chuiko *et al*., 1997), muscle (Valdez Domingos *et al*., 2009) or even in the whole fish head (Stringuetti *et al*., 2008; Rodrigues *et al*., 2011) has been widely used in detecting environmental pollution of fresh (Ferrari *et al*., 2004, 2007) and estuarine waters (Fulton & Key, 2001), particularly as biomarker of OP and carbamate exposure and effect. Despite this widespread global use, this is the first paper studying any cholinesterase from a fish caught in Uruguay.

The goals of this study were: (1) to determine if concentrations of azinphos-methyl which can be found in water bodies as a result of antropic activities are able to induce genotoxic and neurotoxic effects in *A. fasciatus*; (2) to characterize the concentration-response relationship between azinphos-methyl and brain cholinesterase activity; (3) to determine the LC_{50} (48 hours) for this toxicant in the selected species; (4) to investigate if *A. fasciatus* brain cholinesterase activity is recoverable upon inhibition caused by exposure to azinphos-methyl; and (5) to assess the potential of *A. fasciatus* as a sensitive and predictable bioindicator of exposure to organophosphate insecticides.

MATERIAL AND METHODS

Field study

The system under study was the "*Cañada del Dragón*" (Dragon stream) a small stream located in a rural zone of Northwest Montevideo Department (Fig 1). The stream belongs to the Santa Lucia River basin (1435 ha), the potable water source of ca. 1500000 inhabitants (Fig 1,C). Its hydrological regime (13.67 km) is pluvial, with maximum and minimum flows in winter and summer, respectively (Carrasco-Letelier *et al*., 2006). The basin has a mean slope of 0.54%, a maximal elevation of 60 m, a drainage area of 14.67 km^2, and its dominating soils are Argisols and Mollisols (Altamirano *et al*., 1976). As a function of the land use, the basin may be subdivided into three main sub-zones: (1) Sub-urban (stream origins); (2) Agricultural, the largest sub-zone, mostly occupied with fruticulture, and (3) Grassland and wetlands, the downstream fourth of the stream (Fig 1, D). Dose and application frequencies of pesticides in the basin were recorded from systematic interviews with local landowners. Pesticides used are mainly organophosphates (OP) and carbamates, with neat predominance of the OP azinphos-methyl (CAS Nr 86-50-0) (Carrasco-Letelier *et al*., 2006). *A. fasciatus* specimens (Fig. 2) were collected by means of electrofishing (FEG 1000, Sachs Elektrofischfanggerate GmbH) from two sampling sites of Dragon stream: the pre-impacted zone (S1 in Fig. 1, upstream to the farms' zone) and the impacted zone (S2, downstream to the farms' zone) (Fig. 1) and from *Juncal stream* (see below and Fig. 1). Samplings were carried out in two periods: before (September) and after (December) the period of maximal intensity of pesticide applications. Only adult specimens (body weight range 5-9 g; total length 56-77 mm) were used for all studies. The *Cañada del Juncal* (shortly, Juncal stream), an unpolluted small stream that runs close to Dragon steam and having similar flow and total length as it (Fig 1, C), was chosen as the control stream for the field study and as the source of wild fish for all laboratory experiments.

Laboratory study

Animals used and aquaria conditions

We limited our study to adult specimens (body weight range 5-9 g; total length 56-77 mm) of *A. fasciatus*. Wild specimens from Juncal stream were transported alive to the laboratory in plastic cages containing stream water and groups of 9-10 specimens were placed in 60-litre aquaria and acclimated for 15 days. Aquaria conditions were: a 12:12 h light:darkness photoperiod regime, temperature of 18.2 ± 0.4 ºC, pH 7.4 ± 0.1, and 7.2 ± 0.5 mg L^{-1} dissolved oxygen. Commercial fish food (Tetra Pond) was administrated twice a day, with the exception of bioassays days, when feeding was omitted.

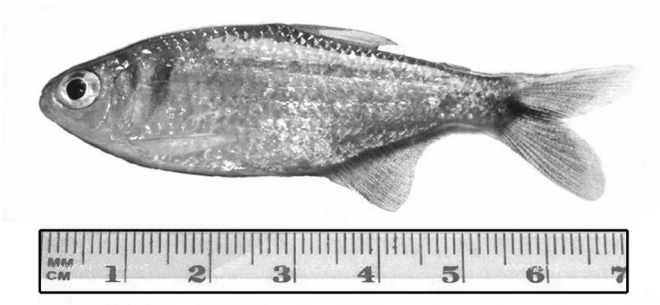

Figure 2 - Adult *Astyanax fasciatus.*

Mortality and enzyme inhibition bioassays

The pesticide selected for the laboratory study was azinphos-methyl because it resulted to be the most intensively and extensively used pesticide in the whole Dragon stream basin. Static bioassays, with an exposition time of 48 hours and including a control group (clean aquaria water) were done by duplicate. Azinphos-methyl (99.0% purity) was purchased from Chem. Service (West Chester, PA, USA) and aqueous solutions prepared as described by Ferrari *et al*., (2004). The following final nominal concentrations of the toxicant were applied to aquaria: 0.001, 0.01, 0.1, 1.0, 2.0, or 4.0 mg L^{-1}. Dilutions in water were added in the smallest possible volume into the physical center of aquaria and immediately mixed with a glass rod. Measured test concentrations departed less than 6% from nominal values. At the aquaria water pH used in this study (7.4), azinphos methyl hydrolysis is known to be very slow (t ½ = 37 d) (Extoxnet, 1979; Erickson & Turner, 2003) and therefore, toxicant decay depended mainly on aqueous photolysis (Erickson & Turner, 2003) which under maximal permanent light would determine a t ½ of 76.7 h. Under the light intensity and photoperiod employed in our laboratory (12:12), total, integrated t ½ of azinphos-methyl in water was calculated to be approximately 10 days. The 48 h-decay was therefore estimated as 5% and thus a reposition time of 48 h was chosen. Nine or ten adults (3.14 ± 0.31 g and 6.07 ± 0.16 cm, mean ± SD) per aquaria were exposed to 0.001, 0.01, 0.1, 1.0, 2.0, or 4.0 mg L^{-1} final nominal concentration (CCF) of azinphos-methyl.

Brain cholinesterases recovery experiments

In separate experiments, brain cholinesterase recovery assays were carried out under similar conditions as above, in order to determine the recovery time of *A. fasciatus* brain AChE once the inhibitor is no longer present in the aquarium. Groups of six or seven adult *A. fasciatus* specimens per aquaria were used. One control group was exposed for 48h to normal clean aquarium water whereas five groups were exposed for 48 h to 0.8 mg L^{-1} azinphos-methyl ccf. After 48 h exposure time, the control reference group and one exposed group (recovery time zero, RT0) were simultaneously sacrificed. The remaining exposed groups were transferred to toxicant-free aquaria and sacrificed after 2, 4, 6 and 10 days. Cholinesterase activities found after the different recovery times (see below) were expressed as percent of control group enzyme activity.

Tissue treatment and brain cholinesterases biochemical determination

Fishes were submitted to a mild anesthesia with tricaine methanesulfonate (MS-222) at a fcc of 135-150 mg L^{-1} in the aquarium water, and killed by decapitation. The central nervous system was immediately dissected out on a cold plate, meninges and main blood vessels eliminated, tissue weighted and immediately homogenized (5% w/v) on ice in 0.1M sodium phosphate buffer (pH 8.0) containing 0.5% Triton X-100 (Rodríguez-Ithurralde *et al*., 1997; Oliveira *et al*., 2007) using an Ultrasonic Homogenizer 4710 series (Cole Palmer Instrument Co). When necessary, extracted tissue was kept at -80ºC for 1 month, without noticeable enzymatic activity loss (Karlsson *et al*., 1984). Total cholinesterases and AChE activities in whole brain were determined according to the Karlsson *et al*. (1984) modification of the method of Ellman *et al*. (1961). All biochemical determinations were run in triplicate. Kinetic measurements were carried out at 22±1ºC with acetylthiocholine iodide (AcSCh, Sigma) as a substrate and 4,4'-dithiodipyridine (PDS, Sigma) as the chromophore. The thiocholine formed by the reaction was allowed to react with the chromophore, producing 4-thiopyridine, which has a maximal absorbance at 324 nm (Karlsson *et al*., 1984). Quartz cuvettes (1 mL) were used, where 0.85 mL enzyme (homogenate) and 0.1 mL chromophore were added and mixed. The spectrophotometer (Shimadzu Model UV-2401 PC) was adjusted to zero, and when 50 µL substrate was added to the sample cuvette and mixed, the increase in absorbance, usually linear, was recorded for 60 seconds. Enzymatic activity was corrected for spontaneous substrate hydrolysis and expressed as specific activity (nmol of hydrolyzed AcSCh.min^{-1}.mg^{-1} of protein, i.e., Units.mg^{-1} of protein). To determine AChE activity, a final concentration of $2x10^{-5}$M ethopropazine (Rodríguez-Ithurralde *et al*., 1997) was used to inhibit BuChE in a separate series of assays. Purified AChE from electric eel (Sigma) and erythrocyte ghosts prepared from human blood (certified by Hemotherapy Dept.University Hospital, Montevideo) were used as standards (Karlsson *et al*., 1984). In preliminary as well as in control assays, we found that brain cholinesterase activity was not significantly different when enzyme assays were performed either 30 min after capture or upon a 15-day aquarium acclimation period. Also, fishes exposed for one h to the anesthetic MS-222, exhibited brain AChE activities that were indistinguishable from those obtained from control fishes non-exposed to the anesthetic. Proteins were assayed according to Lowry *et al*. (1951) with bovine serum albumin as standard.

Micronuclei test in erythrocytes

Micronuclei test was carried out according to Campana *et al*. (1999) including a fish group exposed to 5 mg L^{-1} of cyclophosphamide as a positive control for the assay. Once finalized exposition time, blood samples were obtained by direct heart puncture with a heparinized syringe. After 24 hours, slides were fixed in absolute methanol for 10 min, and air dried. The next day, slides were stained with 3% Giemsa solution for 10 min. From each animal, 1000 erythrocytes were analyzed under 1000x magnification to determine micronuclei frequency, which was expressed as number of micronuclei per 1000 cells.

Statistical analysis

LC_{50} calculations were done following the trimmed Spearman-Karber method (Hamilton *et al*., 1977) as recommended by the Ecological Exposure Research Division of US Environmental Protection Agency (US-EPA). Normality and variance homogeneity were analyzed by Kolmogorov-Smirnov and Bartlett tests, respectively. The differences among groups were assessed with one-way ANOVA or Kruskal-Wallis tests, and statistical significance was confirmed by Post Hoc test ($p < 0.05$) unless otherwise indicated.

RESULTS

Behavioral changes and mortality (LC_{50}) upon laboratory exposure to azinphos-methyl

Acute exposure of fishes to toxicant nominal concentrations ranging between 1 and 4 mg L^{-1} elicited signs of OP intoxication that appeared after different delays according to toxicant concentration. Upon 4 mg L^{-1}, behavioral changes started during the first 30 min, whereas at 1-2 mg L^{-1}, alterations appeared after one hour of delay. Intoxication signs included: decrease in spontaneous locomotor activity and spontaneous feeding, increase in respiratory rate and/or amplitude, loss of normal posture, including loss of cephalo-caudal horizontality, lateral tilts, erratic and/or spiral swimming, and ocular haemorrhage. Remarkably, fishes hit each other and tank walls and finally stayed motionless close to the water surface, exhibiting a marked orange coloration in caudal fins. Death delay also changed with toxicant concentration: at 4 mg L^{-1}, 79% of individuals died upon between 1 and 24 hours; while at 1 or 2 mg L^{-1}, percentages of 24 h-mortality were lower, 16 and 40 % respectively (Fig. 3,A).

Azinphos-methyl lethal concentration (LC_{50}) for *Astyanax fasciatus,* as estimated according to the trimmed Spearman-Karber method (Hamilton *et al*., 1977) was 2.31 mg L^{-1}, with 95% confidence limits of 3.11 and 1.72 mg L^{-1}.

Effects of azinphos-methyl on micronuclei frequency

On the other hand, micronuclei frequency (MN) as determined upon the different treatments is summarized in Table 2. Only the positive control, 5 mg L^{-1} of cyclophosphamide caused a significant increase in the frequency of micronucleated erythrocytes, whereas AChE enzymatic activity after cyclophosphamide exposure was practically the same as that in control group ($p<0, 05$).

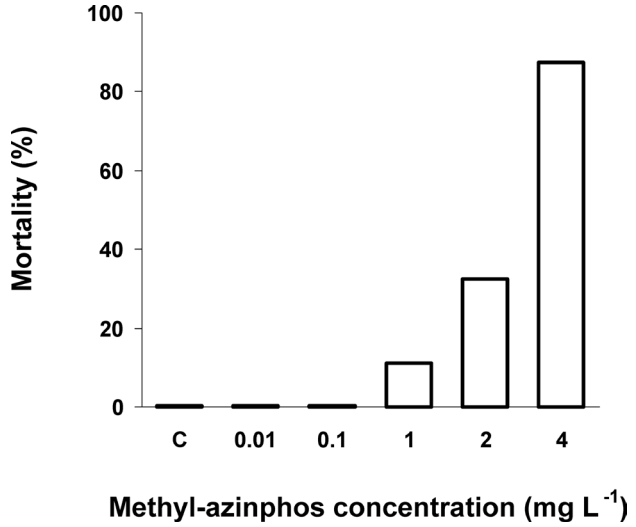

Figure 3 - Results from static, 48 h, paired bioassays illustrating *Astyanax fasciatus* mortality versus azinphos-methyl concentration. Bars represent mean mortality ± SD of fish groups exposed to different toxicant concentrations.

Brain AChE inhibition versus toxicant concentration

When groups of adult *A. fasciatus* were exposed to azinphos-methyl for 48 h, concentration-dependent decreases in brain AChE specific activity were found. Table 1 shows *A. fasciatus* non-inhibited brain AChE specific activity and inhibitions found upon exposure to five different concentrations of azinphos-methyl. In a parallel series of exposure experiments similar to those represented by Table 1, but using six different concentrations of the toxicant plus the control (not shown), AChE activity (expressed as percent of control activity) exhibited an inhibition which increased linearly with respect to the logarithm of toxicant concentration. This occurred between concentrations ranging between 0.001 and 1 mg L^{-1} of the toxicant. At higher concentrations, inhibition was already close to 85-97 % of control activity, with practically no further significant changes in % inhibition. It was also remarkable to find that *A. fasciatus* tolerated about 75% inhibition of its brain cholinesterase activity without lethality. Thus, we find that exposure to a wide range of toxicant concentrations caused graded changes in inhibition and in clinical signs of neurotoxicity, without mortality.

Table 1 - Brain AChE specific activity (mean ± S.D.) in groups of *A. fasciatus* exposed to different concentrations of azinphos-methyl for 48 h. Column N shows total number of individuals per treatment condition (19-20); groups marked with different superindex letters in the right-hand column were found significantly different to each other, while those denoted with the same letter were not, according to Tukey´s Test ($p< 0.05$).

Azinphos-methyl (mg L^{-1})	N	AChE specific activity (Units.mg^{-1} protein)	Inhibition (% of control)
Control	19	60.38 ± 5.53	0^a
0.01	20	44.49 ± 3.68	26^b
0.10	20	27.07 ± 5.06	55^c
1.00	19	9.45 ± 2.63	84^d
2.00	20	9.02 ± 2.10	85^d
4.00	19	8.28 ± 1.45	87^d

Table 2 - Frequency of erythrocyte micronuclei in *A. fasciatus* exposed for 48 hours to either azinphos-methyl or cyclophosphamide. N, number of animals per experimental group; MNF, micronuclei frequency (mean ± standard deviation); different letters (MNF^a, MNF^b) indicate significant differences between groups ($p< 0.05$).

Toxicant used	Final toxicant concentration (mg L^{-1})	N	MNF
Cyclo-phosphamide	5.0	6	5.33 ± 1.0^a
Azinphos-methyl	0.0	10	0.10 ± 0.3^b
	1.0	8	0.40 ± 0.5^b
	2.0	10	0.10 ± 0.3^b
	4.0	10	0.20 ± 0.4^b

Brain AChE recovery experiments

In the experiments designed to investigate brain AChE recovery upon OP exposure, groups of fishes were exposed to 0.8 mg L^{-1} azinphos-methyl for 48 h, and immediately later returned to normal clean aquarium water, as explained in detail in the Methods section. A gradual but remarkable recovery of brain AChE specific activity was found (Fig. 4). Importantly, recovery of brain AChE reached about 80% of control, non-inhibited activity in ten days (Fig. 4).

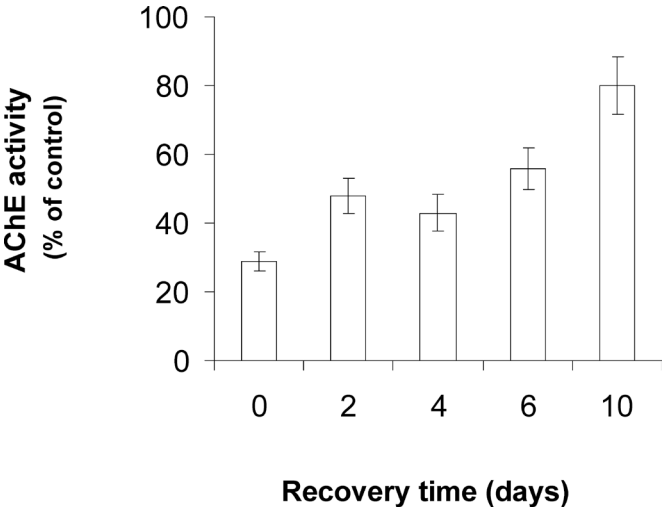

Figure 4 - Recovery of *A. fasciatus* brain AChE activity after exposure to azinphos-methyl. Fishes captured from the non-polluted Juncal stream were separated in six groups of 7-8 fishes per aquarium and acclimated for 15 days. The control group, from which 100% control AChE activity was calculated, was kept for 48 h in usual aquarium water conditions (without toxicant, light:darkness cycle 12:12 h), whereas the other five groups started their 48 h exposure to 1 mg L^{-1} (ccf) azinphos-methyl. At recovery time 0 (zero), i.e., after 48 h of toxicant action, the Control group and one of the exposed groups ("zero recovery") were sacrificed, whereas the remaining four groups were placed in normal, toxicant-free aquarium conditions and allowed to recover for 2, 4, 6, or 10 days. All brains were removed and assayed in triplicate for AChE. Enzyme activities were expressed as percent of control group AChE activity (mean ± S.D.).

Field study: time- and space-related changes in a biomarker: brain AChE

As a result of interviews with local landowners, we established a list of active compounds used in the Dragon stream basin which agreed well with data resulting from previous interviews (Carrasco-Letelier *et al*., 2006) and which indicated that azinphos-methyl was the most extensively used OP in the region. In order to detect **time-dependent** field changes in pesticide effects on Dragon stream fish biochemical markers, we took into account that the peak of OP application was scheduled to be -according to landowners application plans- between October and November. Thus, we performed our samplings in two periods: before and after the peak of OP application, i.e., in the months of September and December.

On the other hand, in order to investigate the occurrence of spatial-dependent pesticide impacts, we decided to carry out samplings in three sampling points (as shown in Fig. 2): a control point at Juncal stream (*Cañada del Juncal* in Fig. 2,C), and two zones of the Dragón stream: a pre-impacted zone and an impacted zone (S1 and S2, respectively, in Fig. 2,D). When September samplings were carried out, *A. fasciatus* specimens were present at all sampling points (control, S1 and S2), and therefore their enzyme activities could be compared (Fig. 5). However, in samplings performed in December, the species was only present at control place and at S2 (Fig. 5) but not at S1. When we compared brain cholinesterase activities from samples collected exclusively during September, we found that those taken from the control point and from Dragon stream pre-impacted zone (S1) were not significantly different, whereas fishes caught at the impacted zone (S2) presented activities approximately 32% lower than S1 and controls. In other words, in September, brain AChE specific activity in fishes from S1 was similar to that found in fishes from the control stream, but activity dropped remarkably and significantly (Student's test; p=0.00029) from S1 to S2 (downstream to the fruit-tree farm zone). On the other hand, when we analyzed temporal changes on AChE (December vs. September), and we compared samples collected at the same point (S2, impacted zone) but at different months (Fig. 5), December samples showed brain AChE specific activities approximately 30% higher than September samples. The reference site (Juncal stream) exhibited AChE increases similar to those detected in S2.

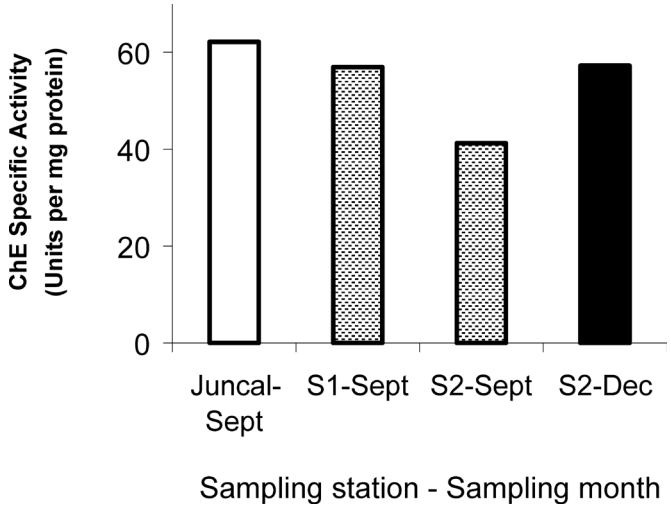

Figure 5 - Field location-dependent changes in *A. fasciatus* brain cholinesterases (ChE) specific activities. Fish sampling points were indicated in Fig. 1, as "Cañada del Juncal" (Juncal stream) in Fig. 1C and as S1 and S2 in Fig.1D. Bars represent brain total ChE specific activities (mean ± S.D.) of fish groups collected from either the Juncal stream (open bar), S1 sampling point (stippled bars) or S2 sampling point (black bar), as depicted in Fig.1D. The three left-hand side bars show brain enzyme activities from fishes collected in September (Sept) whereas the black bar to the right illustrates activities from fishes captured in December (Dec). Brain ChE specific activities were expressed as nmoles of AcSCh hydrolyzed.min^{-1}.mg $^{-1}$ of protein.

DISCUSSION

Behavioral changes, mortality (LC_{50}) and micronuclei test upon exposure to azinphos-methyl

The behavioral changes that we recorded upon *A. fasciatus* exposure to azinphos-methyl were qualitatively similar to both those found in goldfish under the same toxicant (Ferrari *et al*. 2004) and those seen in *Corydoras paleatus* and *Anguilla anguilla* upon exposure to fenitrothion (Sancho et al., 1998; Sarikaya et al., 2004), an OP widely used against insect pests and mites. Taken together, these changes are typical signs of behavioral impairment caused by inhibition of brain cholinesterases in teleosts (Sandahl *et al*., 2005). At sublethal concentrations, anticholinesterases impair several important physiological and behavioral processes including swimming and feeding performances, predator avoidance, migration, learning and memory, as well as conspecific social interactions (Sandahl *et al*., 2005).

The LC_{50} of 2.31 mg L^{-1} (48h) that we found for azinphos-methyl appears to indicate that *A. fasciatus* is less tolerant to the OP than a number of freshwater species, as *Carassius auratus* (LC_{50} 2.0-8.2 mg L^{-1}, Ferrari *et al*., 2004) or *Ictalurus punctatus* (LC_{50} 3.2-3.5 mg L^{-1}, Morton *et al*. (1997) and, on the other hand, 1-3 orders of magnitude more tolerant to this OP than *Daphnia magna* (LC_{50} 1.6 µg L^{-1}) and most fishes, including, for instance, *Onchorhynchus mykiss* (LC_{50} 5.5 µg L^{-1}), *Micropterus salmonoides* (25 µg L^{-1}), *Lepomis macrochirus* (25 µg L^{-1}), *Pimephales promelas* (64-93 µg L^{-1}), *Notemigonus crysoleucas* (100 µg L^{-1}) and *Poecilia reticulata* (120 µg L^{-1}) (see Morton *et al*., 1997 and WHO-FAO Data Sheet on Pesticides Nº59, for details). However, extreme care must be taken in these comparisons, since concentrations that we report here are nominal, as explained in Methods section. The 48 h-renewal time that we used in the static assay appears to be adequate, since less than 5% degradation was calculated for the toxicant to occur in the period at pH (7.4) and photoperiod employed in our study, as explained in the Methods section.

The negative results of the micronuclei test appear to suggest that azinphos-methyl is not significantly genotoxic for

A. fasciatus, which is in line with the WHO-FAO Datasheet on Pesticides Nº 59. The method is usually considered an useful genotoxicity indicator in fishes (Campana *et al*., 1999), although Moreira *et al*. (2010) stated that the species used in our study commonly displays low micronuclei frequencies.

Brain cholinesterases specific activities in Astyanax

In *A. fasciatus* specimens collected from the non-polluted Juncal stream, we found a brain cholinesterase specific activity of 62.2 ± 5.1 $U.mg^{-1}$ protein, of which 97.7% was AChE, as shown by our BuChE inhibition experiments. This AChE:total cholinesterases ratio is common in most seawater (Galgani *et al*., 1992) and freshwater (Dembélé *et al*., 1999) teleosts, although brains of some species of fish have been reported to contain substantial amounts of both AChE and BuChE (Sandahl, 2009). It is worth noting that we employed Triton X-100-containing whole homogenates in all our AChE assays, since particulate and masked activities are lost otherwise (Rodríguez-Ithurralde *et al*., 1997; Oliveira *et al*., 2007). We were unable to find papers on *Astyanax* **brain** cholinesterases. Shulz & Martins-Junior (2000) recommended *A. fasciatus* for biomonitoring, and Moreira *et al*. (2010) studied effects of capture methods on its genotoxicity biomarkers, but they did not analyzed its cholinesterases. In Asyanax sp. **muscle**, Akaishi *et al*. (2004) found an AChE activity of about 260 $Units.mg^{-1}$ protein (for a review on AChE as fish biomarker, see Oliveira Ribeiro & Silva de Assis, 2005), whereas in *Poecilia reticulata* **head**, Stringuetti *et al*.(2008) got 149.7 $U.mg^{-1}$ protein (at 30ºC), and in *Cnesterodon decemmaculatus* brain, de la Torre *et al*. (2002) found 249 $U.mg^{-1}$ protein. Brain AChE values vary 15-fold (Chuiko *et al*., 1997) or even more among species, e.g., 7449 $U.mg^{-1}$ protein in *Limanda limanda* (Galgani *et al*., 1992), 390-1600 $U.mg^{-1}$ protein in *Cyprinus carpio* (Dembélé *et al*., 1999; de la Torre *et al*., 2002) and 85.5 $U.mg^{-1}$ protein in rainbow trout (Sturm *et al*., 2007), but are much more consistent than muscle AChE values when data from different authors studying the same species or genera are compared (Chuiko *et al*., 1997).

Sensitivity, concentration-response relationship and recovery of Astyanax brain AChE upon fish exposure to azinphos-methyl

A remarkable original finding of this work is that *A. fasciatus,* despite its good behavioral and vital tolerance to azinphos-methyl, possesses a brain AChE that is readily inhibited by fish exposure to relatively low toxicant concentrations, and which responds predictably and quite linearly to a wide range of OP concentrations. Since brain cholinesterase inhibitions close to 80% are frequently associated with high lethality (Fulton & Key, 2001), the absence of mortality encountered after exposures to toxicant concentrations causing brain AChE inhibitions of 75% is also of great interest. In fact, this is very convenient for monitoring purposes, since most useful species for biomonitoring would be those exhibiting concentration-related changes of a biomarker through a wide range of toxicant concentrations (Chambers *et al*., 2002). Another novel important finding of this study was the velocity at which *A. fasciatus* brain AChE is recovered after toxicant is withdrawn, i.e., reaching 80% of normal enzymatic activity in 10 days. This is noteworthy, since recovery of brain cholinesterase activities upon exposure to OP pesticides has been found to be protracted in most fish species, usually taking several weeks (Sancho, 1998; Ferrari *et al*., 2004, 2007; Cong *et al*., 2008). *Cyprinus carpio*, the quickest in this matter reported to date, takes about 15 days (Dembelé *et al*., 1999). As we have stated in the *Methods* section and discussed in connection with the LC_{50} (48 h), the theoretical decay in azinphos-methyl concentration for the exposure and recovery experiments, was calculated by us to be 5% or less in the 48 h. This figure took into consideration the main causes of azinphos-methyl degradation in our experiments, i.e., hydrolysis and aqueous photolysis (Extoxnet, 1979; Erickson & Turner, 2003), the time when fishes were returned to the clear water in recovery experiments. Since OP:s behave as irreversible cholinesterase inhibitors (Karlsson *et al*., 1984) the main mechanism of enzyme activity recovery must be *de novo* synthesis of the protein, as postulated by Ferrari *et al*. (2004) in the goldfish. This rapid AChE dynamics found in the central nervous system of our *lambarí* looks as very convenient for biomonitoring purposes, because in this way decreased AChE levels found at any sampling time are a reliable marker of recent exposure to environmental anti-cholinesterase xenobiotics. Moreover, since the species is non-migratory, it would also indicate geographic proximity to a xenobiotic source.

Biomarker spatial gradients detected in fish collected from Dragon small stream

Another interesting finding of this study is the high sensitivity of *A. fasciatus* to "bio-indicate" the presence of environmental xenobiotics with anti-cholinesterase activity. In the Juncal stream study area, the most plausible cause of decreased brain AChE activity found in fishes captured at S2 sampling point (Fig 1,D) with respect to those from S1, i.e., after the Dragon stream crosses the intensive fruticulture area (Fig.1,D), is that the transit across the farming zone impacted the stream with pollutants from adjacent application sites. OP are transported from application sites to river tributaries in sufficient amount as to inhibit cholinesterases of fishes present in the stream (Schulz, 2001, 2004; Sturm *et al*., 2007) and azinphos-methyl is very stable in water below pH 10.0 (Extoxnet, 1979; Erickson & Turner, 2003). Edge-of-the-field runoff (and in much lesser extent spraydrift) are important routes of azinphos-methyl and other OP entry into streams (Schulz, 2001; Sturm *et al*., 2007). OP from spraydrift usually resides in the water phase, while pesticides in contaminated runoff are to a large extent associated with suspended particles (**Schulz, 2001;** Gavilan *et al*., 2001; Barra *et al*., 2005; Sturm *et al*., 2007). Azinphos-methyl is, by-far, the most used pesticide at Dragon stream basin (Carrasco-Letelier *et al*., 2006 and this study), but obviously other pesticides with anticholinesterase

activity (OP, carbamates), and even other pollutants (Valdez-Domingos *et al*., 2009) might contribute to changes found in fish cholinesterases. For instance, fishes collected from the S2 site (downstream to the farm area) in September show enzyme inhibitions very similar to those seen in specimens exposed to 0.01 mg L^{-1} azinphos-methyl in the laboratory bioassay. However, this fact does not mean that fishes captured close to S2 were exposed to an azinphos-methyl concentration close to 0.01 mg L^{-1}, obviously. Field data must be interpreted with extreme caution, since due to synergic, additive and antagonist responses, similar biomarker values can be achieved by different combinations of xenobiotics (Sandahl *et al*., 2005; Sturm *et al*., 2007; Laetz *et al*., 2009). In fact, many frequent pollutants have been shown to cause decreases or even increases in the activity of fish muscle cholinesterases, including heavy metals and hydrocarbons (Podolska & Napierska, 2006; Valdez-Domingos *et al*., 2009).

Time-dependent biomarker changes at Dragon small stream

Brain cholinesterase activities of fishes collected from S2 (impacted zone) in December were about 32% higher than activities found in the same place in September, reaching practically S1 (pre-impact) September levels (Fig. 5). As our electric-fishing was unable to collect *Astyanax* specimens at S1 in December, direct S2-S1 comparisons were impossible. However, since temporal changes in enzyme activity found in fishes from S2 were accompanied by similar changes in specimens collected from the control sampling site (Juncal stream, not shown), time-dependent changes at S2 seem to be due to seasonal factors in a basin characterized by absence of rainfall after the main application period. Fish muscle (Valdez Domingos *et al*., 2009) and brain (Durieux *et al*., 2010) cholinesterases are responsive to natural factors, as seasonal changes in temperature and photoperiod practically all species studied have shown a strong positive relationship between water temperature and solstice photoperiod with brain AChE activity (Galgani *et al*., 1992; Chuiko *et al*, 1997; Cong *et al*., 2008; Durieux *et al*., 2010). In our field study, both increases in temperature and maximal photoperiod typical of December Summer Solstice in the Southern Hemisphere, plus the absence of rainfall for months after application (common in Uruguay) may fully explain why stream fish cholinesterases may remain non-impacted for months after the main OP application period. In fact, toxicants need rainfall to reach water courses in meaningful amounts, since rainfall-induced runoff of sediment-transported pesticides into small streams is the main mechanism for river pollution with azinphos-methyl (Schulz, 2001).

Possible Ecotoxicological value of Astyanax cholinesterases

A great number of fish species have been analyzed with the purpose of developing reliable ecotoxicological methods using brain or muscle cholinesterase inhibition as a biomarker (Chambers *et al*., 2002), including bluegill (*Lepomis macrochirus*), goldfish (*Carassius auratus*), wild *Poecilia reticulata* (Stringuetti *et al*., 2008), killifish (*Fundulus heteroclitus*), zebrafish (*Danio rerio*, Roex *et al*., 2003), snakehead fish (Cong *et al*., 2008), *Astyanax sp.*, *Hoplias malabaricus,* etc. (for a review, see Oliveira Ribeiro & Silva de Assis, 2005). We think that *A. fasciatus* compares favorably in many aspects with most of the above-mentioned examples. In fact, we have shown that this "lambarí" (Moreira *et al*., 2010) or "mojarra" is able to exhibit graded, replicable, concentration-dependent changes in brain cholinesterases when exposed to a wide range of environmentally realistic OP concentrations. Importantly, this occurs before fish behavior is severely compromised, so indicator organisms can survive for many hours at harsh pesticide concentrations (Shultz & Martins-Junior, 2000). This is particularly relevant when selecting a sentinel species, because it is well known that anticholinesterases induce, in most fishes, behavioral changes that may affect survival under field conditions (Ferrari *et al*., 2004, 2007; Chambers *et al*., 2002). As stated for *C. decemmaculatus* (de la Torre *et al*., 2002), *A. fasciatus* AChE may be considered an useful and sensitive biomarker even at polluted water bodies, where exotic species fail to provide a sensitive parameter, illustrating the advantages of considering native species biomarkers in monitoring programs (de la Torre *et al*., 2002, 2007). In addition to all mentioned qualities as an indicator species, its suitability to laboratory conditions is remarkable (Shultz & Martins-Junior, 2000). Moreover, it has a central role in riverine food webs and it is relatively tolerant to stress and to varied types of environmental degradation (Shultz & Martins-Junior, 2000; Moreira el al., 2010).

A. fasciatus has already been proposed by various authors as a potential bio-indicator species to monitor water quality (Shultz & Martins-Junior, 2000; Moreira *et al*., 2010) including porphyrin profiles as markers of anthropogenic pollution (Carrasco-Leteleir *et al*., 2006). The body of our data suggests that the species possesses points of contact with an ideal indicator of anticholinesterase compounds. For comparison purposes among different hydrological systems and countries, the abundance and the extremely wide distribution in America of the species, despite of its convenient non-migratory character, are other relevant advantages of *A. fasciatus* (Moreira *et al*., 2010). Moreover, it is worth noting that although biomarker assessment needs a destructive technique, the abundance and widespread distribution of this characid should allow researchers and the general public to accept the minor environmental impact that may result from its sampling for bio-surveillance monitoring programs (Carrasco- Leteleir *et al*., 2006; Moreira *et al*., 2010).

Concluding remarks

In conclusion, we have found that when *A. fasciatus* is exposed under laboratory conditions to environmentally realistic concentrations of the OP pesticide azinphos-methyl, it exhibits clear behavioral and biochemical signs of neurotoxicity, without noticeable alteration of the micronuclei

index. We have determined an LC_{50} (48h) of 2.31 mg L^{-1} for azinphos-methyl. We have measured total cholinesterases, BuChE and AChE from any *Astyanax* brain for the first time, and shown that in *A. fasciatus* brain, AChE is approximately 98% of total brain cholinesterases. We demonstrated concentration-dependent biochemically detectable inhibitions of brain AChE along a wide range of toxicant concentrations that do not determine death of the organism, which occurs with brain AChE inhibitions of 80 % or higher. We have characterized the concentration-response relationship between azinphos-methyl and brain AChE activity and, importantly, shown unequivocally that its activity is able to recover rapidly (80% of normal in ten days) once the toxicant is retired, due to *de novo* synthesis of the protein. Our field study at the Dragon stream basin showed significant space-dependent inhibitions of about 32 % in brain cholinesterase activities after the stream crosses the zone impacted by intensive farm activity. The body of our findings supports the view that *A. fasciatus* AChE is a sensitive and predictable biomarker of exposure to organophosphate insecticides and carbamates, which may be very useful in regional ecotoxicological bio-surveillance programs. Our results reinforce the view that this characid reunite a wide number of qualities desirable in a sentinel organism for freshwater monitoring programs throughout most Southern America and in particular within freshwater systems of Uruguay.

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REFERENCES

ALTAMIRANO, A., DA SILVA, H., ECHEVARRÍA, A., PANARIO, D. & PUENTES, R., 1976, Descripción de las unidades de suelos. Carta de Reconocimiento de Suelos del Uruguay. Tomo III: Dirección de Suelos y Fertilizantes, Ministerio de Agricultura y Pesca, Montevideo, Uruguay.

AKAISHI, F. M., SILVA DE ASSIS, H. C., JACOBI, S.C., EIRAS-STOFELLA, S.D. COUTENAY, S.C., LIMA, E. F., WAGENER, A. L. R., SCOFIELD, A.L. & OLIVEIRA RIBEIRO, C. A., 2004, Morphological and neurobiological findings in tropical freshwater fish (*Astyanax* sp.) after waterborne and acute exposure to water soluble fraction (WSF) of crude oil. *Arch. Environ. Contam. Toxicol*., 46 (2): 244-253. doi: 10.1007/s00244-003-2260-1.

BARRA, R., COLOMBO, J.C., EGUREN, G., GAMBOA, N., JARDIM, W. & MENDOZA, G., 2005, Persistent Organic Pollutants (POPs) in Eastern and Western South American Countries. *Rev. Environ. Contam. Toxicol*., 185: 1-33.

CAMPANA, M., PANZERI, A., MORENO, V. & DULOUT, F., 1999, Genotoxic evaluation of the pyrethroid lambda-cyhalothrin using the micronucleus test in erythrocytes of the fish *Cheirodon interruptus interruptus*. *Mutat. Res*., 438: 155-161. doi: 10.1016/S1383-5718(98)00167-3.

CARRASCO-LETELIER, L., EGUREN, G., TEIXEIRA DE MELLO, F. & GROVES, P.A., 2006, Preliminary field study of hepatic porphyrin profiles of *Astyanax fasciatus* (*Teleostei, Characiformes*) to define anthropogenic pollution. *Chemosphere,* 62(8): 1245–1252. doi: 10.1016/j.chemosphere.2005.07.005.

CASTRO, R. M. C. & CASATTI, L., 1997, The fish fauna from a small forest stream of the upper Parana River basin, southeastern Brazil. *Ichthyiol. Explorat. Freshwat.,* 7: 337-352.

CHAMBERS, J. E., BOONE, J.S., CARR, R. L., CHAMBERS, H.W. & STRAUS, D. L., 2002, Biomarkers as predictors in health and ecological risk assessment. *Hum. Ecol. Risk Assess*. 8(1), 165-176. doi: 10.1080/20028091056809.

CHUIKO, G. M., ZHELNIN, Y.Y. & POD'GORNAYA, V. A, 1997, Seasonal fluctuations in brain acetylcholinesterase activity and soluble protein content in roach (*Rutilus rutilus* L.): a freshwater fish from Northwest Russia. *Comp. Biochem. Physiol. Part C: Pharmacol., Toxicol. & Endocrin*., 117(3): 251-257. doi: 10.1016/S0742-8413(97)00068-6.

CONG, V.N., PHUAONG, N.T, & BAYLEY, M., 2008, Brain acetylcholinesterase response in the snakehead fish *(Channa striata*) after field exposure to duazinon. *Ecotoxicol. Environm. Saf.*, 71(2): 314-318. doi: 10.1016/j.ecoenv.2008.04.005.

de la TORRE, F. R., FERRARI, L. & SALIBIÁN, A., 2002, Freshwater pollution biomarker: response of brain acetylcholinesterase activity in two fish species. *Comp. Biochem. Physiol. Part C: Toxicol. & Pharmacol*., 131(3): 271-280. doi: 10.1016/S1532-0456(02)00014-5.

de la TORRE, F. R., SALIBIÁN, A. & FERRARI, L., 2007, Assessment of the pollution impact on biomarkers of effect of a freshwater fish. *Chemosphere*, 68(8): 1582-1590. doi: 10.1016/j.chemosphere.2007.02.033.

DEMBÉLÉ, K., HAUBRUGE, E., & GASPAR, C., 1999, Recovery of acetylcholinesterase activity in the common carp (*Cyprinus carpio* L.) after inhibition by organophosphate and carbamate compounds. *Bull. Environ. Contam. Toxicol*., 62(6): 731-742. doi: 10.1007/s001289900934.

DURIEUX, E. D. H., FARVER, T. R., FITZGERALD, P. S., EDER, K.J. & OSTRACH, D. J., 2010, Natural factors to consider when using acetylcholinesterase activity in young for the year stripped-Bass (*Morone saxatilis*). *Fish Physiol. Biochem.* doi: 10.1007/s10695-010-9412.

ELLMAN G. L., COURTNEY, K. D., ANDRES, V. & FEATHERSTONE, R. M., 1961, A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.,* 7(2): 88-95. doi: 10.1016/0006-2952(61)90145-9.

ERICKSON, W. & TURNER, L., 2003, Azinphos methyl- Analysis of risk to endangered and threatened salmon and steelhead. US-EPA. http://www.epa.gov/espp/litstatus/effects/azinphos_methyl_analysis_final.pdf

EXTOXNET: Extension Toxicology Network, 1979, Azinphos methyl. http://pmep.cce.cornell.edu/profiles/extoxnet/24d-captan/azinphos-methyl-ext.html.

FERRARI, A., VENTURINO, A. & PECHEN DE D'ANGELO, A., 2004, Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphos-methyl, parathion and carbaryl exposure in the goldfish (*Carassius auratus*). *Ecotoxicol. Environm. Saf*., 57(3): 420-425. doi: 10.1016/S0147-6513(02)00069-6.

FERRARI, A., VENTURINO, A. & PECHEN DE D'ANGELO, A., 2007, Muscular and brain cholinesterase sensitivities to azinphos-methyl and carbaryl in the juvenile rainbow trout *Oncorhynchus mykiss*. *Comp. Biochem. Physiol. Part C: Toxicol. & Pharmacol*.,

146(3): 308-313. doi: 10.1016/j.cbpc.2007.04.002.

FULTON, M. H. & KEY, P. B., 2001, Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects (review). *Environm. Toxicol. Chem.,* 20(1): 37-45. doi: 10.1002/etc.5620200104.

GALGANI, F., BOCQUENÉ, G. & CADIU, Y., 1992, Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. *Mar. Ecol. Prog. Ser*. 91: 77-82.

GAVILAN, J. F., BARRA, R., FOSSI, M.C., CASINI, S., SALINAS, G., PARRA, O. & FOCARDI, S., 2001, Biochemical biomarkers in fish from different river systems reflect exposure to a variety of anthropogenic stressors. *Bull. Environ. Contam. Toxicol.,* 66(4): 476–483. doi: 10.1007/s00128-001-0031-0.

HAMILTON, M.A., RUSSO, R.C, & THURSTON, R.V., 1977, Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol*., 11(7): 714-719. doi: 10.1021/es60130a004.

HAYDEN, K. M., NORTON, M., DARCEY, D., OSTBYE, T., ZANDI, P., BREITNER, J. & WELSH-BOHMER, K.A., 2010, Occupational exposure to pesticides increases the risk of incident Alzheimer's Disease. The Cache County study. *Neurol.*, 74(19): 1524–1530. doi:10.1212/WNL.0b013e3181dd4423.

KARLSSON, E., MBUGUA, P. M. & RODRÍGUEZ-ITHURRALDE, D., 1984, Fasciculins, anticholinesterase toxins from the venom of the green mamba *Dendroaspis angusticeps*. *J. Physiol. (Paris),* 79: 232-240.

LAETZ, C. A., BALDWIN, D. H., COLLEIR, T. K., HEBERT, V., STARK, J. D. & SCHOLZ, N., 2009, The synergistic toxicity of pesticide mixtures: implications for risk assessment and the conservation of endangered Pacific salmon *Env. Health. Perspect*. 117(3): 348-353. doi: 10.1289/ehp.0800096.

LIZAMA, M. A. P. & AMBRÓSIO, A.M., 2002, Condition factor in nine species of fish of the Characidae family in the upper Paraná river floodplain, Brazil. *Braz. J. Biol*., 62(1): 113-124. doi: 10.1590/S1519-69842002000100014.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. & RANDALL, R., 1951, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* 193(1): 265-275.

MOREIRA, T. N., NUNES, E. A., LEAL, M. E., SCHULZ U. H., & DE LEMOS, C. T., 2010, Influência dos métodos de captura de peixes na availaçào genotóxica utilizando diferentes tecidos de *Asyanax fasciatus* (Osteichthyes, Characidae). *J. Braz. Soc. Ecotox*., 5: 1-7. doi: 10.5132/jbse.2010.01.001.

MORTON, M. G., MAYER, F. L., DICKSON, K. L., WALER, W. T. & MOORE, J. C., 1997, Acute and chronic toxicity of azinphos-methyl to two estuarine species, *Mysidopsis bahia* and *Cyprinodon variegatus*. *Arch. Environ. Contam Toxicol,* 32(4): 436-441. doi: 10.1007/s002449900210.

OLIVERA, S., RODRÍGUEZ-ITHURRALDE, D. & HENLEY, J. M., 2003, Acetylcholinesterase promotes neurite elongation, synapse formation and surface expression of AMPA receptors in hippocampal neurons. *Mol. Cell. Neurosci.,* 23(1): 96-106. doi: 10.1016/S1044-7431(03)00021-6.

OLIVEIRA RIBEIRO, C. A. & SILVA DE ASSIS, H. C., 2005, AChE inhibition as a biomarker for pollutant contamination in tropical aquatic ecosystems, Chapter-10, pp. 103-124. In: *Recent Trends in the Acetylcholinesterase System*, Parveen, M. & Kumar, S., Eds. IOS Press.

OLIVEIRA, M. M., SILVA FILHO, M., CUNHA BASTOS, V. L. F., FERNANDES, F., & CUNHA BASTOS, J., 2007, Brain acetylcholinesterase as a marine pesticide biomarker using Brazilian fishes. *Marine Environ. Res.,* 63(4): 303-312. doi: 10.1016/j.marenvres.2006.10.002.

PODOLSKA, M. & NAPIERSKA, D., 2006, Acetylcholinesterase activity in hosts (herring *Clupea harengus*) and parasites (*Anisakis simplex* larvae) from the southern Baltic. ICES *J. Mar. Sci.,* 63(1): 161-168. doi: 10.1016/j.icesjms.2005.08.001.

RINGUELET, R.A., ARAMBURU, R.M., & ARAMBURU, A.A., 1967, Los peces argentinos de agua dulce. Comisión de Investigación Científica, Provincia de Buenos Aires, La Plata, Argentina. 600 pp.

ROEX, E. W. M., KEIJZERZ, R., & VAN GESTEL, C. A. M., 2003, Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, *Danio rerio*, after chronic exposure to parathion. *Aquat. Toxicol*., 64(4): 451-460. doi: 10.1016/S0166-445X(03)00100-0.

RODRIGUES, S. R., CALDEIRA, C., CASTRO, B. B., GONÇALVES, F., NUNES, B. & ANTUNES, S.C., 2011, Cholinesterase (ChE) inhibition in pumpkinseed (*Lepomis gibbosus*) as environmental biomarker: ChE characterization and potential neurotoxic effects of xenobiotics. *Pestic. Biochem. Physiol*., 99(2): 181-188. doi: 10.1016/j.pestbp.2010.12.002.

RODRIGUEZ-ITHURRALDE, D., OLIVERA, S., VINCENT. & MARURI, A.,1997, *In vivo* and *in vitro* studies of glycine- and glutamate-evoked acetylcholinesterase release from spinal motor neurones: implications for amyotrophic lateral sclerosis/motor neurone disease pathogenesis. *J. Neurol. Sci.,* 152 (S1): 54-61. doi: 10.1016/S0022-510X(97)00245-1.

SANCHO, E., FERRANDO, M.D., TEN, A., LLEO, C. & ANDREU-MOLINER, E., 1998, Sub lethal bio-concentration of fenitrothion in the blood and brain of the European eel. *Bull. Environ. Contam. Toxicol.,* 60(5): 809-815. doi: 10.1007/s001289900699.

SANDAHL, J. F., BALDWIN, D. H., JENKINS, J. J., & SCHOLZ, L., 2005, Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in *Coho salmon* exposed to chlorpyriphos. *Environm. Toxicol. Chem*. 24(1): 136-145. doi: 10.1897/04-195R.1

SARIKAYA, R., SELVI, M. & ERKOC, F., 2004, Investigation of acute toxicity of fenitrothion on peppered corydoras (Corydoras paleatus) (Jenyns, 1842). *Chemosphere* 56(7): 697-700. doi: 10.1016/j.chemosphere.2004.04.008.

SCHULZ, R., 2001, Rainfall-induced sediment and pesticide input from orchards into the Lourens River, Western Cape, South Africa: importance of a single event. *Water Res*. 35(8): 1869-1876. doi: 10.1016/S0043-1354(00)00458-9.

SCHULZ, R., 2004, Field studies on exposure, effects, and risk mitigation of aquatic non-point-source insecticide pollution. A Review. *J. Environ. Qual.,* 33(2): 419-448. doi:10.2134/jeq2004.4190.

SCHULTZ, U.H.& MARTINS-JUNIOR, H., 2000, *Astyanax fasciatus* as bioindicator of water pollution of Rio dos Sinos, RS, Brazil. *Braz. J. Biol*., 61: 615–622. doi: 10.1590/S1519-69842001000400010.

STRINGHETTI, C., GUILHERMINO, L. & DA SILVE, E. M., 2008, Cholinesterase activity in the head of wild *Poecilia reticulata* from Bahia, Brazil: biochemical characterization, effects of sample storage and normal range of activity. *J. Braz. Soc. Ecotoxicol.,* 3:57-63. doi: 10.5132/jbse.2008.01.009.

STURM, A., RADAU, T.S., HAHN, T. & SCHULZ, R., 2007, Inhibition of rainbow trout acetylcholinesterase by aqueous and suspended particle-associated organophosphorous insecticides. *Chemosphere*, 68(4): 605-612. doi: 10.1016/j.chemosphere.2004.04.008.

VALDEZ DOMINGOS, F.X., ASSIS H.C.S, SILVA, M.D., DAMIAN, R.C., ALMEIDA, AI.M., CESTARI, M.M. RANDI, M. & OLIVEIRA RIBEIRO, C.A., 2009, Anthropic impact evaluation of two Brazilian estuaries through biomarkers in fish. *J. Braz. Soc. Ecotoxicol.*, 4: 21-30. doi: 10.5132/jbse.2009.01.004.

WHO-FAO Data sheet on Pesticides Nº 59. Azinphos-methyl (VBC/PDS/DS/85.59), http://www.inchem.org/documents/pds.