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Alterations in the general condition, biochemical parameters and locomotor activity in *Cnesterodon decemmaculatus* exposed to commercial formulations of chlorpyrifos, glyphosate and their mixtures

Alejo Fabian Bonifacio[a], Jimena Cazenave[b], Carla Bacchetta[b], María Laura Ballesteros[a], María de los Ángeles Bistoni[b], María Valeria Amé[c], Lidwina Bertrand[c], Andrea Cecilia Hued[a,*]

[a] *Instituto de Diversidad y Ecología Animal (IDEA), CONICET and Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, Córdoba CP 5000, Argentina*
[b] *Laboratorio de Ictiología, Instituto Nacional de Limnología (INALI-CONICET-UNL), Paraje El Pozo, Ciudad Universitaria UNL, 3000 Santa Fe, Argentina*
[c] *Universidad Nacional de Córdoba – CONICET, Facultad Ciencias Químicas, Dto. Bioquímica Clínica – CIBICI, Haya de la Torre esq. Medina Allende, Ciudad Universitaria, Córdoba CP 5000, Argentina*

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ABSTRACT

The Pampean region, an extensive area of South America is continuously impacted by agricultural activities and the pesticides related to them like chlorpyrifos and glyphosate. Both pesticides have been registered in freshwater bodies of the region. One of the most abundant and widely distributed fish species in Pampean streams is *Cnesterodon decemmaculatus*, which have to cope with this altered scenario.

In the present study the toxicity of Clorfox® and Roundup Max®, the commercial formulations of chlorpyrifos and glyphosate, respectively, and their mixture where evaluated using a set of biomarkers at different biological organization levels in fish exposed to relevant environmentally pesticides concentrations. Somatic indexes such as the condition factor (*K*), and the hepato-somatic index (HSI), the locomotor activity through the distance traveled and the average speed, the enzymatic activities of acetylcholinesterase (AChE) in brain and muscle, catalase (CAT) in muscle and liver, glutathione-S-transferase (GST) in brain, liver, muscle and gills, aspartate amino-transferase (AST), alanine amino-transferase (ALT), AST/ALT ratio and alkaline phosphatase (ALP) in liver were measured on *C. decemmaculatus*. Adult females were exposed during 6 weeks to the following concentrations: 0.0084 μl/l and 0.00084 μl/l of Clorfox (CF), 0.2 and 2 mg/l of Roundup Max (RM) and all the combinations of these concentrations. The CF exposure caused a decrease in the condition factor and in the locomotor activity parameters and induced an increase brain AChE, liver CAT activity and AST/ALT ratio. On the other hand, the exposure to RM produced a decrease in liver GST, AST/ALT ratio and ALP activity. Finally, some pesticide combinations decrease general condition and liver GST activities, and increase brain GST and liver ALP activities. Different responses in biomarkers were observed in mixtures treatments, reflecting the complex interactions between these toxics and suggesting a suppressive action of RM on CF effects.

Since the concentrations we tested are environmentally relevant and the overall fish health condition was affected, the presence of these pesticides in freshwater systems could impose a risk for populations by causing deleterious effects on *C. decemmaculatus* in Pampean region.

1. Introduction

The Pampean region is an extensive area located in South America covering 750,000 km^2 and including territories of three countries: Argentina, Uruguay and Brasil. The high demographic and agricultural production in this area become the Pampean basins

* Corresponding author. Tel.: +54 351 4332102/03x110–112.
E-mail address: achued@efn.uncor.edu (A.C. Hued).

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into severely affected water bodies (Rodrigues Capítulo et al., 2010). Particularly, in the recent decades the increase of cultivated areas together with the intensive use of pesticides, have greatly contributed to impair the quality of water resources. This impairment affects the structure and function of natural communities, resulting in impacts at different levels of biological organization (molecular, biochemical, physiological, histological, reproductive, etc.) (Grisolia, 2002; van der Oost et al., 2003). Therefore, it is important to study the sensitivity of non-target species such as fish.

The ten spotted livebearer fish, *Cnesterodon decemmaculatus* (Poeciliidae, Cyprinodontiformes) is a small size native fish whose distribution pattern corresponds entirely to the full extension of the Pampean region (Liotta, 2005; Lucinda, 2005). Since *C. decemmaculatus* can be found in pristine as well as in severely degraded habitats, it has been used as tolerant species in a biotic index in order to evaluate the water quality (Hued and Bistoni, 2005; Maggioni et al., 2012). This fish has also been used as a laboratory model to evaluate the effect of toxic substances through the application of different biomarkers under acute toxicity test (Menéndez-Helman et al., 2012; Mastrángelo and Ferrari, 2013; Vera-Candioti et al., 2013). However, under natural environmental conditions, *C. decemmaculatus* is exposed to chronic and sublethal concentrations of a wide variety of natural and anthropogenic compounds. In order to approach to the real field situation, emerge as alternatives the long term bioassays using sublethal concentrations of pesticides and their mixtures.

Nowadays, one of the most widely agrochemical used is glyphosate (N-[phosphonomethyl] glycine), a broad-spectrum, non-selective, postemergent herbicide. In the Pampean region, as in other parts of the world, where the highest productivity corresponds to genetically modified glyphosate-resistant soybean, raises concerns about the effects that this herbicide could lead to non-target organisms. This becomes important when considering the levels of glyphosate registered in water of Pampean rivers (0.1–0.7 mg/l) (Peruzzo et al., 2008). Furthermore, Annett et al. (2014) mentioned these values as one of the highest concentrations registered in freshwater systems.

Another pesticide commonly applied during the soybean cycle is chlorpyrifos (O,O-dimethyl-O-[3,5,6-trichloro-2-pyridinyl] phosphorothioate) (Micucci and Taboada, 2006). It is a broad-spectrum organophosphate insecticide, registered in streams of the Pampean region at concentrations up to 10 μg/l (Marino and Ronco, 2005; Aparicio et al., 2013).

Commercial formulations of glyphosate and chlorpyrifos are applied together with other insecticides and fungicides, in order to assure the profitability of the soybean crop (Pazos, 2008). They could reach water bodies by surface runoff on cultivated land. Thus, it is extremely important to consider not only the toxicity of each particular pesticide but also the effects of their mixture on native biota.

In this sense, biomarkers measured in organisms exposed to such conditions, arise as tools to understand how an organism responds to a given toxic situation in both natural and control laboratory conditions. A multibiomarker approach including different levels of biological organization gives an integral view of deleterious effects on native fish such as *C. decemmaculatus*. Many ecologically relevant fish behaviors could be easily quantified and have been proposed as indicators of toxicity in environmental pollution monitoring (Scott and Sloman, 2004; Kavitha and Venkateswara Rao, 2007). Therefore, disruption of the normal behavioral patterns could indicate detrimental alterations with significant consequences for survival of the individual and at last for the fish species populations. These alterations could also be related to an impairment of the general health condition of fish, thereby the assessment of somatic biomarkers gives a first approach to evaluate the effects of xenobiotics on organisms. Among these biomarkers, the condition factor (K) and the hepatosomatic index (HSI) can be used in both field and laboratory assays (de la Torre et al., 2005; Menéndez-Helman et al., 2015, 2012).

At the present, several enzymes related to biotransformation and detoxification of xenobiotics, are currently used in ecotoxicological research (Bebe and Panemangalore, 2003; Ozcan Oruc et al., 2004). Catalase (CAT) and glutathione-S-transferase (GST) are among the most used antioxidant and biotransformation enzymes, respectively (Di Giulio and Hinton, 2008). The activity of them can be increased or inhibited under conditions of chemical stress and these responses are dependent on the intensity and duration of exposure, as well as on the susceptibility of the different species.

Among others useful enzymatic biomarkers, the hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST), catalyze the interconversion of amino acids and α-ketoacids by transfer of amino groups (van der Oost et al., 2003), an essential function in the synthesis or degradation of proteins. Both enzymes are in great amounts in hepatocites, due to their metabolic complexity. When hepatic damages occur, they can leakage in to the plasma, and then they could be measured to determine such damages (Dufour, 2010). On the other hand, ALP is an enzyme that release inorganic phosphate from an organic phosphate ester with the concomitant production of an alcohol (Bishop et al., 2010). In normal conditions ALP shows a minimum activity in liver tissue.

According to the increasing environmental problems of the Pampean region related to the intensive use of pesticides in agriculture areas, the main goal of our work was to evaluate the use of a native fish species, *C. decemmaculatus* as indicator of the effects of the commercial formulations of glyphosate, chlorpyrifos, and their mixture. Through our study we proposed a set of biomarkers at different biological levels to be use in field biomonitoring studies.

2. Materials and methods

2.1. Bioindicators

Adult females of *C. decemmaculatus* were used for the experiments based on their suitability for laboratory studies. Individuals were collected using a dip net of 1 mm mesh size from a site on Yuspe River (64°83′20″ W; 31°81′70″ S) (Córdoba, Argentina). This site has been used as a reference location for fish collection according to previous water quality assessment carried out by Hued and Bistoni (2005).

Fish were transported to the laboratory in water tanks (20-L) and acclimated for 15 days before the experiments. They were maintained in a 120 L aerated glass aquarium containing dechlorinated tap water in a temperature controlled room at 21 ± 1 °C and under a light-to-dark cycle of 12 h:12 h. During the acclimatization period fish were fed twice a day with commercial fish food (Tetra Min®) and chironomidae larvae (Tetra Bloodworms®).

2.2. Chemicals

Toxicity tests were performed to evaluate the toxicity of commercial chlorpyrifos based insecticide and glyphosate based herbicide on *C. decemmaculatus*. Commercial chlorpyrifos formulation was Clorfox® (CF) (Gleba, SA, Argentina) which contains 48% of the active ingredient (0,0-diethyl 0-[3,5,6-trichloro-2-pyridinyl] phosphorothioate) and 52% as surfactants. Commercial glyphosate formulation was Roundup Max® (RM) (Monsanto, Argentina) which contains 67.9% of N-(phosphonomethyl) glycine as the active ingredient and 32.1% as surfactants. To perform the bioassay, a stock solution of RM dissolved in distilled water was prepared due to the hydrophilic nature of glyphosate, ($\log K_{ow} = -3.2$ to -2.8) (CCME, 2012). The stock solution of CF was made in acetone, because of the

Table 1
Pesticide concentration in exposure media at 0 h and 24 h (T0 and T24) ($n = 9$). The values are expressed as means ± deviation.

Pesticide value	Chlorpyrifos (μg/l)	
	T0	T24
Control	<DL	<DL
0.00084 μl/l of Clorfox	0.196 ± 0.020	<DL
0.0084 μl/l of Clorfox	1.022 ± 0.031	0.135 ± 0.004
Pesticide value	**Glyphosate (mg/l)**	
	T0	T24
Control	<DL	<DL
0.2 mg/l of Roundup Max	0.303 ± 0.005	0.203 ± 0.041
2 mg/l of Roundup Max	1.613 ± 0.033	1.332 ± 0.097

moderate hydrophilic nature of chlorpyrifos, ($\log K_{ow} = 3.31–5.27$) (CCME, 2008), and more diluted solutions were prepared with distilled water for daily changes of water.

The concentrations of chlorpyrifos and glyphosate were measured in the aquarium water at 0 and 24 h by triplicate, at second, fourth and sixth week of the bioassessment (before water replacement). For glyphosate determination by high performance liquid chromatography, water samples were derivatized with p-toluenesulfonyl chloride under alkaline conditions and an aliquot of the reaction mixture was injected into RP 18 column (5 μm particle size, length × I.D: 25 cm × 4.6 mm, Supelco) with a mobile phase consisting of 0.2 M phosphate buffer (pH 2.30) acetonitrile (85:15, v/v) and detection at 240 nm (Kawai et al., 1991). Chlorpyrifos was extracted from water samples by solid phase extraction as described by Bonansea et al. (2013). The extracts were analyzed by an Agilent 6890 gas chromatograph (Santa Clara, CA, USA) equipped with a microelectron capture detector and a Varian VF-5 ms 30 m × 0.25 mm × 0.25 μm capillary column (Palo Alto, CA, USA) to separate and identify the pesticide residue (Maggioni et al., 2012). The quantification of both pesticides was performed using external standards (Sigma–Aldrich). Recoveries of the complete analytical technique were obtained by the laboratory fortified sample method reaching 98%. The obtained limits of the detection and quantification in aquarium water were 0.03 and 0.1 mg/l for glyphosate and 0.001 and 0.002 μg/l for chlorpyrifos, respectively .

2.3. Bioassays

Fish were exposed for 42 days to the following nominal concentrations: 0 μl CF/l + 0 mg RM/l (C = control group), 0.00084 and 0.0084 μl/l of Clorfox (CF) (T1 and T2), 0.2 and 2 mg/l of Roundup Max (RM) (T3 and T4) and the mixture of these concentrations as follows: 0.00084 μl CF/l + 0.2 mg RM/l; 0.00084 μl CF/l + 2 mg RM/l; 0.0084 μl CF/l + 0.2 mg RM/l and 0.0084 μl CF/l + 2 mg RM/l (T5, T6, T7 and T8, respectively). All the concentrations used were environmentally relevant.

Ten individuals per treatment were randomly assigned in 5-L aerated glass aquaria. All the bioassay was performed by triplicate. Individuals were fed with 2.4 mg per fish twice a day along the experiment with commercial fish food. Water of each aquarium was completely renewed every 24 h. The wastewaters generated from bioassays were dismissed by activated carbon filtration.

2.4. Locomotor activity

After the exposure period, each fish was transferred individually into a recording aquarium (30 cm width × 9 cm depth × 25 cm height), containing 4 l of dechlorinated tap water. Two minutes after the fish was introduced into the tank, the locomotor activity of each fish was continuously registered along 8 min. In order to assess if each pesticides and their mixture affect the locomotor activity, it was determined the average speed (m/s) and distance traveled (m) of each individual. These parameters were obtained at the end of each trial from video films, through the video-tracking software (ANY-Maze® Stoelting Co, USA). For the analysis of these behavioral parameters the total recording time was divided in four segments of 120 s in order to take into account the behavioral variability across the time.

2.5. Somatic indexes

After the locomotor activity assessment each individual was killed by severing the spinal cord behind the opercula and dissected. Body weight (g) and standard length (mm) were determined in order to estimate the Fulton condition factor (K). Liver, gills, muscle and brain from individuals of each treatment and control group were removed. Each liver was weighted to calculate the hepatosomatic index (HSI) (Goede and Barton, 1990).

2.6. Acetylcholinesterase, catalase and glutathione-S-transferase activities

Enzyme extracts from each organ were prepared from individual fish according to Cazenave et al. (2006). Briefly, organs were homogenized in 0.1 M potassium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (DTE) using a glass homogenizer (Potter Elvehjem), affording a tissue weight of ca. 10% per volume. The samples were centrifuged at 6900 × g and 4 °C for 10 min to separate cell debris and the supernatant, using refrigerated centrifuge. Acetylcholinesterase activity (AChE) was measured only in brain and muscle homogenates at 412 nm according to Ellman et al. (1961) using acetylthiocholine iodide as substrate and dithiobisnitrobenzoic acid (DTNB). Catalase activity (CAT) was determined according to Beutler (1982) at 240 nm using H_2O_2 as substrate. The activity of glutathione-S-transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at 340 nm, according to Habig et al. (1974). All the determinations were carried out by triplicate using a multi-plate reader Biotek®, equipped with a multiple cell holder and temperature control. The enzymatic activity was calculated in terms of the protein content of the sample (Bradford, 1976), and is reported in mU (mg prot)$^{-1}$. Each enzymatic assay was carried out by triplicate.

2.7. Transaminases and alkaline phosphatase

Liver from each individual fish was homogenized in phosphate buffer (pH 7.4). The homogenate was centrifuged at 15,000 × g at 4 °C for 10 min, supernatant collected, and stored at −80 °C for enzymatic studies. Aspartate aminotransferase (AST) (L-Aspartate-2-oxaloglutarate aminotransferase) and alanine aminotransferase (ALT) (L-Alanine-2-oxaloglutarate aminotransferase) activities were estimated according to Reitman and Frankel (1957). The reaction mixture contained 2 mmol l^{-1} of α-ketoglutarate and AST and ALT specific substrates (100 and 200 mmol l^{-1} of aspartate and alanine l-1, respectively), in buffer phosphate (100 mM pH 7.4). The reaction was started by adding aliquots of the supernatant; after 30 min of incubation, the 2,4-dinitrophenylhydrazine reagent was added and the colored product was measured spectrophotometrically at 505 nm. Alkaline phosphatase (ALP) orthophosphoric monoester phosphohydrolase activity was determined colorimetrically using a commercial kit (Wiener Lab) (Bacchetta et al., 2011). The enzymatic activity was calculated in terms of the protein content of the sample (Bradford, 1976), and is reported in mU (mg prot)$^{-1}$.

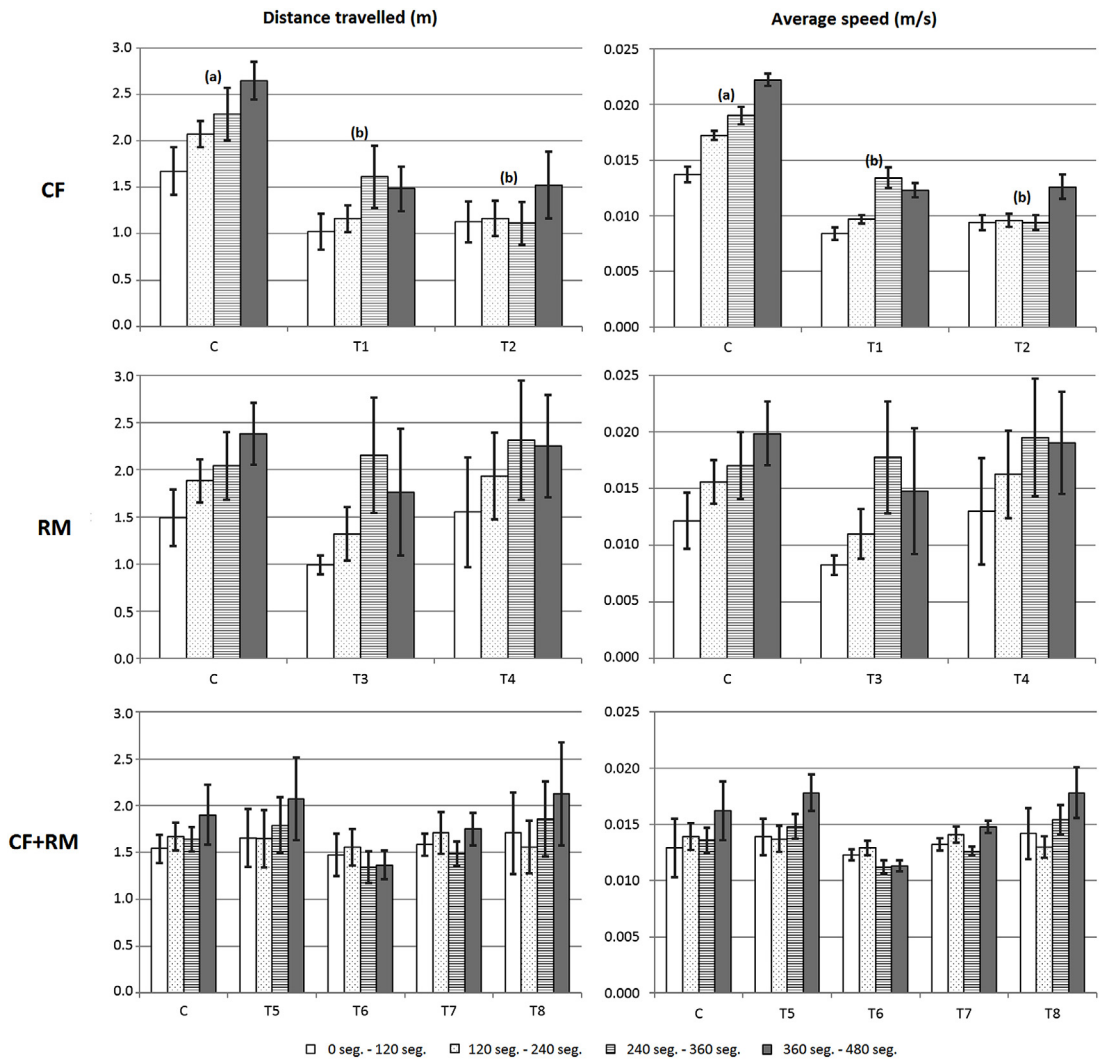

Fig. 1. Variables of locomotor activity recorded in *C. decemmaculatus* exposed to CF, RM and their mixtures ($n = 30$). References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084 μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 – 0.0084 μl CF/l + 0.2 mg RM/l. T8 – 0.0084 μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences ($p < 0.05$).

2.8. Statistical analysis

Data distributions were analyzed using the Shapiro–Wilks index (Sokal and Rohlf, 1999). To compare the biological parameters among different treatments, Kruskal–Wallis test (Sokal and Rohlf, 1999) was performed followed by a Dunn's multiple comparison test. Locomotor activity was analyzed through repeated-measures analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. Statistical analyses were performed using Infostat Software Package (Infostat, 2014).

3. Results

3.1. Concentrations of chlorpyrifos and glyphosate in water

Chlorpyrifos and glyphosate levels were below the detection and quantification limit (LOD) in the control groups at 0 and 24 h. Concentrations of chlorpyrifos showed a drop of 100% and 87% at 24 h, for the lowest and the highest concentrations, respectively (Table 1). On the other hand, in the RM treatments, glyphosate levels drop 33% and 18% at 24 h of exposure, for the lowest and the highest concentrations, respectively (Table 1).

3.2. Locomotor activity

The behavioral parameters significantly differed across time showing the same variation pattern at both control and treatment groups. No interactions between time and treatments were registered. Distance traveled and the average speed, were significantly reduced in those individuals exposed to 0.00084 and 0.0084 μl/l of CF, while RM and the mixtures of pesticides did not affect the locomotor activity of *C. decemmaculatus* (Fig. 1).

3.3. Somatic indexes

After the chronic exposure to pesticides, the Fulton Condition Factor (K) showed a significant decrease at the highest concentration of CF (0.0084 μl/l). On the other hand, this index did not present significant differences among RM concentrations. In the pesticide mixtures only when both pesticides were in the high concentration the K was reduced. The HSI did not differ between treatments and control group (Table 2).

3.4. Acetylcholinesterase activity

The brain AChE activity was significantly increased in those individuals exposed to both concentrations of CF (0.00084 and

Table 2
Fulton condition factor (*K*) and hepatic somatic index (HSI) of *C. decemmaculatus* after exposure to CF, RM and their mixtures ($n = 30$).

Pesticides	Treatment	*K*	HSI
CF	C	1.60 ± 0.03[a]	1.38 ± 0.07
	T1	1.59 ± 0.04[a]	1.39 ± 0.10
	T2	1.48 ± 0.04[b]	1.30 ± 0.09
RM	C	1.60 ± 0.03	1.38 ± 0.07
	T3	1.54 ± 0.06	1.40 ± 0.11
	T4	1.53 ± 0.03	1.10 ± 0.08
CF + RM	C	1.60 ± 0.03[a]	1.38 ± 0.07
	T5	1.55 ± 0.05[a,b]	1.26 ± 0.15
	T6	1.66 ± 0.07[a]	1.21 ± 0.08
	T7	1.58 ± 0.09[a,b]	1.37 ± 0.13
	T8	1.41 ± 0.04[b]	0.96 ± 0.12

References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 – 0.0084 μl CF/l + 0.2 mg RM/l. T8 – 0.0084 μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences ($p < 0.05$).

Table 3
Acetylcholinesterase activity (mU/mg prot) in brain and muscle of *C. decemmaculatus* exposed to CF, RM and their mixtures ($n = 15$).

Pesticides	Treatment	AChE	
		Brain	Muscle
CF	C	1703.6 ± 471.5[a]	124.2 ± 13.8
	T1	6575.8 ± 1195.6[b]	112.2 ± 29.4
	T2	6541.7 ± 1259.7[b]	168.0 ± 39.0
RM	C	1973.6 ± 535.7	97.2 ± 9.6
	T3	3063.5 ± 667.1	127.8 ± 27.6
	T4	2765.4 ± 493.1	113.4 ± 16.8
CF + RM	C	1750.4 ± 385.1	123.6 ± 16.2
	T5	1610.1 ± 225.0	136.8 ± 18.0
	T6	2517.1 ± 562.7	58.8 ± 4.8
	T7	2499.1 ± 365.9	113.4 ± 34.8
	T8	1736.1 ± 16.8	168.0 ± 20.4

References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084 μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 – 0.0084 μl CF/l + 0.2 mg RM/l. T8 – 0.0084 μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences ($p < 0.05$).

0.0084 μl/l), whereas the exposure at RM and the mixtures of both pesticides did not affect the AChE activity of *C. decemmaculatus*. On the other hand, none of the treatments significantly affect the muscle AChE activity (Table 3).

3.5. Antioxidant enzyme activity

Fish exposed to CF showed a significant concentration-dependent increase of CAT activity in liver at 0.00084 and 0.0084 μl CF/l respect to the control group (Table 4). The exposure to RM and to the mixture of both pesticides did not cause changes in the CAT activity in liver and muscle. Finally, the CAT activity in gills and brain could not be estimated because it was always below the detection limits of the method.

The GST activity was not affected by CF for all the studied organs, although it could be observed a trend to an activity decrease in liver and an activity increase in gills (Table 5). In fish exposed to RM alone there was a significant inhibition of GST in liver respect to the control group showing the highest inhibition at 0.2 mg RM/l, whereas a trend to an activity decrease in brain was observed (Table 5). In gills and muscle the GST activity did not show significant changes. On the other hand, fish exposed to 0.00084 μl CF/l + 0.2 mg RM/l and 0.0084 μl CF/l + 2 mg RM/l showed a GST inhibition in liver respect to the control group. Moreover, in brain, the mixture of 0.0084 μl CF/l + 0.2 mg RM/l caused a significant increase of GST

Table 4
Catalase activity (mU/mg prot) in liver and muscle of *C. decemmaculatus* exposed to CF, RM and their mixtures ($n = 6$).

Pesticides	Treatment	Catalase	
		Liver	Muscle
CF	C	11,332.3 ± 1006.7[a]	479.3 ± 39.4
	T1	14,039.6 ± 2042.6[b]	467.8 ± 80.9
	T2	21,970.8 ± 2241.4[c]	483.8 ± 69.8
RM	C	4659.0 ± 298.1	387.6 ± 72.1
	T3	3723.8 ± 501.1	524.7 ± 379.0
	T4	5500.3 ± 4838.4	266.8 ± 49.1
CF + RM	C	12,993.0 ± 946.3	225.1 ± 24.5
	T5	19,820.1 ± 1202.9	371.7 ± 57.3
	T6	10,761.6 ± 2921.8	293.5 ± 64.9
	T7	33,352.4 ± 8711.5	277.0 ± 39.2
	T8	25,959.2 ± 6612.7	244.0 ± 25.9

References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084 μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 – 0.0084 μl CF/l + 0.2 mg RM/l. T8 – 0.0084 μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$).

Table 5
Glutathione-S-transferase activity (mU/mg prot) in brain, gills, liver and muscle of *C. decemmaculatus* exposed to CF, RM and their mixtures ($n = 6$).

Treatment		Glutathione-S-transferase			
		Brain	Gills	Liver	Muscle
CF	C	450.7 ± 57.6	248.6 ± 26.1	1377.7 ± 181.9	59.3 ± 5.2
	T1	597.9 ± 49.8	337.1 ± 25.6	1093.0 ± 287.5	89.4 ± 10.0
	T2	487.2 ± 57.3	346.0 ± 58.0	987.2 ± 136.7	71.2 ± 10.0
RM	C	421.6 ± 62.3	445.0 ± 48.9	911.0 ± 55.8[c]	26.9 ± 2.0
	T3	480.9 ± 71.0	375.6 ± 51.9	381.9 ± 40.8[a]	22.8 ± 2.9
	T4	263.9 ± 68.1	416.2 ± 63.7	635.0 ± 162.4[b]	19.9 ± 2.1
CF + RM	C	275.3 ± 40.9[a,b]	351.1 ± 40.9	844.2 ± 30.9[b,c]	25.4 ± 1.2
	T5	225.0 ± 46.3[a,b]	341.4 ± 46.3	640.5 ± 42.0[a]	35.1 ± 4.6
	T6	374.7 ± 78.8[b,c]	274.8 ± 78.8	713.0 ± 82.9[a,b]	33.0 ± 2.9
	T7	446.0 ± 59.8[c]	327.4 ± 59.8	1275.5 ± 150.0[c]	26.4 ± 1.7
	T8	196.4 ± 17.7[a]	363.0 ± 17.7	667.5 ± 153.3[a]	31.6 ± 8.7

References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084 μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 – 0.0084 μl CF/l + 0.2 mg RM/l. T8 – 0.0084 μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences ($p < 0.05$).

activity respect to the control group whereas this enzyme did not show significantly changes in gills and muscle (Table 5).

3.6. Transaminases and alkaline phosphatase

No significant differences were registered in AST and ALT activity exposed to CF. Conversely, the AST/ALT ratio increased at the highest concentration of CF (Fig. 2). Although the ALP activity did not show significantly differences, it was observed a tendency to increase in those individuals exposed to the highest concentration of CF (Fig. 3).

Although RM exposure did not significantly affect the AST and ALT activity, it was observed that the lowest AST activity corresponds to the lowest RM concentration, whereas the ALT activity tended to increase at the highest RM concentration. On the other hand, the AST/ALT ratio decreased significantly at both treatments respect to the control group (Fig. 2), whereas the ALP activity presented significant differences, showing the lowest value at the lowest RM concentration (Fig. 3).

The pesticide mixtures did not affect the AST and ALT activities and the AST/ALT ratio (Fig. 2). A significant increase in ALP activity was only registered in those individuals exposed to 0.00084 μl CF/l + 0.2 mg RM/l and 0.0084 μl CF/l + 2 mg RM/l (Fig. 3).

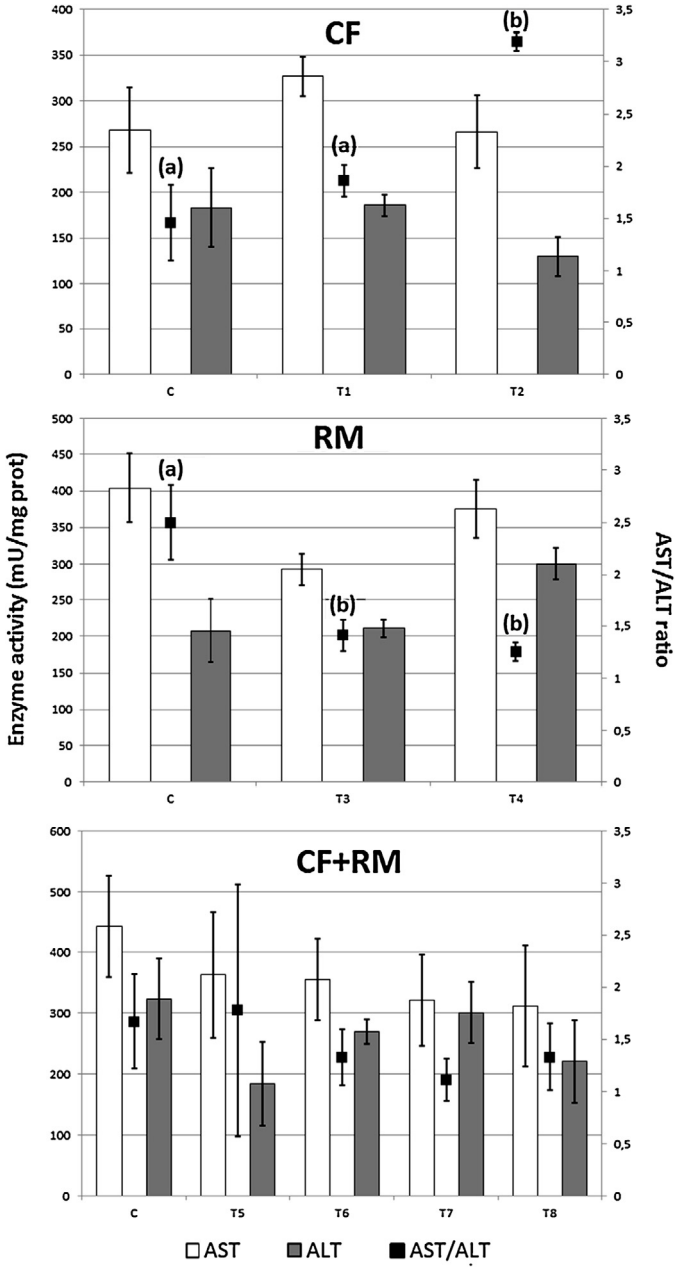

Fig. 2. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) (mU/mg prot) in liver (left vertical axis) and AST/ALT quotient (right vertical axis) in *C. decemmaculatus* exposed to CF, RM and their mixtures ($n = 6$). References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084 μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 – 0.0084 μl CF/l + 0.2 mg RM/l. T8 – μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences ($p < 0.05$).

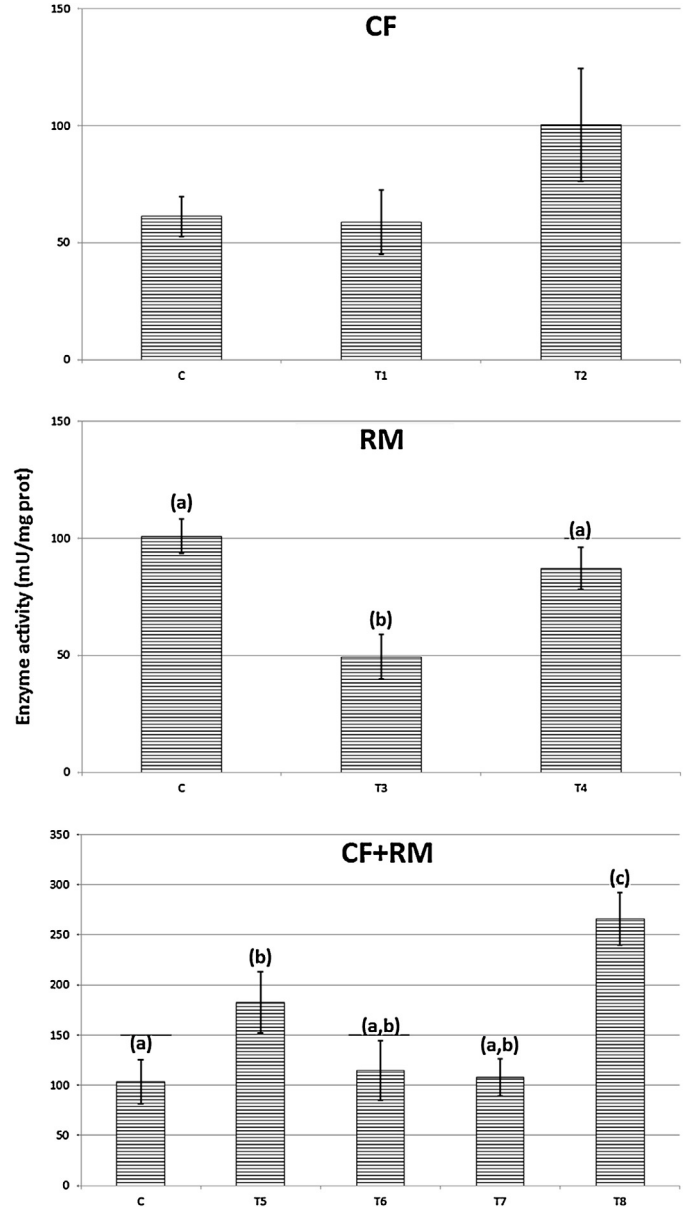

Fig. 3. Phosphatase alkaline (ALP) activity (mU/mg prot) in liver in *C. decemmaculatus* exposed to CF, RM and their mixtures ($n = 6$). References: C – control group. T1 – 0.00084 μl CF/l. T2 – 0.0084 μl CF/l. T3 – 0.2 mg RM/l. T4 – 2 mg RM/l. T5 – 0.00084 μl CF/l + 0.2 mg RM/l. T6 – 0.00084 μl CF/l + 2 mg RM/l. T7 –0.0084 μl CF/l + 0.2 mg RM/l. T8 – 0.0084 μl CF/l + 2 mg RM/l. The values are expressed as means ± standard error. Different letters indicate significant differences ($p < 0.05$).

4. Discussion

Since the distribution pattern of *C. decemmaculatus* covers the entire Pampean region, our results are significant because they are exposed to commercial formulations of chlorpyrifos and glyphosate in their own environment. Through a set of biomarkers at different biological organization we demonstrated the toxicity of Clorfox® and Roundup Max®, and their mixtures at environmentally relevant concentrations (Marino and Ronco, 2005; Peruzzo et al., 2008).

In toxicological studies, behavior is becoming a prominent tool in a wide range of organisms (Kavitha and Venkateswara Rao, 2007). Fish locomotor activity is a simple important tool to estimate deleterious effects of xenobiotics due to their importance on survival. Treatment with CF alone decreased the locomotor activity of *C. decemmaculatus* respect to the control group. Similar results were obtained for juvenile *Oncorhynchus kisutch* exposed to 0.6–2.5 μg/l of chlorpyrifos, after 96 h of exposure (Sandahl et al., 2005), and for *Gambusia affinis* exposed to 60 μg/l during 20 days (Venkateswara Rao et al., 2005). Despite *G. affinis* and *C. decemmaculatus* belongs to the same family, Poeciliidae, they present different LC50 (297 and 105.3 μg/l of chlorpyrifos, respectively) (Deb and Das, 2013; Scalise et al., 2014). This comparison suggests a higher sensitiveness of *C. decemmaculatus*. On the other hand, in our study, individuals exposed to RM only and to the mixture, showed no significant changes in locomotor activity respect to the control group. This suggests that RM decreases the negative effect of CF on locomotor activity.

In our study the condition factor (*K*) was reduced in individuals exposed to the high concentration of CF and to the combination of the high concentrations of both agrochemicals. Variations in *K* have been registered in different studies showing decreasing values under degraded water quality (Foster et al., 2001; Khallaf et al., 2003). Since the exposure to the highest CF concentration affects the general fish health condition whereas RM did not show any effect, the significant *K* reduction in the mixture of the high concentrations of both agrochemicals correspond mainly to the specific effects of CF. In our laboratory tests under controlled dietary conditions, food supply for all exposed organisms was the same, thus the condition factor reflects the adverse conditions imposed by the toxic substances.

Among the somatic indices, the hepatosomatic index (HSI) is a more specific biomarker than condition factor, since it is directly related to the toxic effects on liver. In our study the HSI was not sensitive enough to show differences between the treatments tested. A decrease in this index could indicate a depletion of glycogen reserves due to the energy expenditure to face the toxic event, whereas an increase could reflect pathological changes such as hypertrophy and/or hyperplasia of hepatic cells (Goede and Barton, 1990). Since the loss of glycogen involves a loss of liver weight and events of hyperplasia and hypertrophy lead to a weight increase of this organ, both processes acting together could cause the absence of changes in HSI as we registered from our work.

The two pesticides used in our experiments have been widely recognized as inhibitors of acetylcholinesterase activity in both brain and muscle (Glusczak et al., 2007; Modesto and Martinez, 2010; Sandahl et al., 2005; Xing et al., 2010). According to this, an inhibitory response of AChE from individuals exposed to CF and RM was expected. Our results demonstrated that muscle AChE activity did not change for any treatment compared to the control group. However, the brain AChE activity increased only in those individual exposed to CF alone. In order to explain this enhancement we suggest that fish brain may respond in a compensatory manner by increasing the AChE synthesis to cope with the inhibition caused by the CF. This response could be due to the acetylcholine accumulation in the synaptic cleft and the need to neutralize the neurotransmitter. This argument is consistent with our unpublished studies where individuals of *C. decemmaculatus* exposed to the same concentrations of CF during 48 h, showed significant brain inhibition of AChE. Therefore, *C. decemmaculatus*, after 42 days of CF exposure, shows an adaptive and compensatory response to the inhibition observed at 48 h. Hernández-Moreno et al. (2010) registered a progressive AChE inhibition in *Tinca tinca* exposed to 100 μg/l of carbofuran during 30 days; after this period there was a recovery of the AChE activity despite the exposure continued until 60 days. Although several studies have pointed out the AChE inhibitory effects of glyphosate commercial formulations and to the active principle under 96 h of exposure (Glusczak et al., 2007; Menéndez-Helman et al., 2012; Modesto and Martinez, 2010; Sandrini et al., 2013), our results showed that RM did not affect the brain AChE activity after 42 days. These findings are in agreement with the studies carried out by Rendón-von Osten et al. (2005) in *Gambusia yucatana* exposed to 32 mg/l and Harayashiki et al. (2013) in *Poecilia vivipara* exposed to 0.7 mg/l of glyphosate but only for 96 h. On the other hand, the absence of inhibition or activity increase for all the pesticides mixture respect to the control group suggests an interaction between CF and RM. Rendón-von Osten et al. (2005) found that a commercial formulation of glyphosate decreases the AChE inhibitory activity of chlorpyrifos in *Gambusia yucatana* after 96 h of exposure to the pesticide mixtures. Although we did not expose *C. decemmaculatus* for 48 h to the mixtures, we propose that CF in the presence of RM would not cause the inhibition of AChE. Thus, after 42 days of exposure individuals do not show the compensatory response due to the herbicide suppress the CF inhibitory effects at the first days of the exposure.

Antioxidant enzymes and non-enzymatic systems are essential for the conversion of reactive oxygen species into harmless metabolites and to protect and restore the normal metabolism and cell function (Bebe and Panemangalore, 2003). There is not a general rule that an increase in the concentration of a xenobiotic could induce enzyme activity (Cheung et al., 2001). In the present study, the enzymatic activity showed different responses according to the organ analyzed. The same results have been well documented in the literature (Ballesteros et al., 2009; Lushchak et al., 2009).

Catalase (CAT) is an enzyme located in the cell peroxisomes and converts the hydrogen peroxide into oxygen and water. In the present work, CAT activity was only recorded in liver and muscle of *C. decemmaculatus* at all treatments, even at the control group. Similar results were obtained by Ballesteros et al. (2009) where CAT activity was only registered in the liver of *Jenynsia multidentata*. These authors pointed out that the activity of this enzyme is highest in liver compared with other organs because it is the most important detoxifying organ. The CF exposure significantly affected the CAT activity in liver showing an increase in individuals from both CF treatments. Sharbidre et al. (2011), found the same trend in individuals of *Poecilia reticulata* exposed to chlorpyrifos concentrations ranging between 0.044 and 0.017 mg/l during 96 h. These authors postulated that the increase activity of the enzymes of the antioxidant system, such as CAT, indicates an adaptation of individuals to oxidizing environment, generated by the presence of the pesticide and the efficiency to cope with the negative effects of hydrogen peroxide (Li et al., 2007). On the other hand, RM did not cause significant changes in CAT activity, which agrees with the findings of De Menezes et al. (2011) who did not observe changes in *Rhamdia quelen* exposed to 10 mg/l of Roundup during 8 days. As it was registered for AChE and locomotor activity, none of the pesticide mixtures affected the CAT activity, demonstrating again the suppressive action of glyphosate on chlorpyrifos effects.

One of the most used biomarkers of the biotransformation system is the glutathione-S-transferease (GST). It is involved in phase II of xenobiotic detoxification, adding a glutathione molecule to the products from the phase I mediated by Cytochrome P450. The final result is a more hydrophilic product for subsequent excretion (Winston and Di Giulio, 1991). This enzyme showed no changes in any organ of the fish exposed to CF although a trend toward inhibition of activity in liver was registered. Similar results were obtained by Botté et al. (2012) where the activity of GST in individuals of *Acanthochromis polyacanthus* exposed to concentrations of chlorpyrifos ranging between 1 and 100 μg/l for 96 h remained unchanged. These authors postulate that GST as well as NADPH quinone reductase (NQO1) share, in the promoter region of the gen, an antioxidant response element therefore, chlorpyrifos detoxification could not be entirely carried out via GST but also by NQO1. A similar explanation could be attributed to the lack of changes in GST activity in muscle and gills of fish exposed to all treatments. In fish exposed to RM, there was a significant inhibition as well as increase to the lowest and highest concentrations respectively.

As it is well known liver is the primary organ for detoxification of xenobiotics in the organisms and therefore it can react at the exposure to different kind of contaminants. The induction of GST could be beneficial for the excretion of contaminants from the organism (De Menezes et al., 2011) as well as an inhibition could lead to more serious damage and the accumulation in the tissues. In the literature there is a variable response of GST activity to glyphosate. De Menezes et al. (2011) found inhibition of GST activity in liver of individuals of *Rhamdia quelen* exposed to 0.95 mg/l of Roundup

during 8 days. On the contrary, Modesto and Martinez (2010) found an increase of this enzyme in liver of *Prochilodus lineatus* exposed to 10 mg/l of this herbicide during 96 h. In our study, when fish were exposed to the mixtures, there was an increase of GST activity in brain at high concentration of CF with low concentration or RM and inhibition in liver at low concentration of both pesticides and high concentration of both pesticides. These results could be more associated to the effects of RM more than CF due to the mentioned above.

The variations alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity could indicate toxic effects at cellular level, allowing the specific identification of the liver tissue damage. An activity increased in liver has been related to compensatory mechanisms of tissue damage (Bhattacharya et al., 2008), or to the energy production in animals under stressful conditions through protein catabolism (De Smet and Blust, 2001; David et al., 2004). On the other hand, an activity decreased of aminotransferases in liver has been related to cytolysis processes and the consequent release of them into the blood stream (Bacchetta et al., 2011; Bhattacharya et al., 2008; de Aguiar et al., 2004; Venkateswara Rao, 2006a). In this sense, in *Cyprinus carpio* exposed to 20 and 40 μg/l of chlorpyrifos for 30 days the activities of serum AST and ALT increased (Banaee et al., 2013) whereas Venkateswara Rao (2006b) demonstrated an inhibition of both liver enzymes in *Oreochromis mossambicus* exposed to 17 μg/l of the organophosphate RPR-II (a pesticide-based in monocrotophos) in 3 to 30-day trials. On the other hand, Jiraungkoorskul et al. (2003) reported an activity increased in the plasmatic AST and ALT of *O. niloticus* exposed to 15 mg/l of glyphosate for 3 months. Although there are evidences that these enzymes could vary in fish exposed to CF and RM, in *C. decemmaculatus* the exposure conditions did not affect these hepatic enzymes. Our results suggest that the 42 days of exposure is enough time to activate mechanisms that allow organisms to adapt to the conditions imposed by the toxic used in the experiments. On the other hand, the AST/ALT ratio is widely used as biomarker in clinical medicine, where both enzymes are estimated in plasma. In humans, this ratio is an unquestionable indicator of liver dysfunction (Domanski and Harrison, 2013; Nyblom et al., 2004). On the other hand, this ratio has been measured in fish liver showing both increases and decreases under different stressful conditions (de la Torre et al., 2007, 2005; De La Torre et al., 2000). According to our results the AST/ALT ratio could establish differences between treatments with each pesticide where the individual activity of each enzyme failed to show such variations. However, the interpretation of this index must be accompanied by the observation of the activity of both enzymes separately and requires further detailed analyses to explain how these variations can occur in fish liver.

Another indicator of liver function alteration is ALP enzyme. In the present work, the ALP activity was a sensitive biomarker which values increased at the low RM concentration and at two pesticides mixture treatments, at low concentrations together and at high concentrations together of the both pesticides. Increases in hepatic ALP activity has been also registered in *Puntius conchonius* (Bhattacharya et al., 2008) and *Prochilodus lineatus* (Loteste et al., 2013) exposed to 0.17–0.68 μM of nonylphenol and to 0.075–0.3 μg/l of cypermethrin, respectively, during 96 h. On the other hand, the organophosphates RPR-II lead to a decrease of ALP hepatic activity of *Oreochromis mossambicus* until 30 days of exposure (Venkateswara Rao, 2006b), whereas exposure to 40 μg/l of chlorpyrifos up to 30 days of exposure caused no changes in the hepatic and plasmatic ALP of *Cyprinus carpio* (Banaee et al., 2013). On the other hand, increases in plasmatic ALP activity in *Oreochromis niloticus* exposed to 15 mg/l of glyphosate for 3 months have also been reported (Jiraungkoorskul et al., 2003).

5. Conclusions

According to our results *C. decemmaculatus* could be used as indicator of environmental relevant concentrations of chlorpyrifos and glyphosate. Exposure to CF alone affected the fish general condition, altered behavioral parameters, and AChE activity, modified the AST/ALT ratio, and activated antioxidant mechanisms. On the other hand, RM alone affected AST/ALT ratio and GST and ALP activities. The mixture of both pesticides produced changes in the fish general condition, GST and ALP activities. The absence of significant changes in behavioral parameters, AChE and antioxidant mechanism suggests a suppressive effect of RM on CF action in the fish exposed to mixtures treatments. Since the identification of appropriate biomarkers from native species of fish related to the toxic effects of pesticides is needed, according to our results the above mentioned biomarkers could become useful tools to assess the effects of these pesticides and their mixtures when they reach freshwater systems.

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