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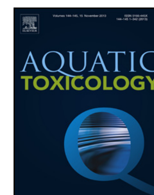

Comparative study of toxicity and biochemical responses induced by sublethal levels of the pesticide azinphosmethyl in two fish species from North-Patagonia, Argentina

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ABSTRACT

Biochemical effects of azinphosmethyl (AZM), an organophosphate pesticide, were determined in gill, brain and muscle tissues of *Odontesthes hatcheri* and *Jenynsia multidentata*. The 96-h toxicity was first assessed, estimating lethal concentrations fifty (LC50) of 7 and 30 $\mu g L^{-1}$ AZM for *O. hatcheri* and *J. multidentata*, respectively. Considering the LC50, sublethal 96-h static exposures were designed for *O. hatcheri* (0.1–0.5 $\mu g L^{-1}$ AZM) and *J. multidentata* (5–10 $\mu g L^{-1}$ AZM) to determine biochemical endpoints. Brain acetylcholinesterase (AchE) was inhibited by AZM in both species, while the buffer enzyme carboxylesterase (CarbE) was not affected in this tissue. Conversely, muscular AchE was not affected but CarbE was augmented by AZM. The enzymes glutathione reductase, glutathione-*S*-transferase and CarbE were significantly inhibited in *O. hatcheri* gills but none of them was affected by AZM in *J. multidentata* gills compared to control. GSH levels were augmented in gills of both species in exposed fish compared to controls and in addition, lipid peroxidation was significantly increased in *O. hatcheri* gills. *Ex vivo* histochemical analysis of ROS by fluorescence microscopy was also performed in *J. multidentata* gills, indicating a significant increase upon exposure to 10 $\mu g L^{-1}$ AZM. Principal component analyses (PCA) were applied, both to the species together or separately. The general analysis demonstrated a clear separation of responses in the two species. For *O. hatcheri*, the variable that explains the major variation in PC1 is gill catalase and brain AchE in PC2. In *J. multidentata* in turn, the variable that explains the major variation in PC1 is brain AchE and total oxyradical scavenging capacity in PC2. The toxicity data and biomarker responses obtained for both species were compared to environmental concentrations of AZM detected in superficial water from different points in the Alto Valle region and risk quotients (RQ) were calculated. This approach indicated probable acute effects for *O. hatcheri* in river and irrigation channels (RQ > 0.1), while the risk was unacceptable in drainage superficial water (RQ > 1). In contrast, *J. multidentata* showed minimal risk in river or channel water (RQ < 0.1) and probable risk in drainage water (RQ = 0.75). We conclude that not only the differential susceptibility of both species to AZM is environmentally relevant, but also that the different biomarkers responding in each case underlie particular pathways stressed by this agrochemical.

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1. Introduction

The Alto Valle of Río Negro and Neuquén in Northern Patagonia is a region of intensive fruit production. Pesticides are widely used to manage the coddling moth *Cydia pomonella* in crops and orchards, being organophosphate (OP) insecticides the main applied. Organophosphates have been detected in superficial and shallow ground water in this fruit-producing region (Loewy et al., 2011). The mechanism of action of OP is based on inhibition of

Abbreviations: ABAP, 2,2′-azobis 2-methylpropionamidine; AchE, acetylcholinesterase; AZM, azinphosmethyl; CarbE, carboxylesterase; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; GR, glutathione reductase; GST, glutathione-S-transferase; OP, organophosphate; PCA, principal component analysis; RQ, risk quotients; TBARS, thiobarbituric acid-reactive substances; TOSC, total oxyradical scavenging capacity; TROLOX, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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the enzyme acetylcholinesterase (AchE; EC 3.1.1.7), while carboxylesterases (CarbE; EC 3.1.1.1) are OP's main detoxification enzymes (Fukuto, 1990). Carboxylesterases are in turn inhibited by OP, becoming alternative targets to protect the organism from AchE inhibition (Jokanović, 2001).

Redox status reflects the dynamic balance between the antioxidant system and pro-oxidants. Exposure to xenobiotics (*i.e.* pesticides) may produce reactive oxygen species (ROS) as a consequence of their metabolism. When ROS overwhelm the cellular antioxidant defense system, they generate oxidative stress. The direct or indirect ROS-mediated damages include peroxidation of membrane fatty acids, DNA base alteration, carbonyl modification of proteins, and loss of sulfhydryl groups leading to enzymes inactivation and/or increased proteolysis. Lipid peroxidation may occur as a consequence of the imbalance between the antioxidant system and the pro-oxidant state generated by pesticide toxicity (Winston and Di Giulio, 1991). It has been reported that lipid peroxidation has a predictive importance as a biomarker for oxidative stress (Lackner, 1998). Endogenous enzymatic and non-enzymatic antioxidants are essential for the conversion of ROS to harmless metabolites as well as to protect and restore normal cellular metabolism and function (Bebe and Panemangalore, 2003). Like other organisms, fish manage elevated levels of ROS with protective ROS-scavenging enzymes such as glutathione reductase (GR; EC 1.8.1.7), catalase (CAT; EC 1.11.1.6), glutathione-*S*-transferase (GST; 2.5.1.18) and non-enzymatic molecules such as reduced glutathione (GSH) (Sharbidre et al., 2011). Glutathione-*S*-transferase also plays an important role in the detoxification of xenobiotics *via* conjugation with GSH. The induction of these enzymes and elevated levels of GSH are beneficial for cellular redox state and provides useful biomarkers of exposure to oxidative stress-inducing chemical contaminants in fish (Van der Oost et al., 2003; Pereira et al., 2013).

Odontesthes hatcheri is an autochthonous fish from Patagonia with relevance from ecological, commercial and sportive aspects. In turn, *Jenynsia multidentata* is recognized as a good model for toxicological studies as an autochthonous South American fish species because it is widely distributed and easy to handle for culture and maintenance in the laboratory (Ballesteros et al., 2011). The aim of this work was to compare the toxicity and biochemical effects of the OP azinphosmethyl (AZM) in these two fish species inhabiting North Patagonia. We have analyzed the main targets as well as detoxifying and antioxidant activities in brain, muscle and gills at sublethal concentrations of the toxicant, to assess their relevance from an environmental point of view and as ecotoxicological bioindicators.

2. Materials and methods

2.1. Animals

The approval for collection of the specimens at the different places, animal treatment, health care and disclaimer for possible environmental impacts was requested to the Application Authority for Law of Fauna in Neuquén Province, through the management of the Center of Applied Ecology of Neuquén.

2.1.1. Odontesthes hatcheri

Juvenile *O. hatcheri* fish were obtained from a small pond connected to Piedra del Aguila reservoir, Limay river, Neuquén (40°16′36″S, 70°39′36″W) and transported to the aquaculture of the Center of Applied Ecology of Neuquén (CEAN), Junín de los Andes. Fish were acclimated in 100-L artificial ponds constantly receiving filtered water from Chimehuin River, with a mean temperature of 8.0 °C. Fish were daily fed 1% of their body weight with formulated food adapted at CEAN to the Patagonian silverside (Hualde et al., 2011). Fish were maintained with a natural photoperiod of 10.5 h L/13.5 h D, corresponding to autumn season at Junín de los Andes.

2.1.2. Jenynsia multidentata

Adult females of the freshwater fish *J. multidentata* were obtained from Pellegrini Lake, Río Negro (38°40′S, 68°00′W) and transported to the laboratory for acclimation in 20-L storage tanks with filtered, dechlorinated, and constantly aerated tap water (mean temperature of 24 °C, photoperiod 12 h L/12 h D). Fish were daily fed with food for cold freshwater fish.

2.2. Toxicity tests

All the toxicity tests were performed in static conditions up to 96 h, with a unique application of AZM in acetone as vehicle at the beginning of the experiments. A high purity-certified standard of azinphosmethyl (98.3% AZM, *S*-(3,4-dihydro-4-oxobenzo[d]-[1,2,3]-triazin-3-ylmethyl) *O*,*O*-dimethyl phosphordithioate, Chem Service Inc. West Chester, PA, USA) was first dissolved in acetone to prepare a stock standard solution that was then diluted in water to the desired concentrations. The exact concentration of AZM in the standard solution was checked by capillary gas chromatography and N-P detection. Final concentration of acetone was kept at 0.05% in all the treatments. Control acetone treatment was included, verifying no toxic effects. Feeding was omitted 24 h prior to and during the exposure period of all toxicity tests.

2.2.1. Acute toxicity tests

Ten fish (approximately 0.8 g total body mass per liter) were housed in each aquarium with a 12 h L/12 h D photoperiod and continuous aeration. The mortality was monitored daily up to 96 h according to the Ecological Effects Test Guidelines (USEPA, 1996). For *O. hatcheri*, AZM concentrations of 0.0, 0.1, 0.3, 1.0, 3.0 and 10 $\mu g\,L^{-1}$ were prepared in aquaria filled with filtered water from Chimehuin River containing 0.3 $g\,L^{-1}$ NaCl, at 20 °C. For *J. multidentata*, AZM concentrations of 0, 5, 10, 15, 25 and 50 $\mu g\,L^{-1}$ were prepared in aquaria filled with dechlorinated tap water at 24 °C.

A logistic model was fitted to 96 h-mortality data using a non linear regression method (Venturino et al., 1992). Data from two experiments with duplicates were used together for fitting the model equation. The LC50 was directly estimated from the fitted equation as one of the model parameters. To estimate the AZM concentrations causing minimal or no lethal effects, the LOEC and NOEC for lethality as endpoint were assessed by a probabilistic approach, calculating the LC10 and LC1 respectively as endpoints from the fitted equation (Crane and Newman, 2000; Liendro et al., 2015).

2.2.2. Sublethal exposure tests for biomarkers assessment and tissue preparation

Odontesthes hatcheri juvenile fish (0.678 ± 0.157 g) were transferred into aquariums with aerated filtered water from Chimehuin River and exposed to 0.0, 0.1 and 0.5 $\mu g\,L^{-1}$ AZM (0.8 g of fish/L of media, USEPA, 1996). Four fish were included in each aquarium, performing treatments by triplicate. The exposures were repeated in two independent assays. *Jenynsia multidentata* adult females (0.45 ± 0.05 g) were transferred into aquariums containing aerated tap water and 0, 5 and 10 $\mu g\,L^{-1}$ AZM in 0.05% acetone (0.8 g of fish/L of media, USEPA, 1996). Four fish were included in each aquarium, performing treatments by quadruplicate. The exposures were repeated in two independent assays.

After exposure, fish of both species were weighed and brain, gills and muscle tissues were extracted from the four fish in each batch and gathered as unique sample for biochemical biomarkers determinations in the respective organs. Tissues were homogenized

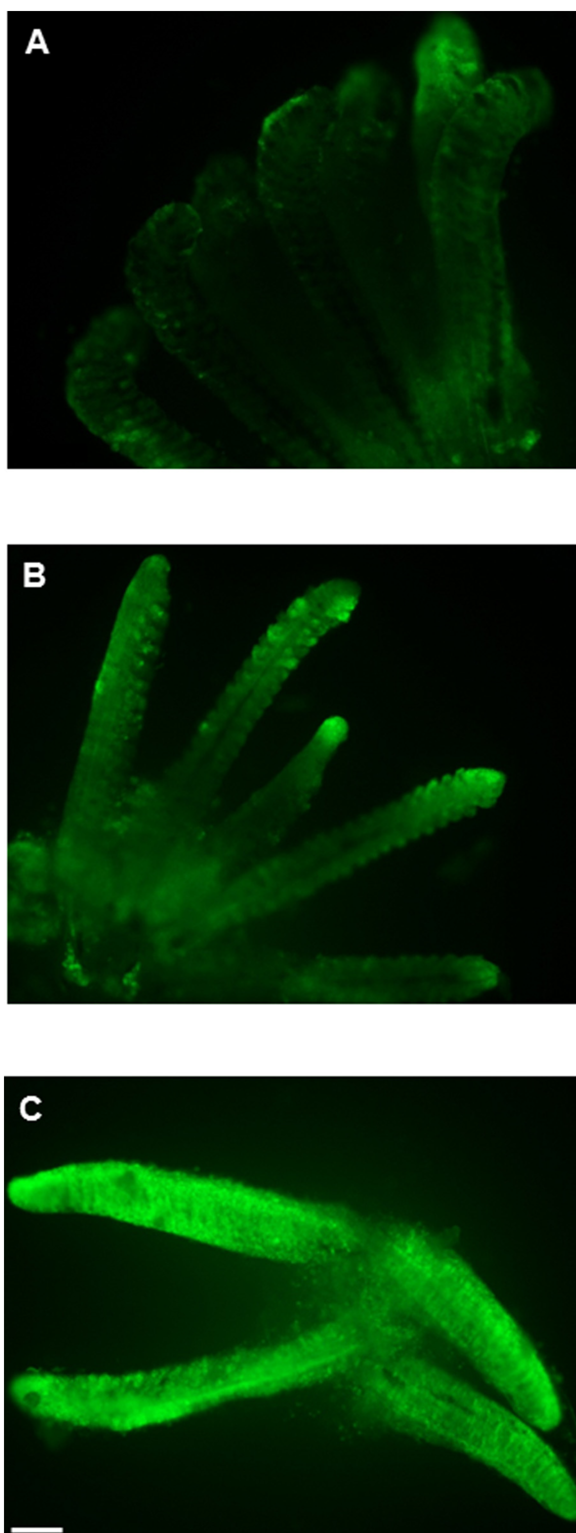

Fig. 1. *Ex vivo* microscopy measurements of ROS fluorescence in *J. multidentata* gills. A-C. Micrographs of *ex vivo J. multidentata* gills after 15 min mounted on a slide with H_2DCF solution. Aspect of the primary lamella exposed to: (A) 0-, (B) 5- and (C) 10 μg L^{-1} AZM. Scale bar = 100 μm.

in 100 mmol L^{-1} potassium phosphate buffer, pH 7.5, containing 6.3 mmol L^{-1} EDTA in a proportion of 10 μL/ mg tissue. Gill samples were split in two aliquots, using one of them to immediately measure GSH, TBARS, ROS and TOSC. The remaining aliquots and brain and muscle samples were centrifuged at 10.000g for 30 min at 4 °C. The resultant supernatant was kept at −20 °C until enzyme determinations.

2.3. Enzymatic analyses

2.3.1. Brain and muscular esterase activities

Resultant supernatants from brain and muscle were used to measure AchE activity by the method of Ellman et al. (1961) using 0.75 mmol L^{-1} acetylthiocholine iodide as substrate. CarbE activity was determined using 1 mmol L^{-1} *p*-nitrophenylbutyrate as substrate (Caballero de Castro et al., 1991).

2.3.2. Gill antioxidant and detoxifying enzymes

GST activity was determined using 2.5 mmol L^{-1} GSH and 0.5 mmol L^{-1} 1-chloro-2, 4-dinitrobenzene (CDNB) dissolved in 1% v/v acetonitrile as substrates, at 340 nm (Habig et al., 1974). For GR activity, 50 mmol L^{-1} oxidized glutathione and 0.21 mmol L^{-1} NADPH were used as substrates for continuous recording of absorbance at 340 nm (modified from Schaedle and Bassham, 1977). For CAT activity, the continuous decrease of absorbance at 240 nm of 25 mmol L^{-1} H_2O_2 as substrate was recorded (modified from Beers and Sizer, 1952). In all cases, the specific activity was calculated by the protein content determined according to the method by Lowry et al. (1951), using bovine serum albumin as standard.

2.4. Non-enzymatic oxidative stress and antioxidant response parameters in gills

2.4.1. GSH and lipid peroxidation

GSH was measured as acid-soluble thiols in 0.2 mL of supernatant from 10% tricholoroacetic acid treatment of sample homogenates, using 1 mL of 1.5 mmol L^{-1} 5,5′ dithio bis 2-nitrobenzoic acid in 0.25 mol L^{-1} sodium phosphate buffer, pH 8. The mixture was incubated during 20 min and absorbance was measured at 412 nm. Acid-soluble thiols were quantified using a calibration curve with pure GSH as standard (Venturino et al., 2001a).

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid-reactive substances (TBARS) (modified from Asakawa and Matsushita, 1979). 250 μL of 25% trichloroacetic acid, 250 μL of 0.72% thiobarbituric acid, 25 μL of 2.78% $FeSO_4{\cdot}7H_2O$, and 25 μL of 0.22% of butylated hydroxytoluene were added to 250 μL of homogenate. The reactants were mixed and incubated for 15 min at 100 °C. After incubation, the mixtures were cooled and measured by spectrofluorometry (excitation λ 517 nm, emission λ 550 nm). TBARS were quantified using a calibration curve with pure 1,1,3,3 tetramethoxypropane as standard.

2.4.2. Fluorometric determination of TOSC and ROS

Total oxyradical scavenging capacity (TOSC) against peroxyl radicals was evaluated through ROS generation in tissue using 2,2′-Azobis 2-methylpropionamidine (ABAP). The ROS generated were determined by fluorescence spectrophotometry using dehydro-2,7-dichlorofluorescein (H_2DCF) as reagent (excitation λ 485 nm, emission λ 520 nm). Intrinsic ROS in the tissues were also measured in the absence of ABAP. TOSC was calculated from the relative area between curves obtained with and without ABAP and referred to TROLOX equivalents using a calibration curve. Tissue ROS were also calculated from the signal area in the absence of ABAP using H_2O_2 as standard (Amado et al., 2009).

2.4.3. Fluorescence microscopy measurements of ROS in J. multidentata gills ex vivo preparations

Microscopy *ex vivo* measurements of ROS in fresh gills of *J. multidentata* were performed after 96 h exposure to different sublethal AZM concentrations. Gill arches from four fish in each treatment

were mounted on a slide with dehydro-2,7-dichlorofluorescin solution and observed under fluorescence microscope at 488 nm excitation/525 nm emission wavelengths. Each preparation was observed during 15 min registering digital images at 3-min intervals. The serial images were analyzed in gray scale (0–255) using the Image J program version 1.49. The average values from 400 image areas of 2304 pixels were calculated for each treatment and plotted as a function of the reaction time to determine the area-under-curve as a relative measure of the ROS generated in 15 min.

2.5. Risk probability assessment for Odontesthes hatcheri and Jenynsia multidentata

The toxicity data and biomarker concentration responses assessed for both species were compared to environmental concentrations of AZM detected in superficial water from different points in the Alto Valle region (Tossi et al., 2009; Loewy et al., 2011) and risk quotients (RQ) were calculated. We calculated a percentile probabilistic risk curve for local species from our published data (Anguiano et al., 2014) and the new data generated in this study. Percentile values for AZM acute toxicity in *O. hatcheri* and *J. multidentata* were estimated and compared to those obtained from a general species probabilistic risk curve (Tossi et al., 2009).

2.6. Statistical analysis

Biochemical parameters were analyzed by one-way ANOVA, followed by *post hoc* Tukey's Test. The three levels of treatments included triplicate samples for *O. hatcheri* (Total N = 9) and quadruplicate samples for *J. multidentata* (Total N = 12). In all cases, each sample was constituted by organs from 4 fish. Data from ROS semi-quantitative analysis by fluorescence microscopy in *ex vivo* preparations were analyzed by Kruskal-Wallis non parametric test. Principal component analyses (PCA) were applied to both species together and separately for each of them in order to determine the contribution of each biomarker response to the variability within each species due to the AZM treatment.

3. Results

3.1. Acute toxicity

The non-linear regression analysis of the acute toxicity data revealed a 96 h-LC50 value of 7.02 $\mu g L^{-1}$ AZM for *O. hatcheri* and 29.86 $\mu g L^{-1}$ for *J. multidentata* (Table 1). The steepness of the mortality curve was higher for *O. hatcheri,* with a slope of 3.32, compared to *J. multidentata* (slope 1.67). The LC1 was calculated from the non-linear regression analysis to estimate a NOEC value, resulting in 1.84 $\mu g L^{-1}$ AZM for *O. hatcheri* and 1.91 $\mu g L^{-1}$ AZM for *J. multidentata*. As an estimation of LOEC, the LC10 was also calculated from the fitted equation in 3.83 $\mu g L^{-1}$ AZM for *O. hatcheri* and 8.01 $\mu g L^{-1}$ AZM for *J. multidentata*.

3.2. Comparative effects of sublethal AZM concentrations in enzymatic activities

3.2.1. Brain and muscular esterase activities

In *O. hatcheri* exposed to 0.5 $\mu g L^{-1}$ AZM, AchE brain activity was inhibited by 50% with respect to the control group (ANOVA, $p < 0.001$). Muscular AchE activity showed a slight dual response depending on AZM concentration, being inhibited by 14% at 0.1 $\mu g L^{-1}$ AZM and increased by 8% at 0.5 $\mu g L^{-1}$ AZM ($p < 0.001$) (Table 2). Exposure to AZM did not produce any significant change in brain CarbE activity, while muscular CarbE activity was significantly increased by 50% in fish exposed to 0.1 $\mu g L^{-1}$ AZM ($p < 0.001$). The exposure of *J. multidentata* to 10 $\mu g L^{-1}$ AZM caused about 42% inhibition in brain AchE activity compared to the control group ($p = 0.033$). No significant effects were observed in muscular AchE activity. In turn, significant increases in CarbE activities were recorded for *J. multidentata* exposed to 10 $\mu g L^{-1}$ AZM in brain (33%, $p = 0.004$) and muscle (25%, $p = 0.034$).

3.2.2. Gill antioxidant and detoxifying enzymes

In *O. hatcheri* gills, a 30% inhibition of GR activity was observed in fish exposed to 0.1 $\mu g L^{-1}$ AZM compared to controls ($p = 0.021$) (Table 3). CAT activity was also inhibited by 0.1 $\mu g L^{-1}$ AZM (46%), but its activity was similar to control fish in individuals exposed to 0.5 $\mu g L^{-1}$ AZM. Exposure to 0.1 $\mu g L^{-1}$ and 0.5 $\mu g L^{-1}$ AZM caused a significant induction of gill GST activity, of about 2.7-fold and 2.1-fold, respectively. The exposure to the OP caused 40–45% of inhibition in CarbE activity. In contrast, no significant effects were found in any of these enzyme activities in the gills of *J. multidentata* exposed to AZM, compared with controls.

3.3. Non-enzymatic oxidative stress responses in gill

Gill tissue from *O. hatcheri* exposed to AZM showed a significant increase of 25% in lipid peroxidation at 0.1 $\mu g L^{-1}$ AZM, with respect to controls ($p < 0.001$) (Table 4). On the other hand, exposure to AZM caused a 2-fold significant increase in GSH in *J. multidentata* gills, independently of the AZM concentration ($p = 0.001$). The total oxyradical scavenging capacity (TOSC) against peroxyl radicals was significantly reduced (65%) in gills from fish exposed to 5 $\mu g L^{-1}$ AZM ($p = 0.017$). Lipid peroxidation showed no relevant changes in gills of *J. multidentata* exposed to AZM.

3.4. Ex vivo fluorescence microscopy measurements of ROS in J. multidentata gills

Exposure of *J. multidentata* to AZM clearly induced ROS production in gills, as demonstrated in freshly excised preparations evaluated by fluorescence microscopy (Fig. 1). Digital analyses of the images indicated a relative increase respect to control, to 119% ± 23% for 5 $\mu g L^{-1}$ AZM (not significant) and to 208% ± 63% ($p = 0.023$) for 10 $\mu g L^{-1}$ AZM, which was consistent with the trend towards an increase observed *in vitro* ROS.

3.5. Principal component analysis

PCA was first performed including both species, to determine the degree of overlap in their responses to AZM treatment. The projection of the case variables demonstrated that the species were clearly separated from each other according to their response variables in the first component (PC1 70.2%, Fig. 2 A). The projection in PC1 allowed us to separate control from treatments in both species. For *J. multidentata*, both AZM concentrations were separated in this component. Treatments were also separated in the second projection (PC2 10.44%). The variables that contributed the most to the first component were: gill GR, CAT and ROS, brain AchE, (lower mean values in *O. hatcheri*), and gill, brain and muscle CarbEs, gill TBARS and GSH (lower mean values in *J. multidentata*). The variables which contributed to the second component were: gill GST and TOSC (lower mean values in *J. multidentata* control group and higher mean values in *O. hatcheri* control group). The variable muscular AchE contributed to both principal components.

We then performed PCA to determine the variability of biomarkers response in each species. As it is shown in Fig. 2B, the first component for *O. hatcheri* explained 74.1% of the variability. The main variables contributing to PC1 were: muscular AchE and gill biomarkers GR, CAT, GSH, TBARS, ROS and TOSC. The second component explained 25.9% of variability, through CarbE in different tissues, brain AchE and gill GST. The projection of the treatments

Table 1
Non-linear regression analysis of the toxicity test for *O. hatcheri* and *J. multidentata.*

	LC50	Steepness	NOEC	LOEC
O. hatcheri	7.02 ± 1.69	3.32 ± 1.91	1.84 (95% CI 0.23–3.73)	3.83 (CI 95%: 1.28–5.93)
J. multidentata	29.86 ± 4.83	1.67 ± 0.47	1.91 (95% CI 0.03–6.98)	8.01 (CI 95%: 0.84–17.25)

Toxicological parameters estimated from the acute 96 h toxicity tests performed in static conditions with different concentrations of AZM (0.0, 0.1, 0.3, 1.0, 3.0 and 10 $\mu g\,L^{-1}$ for *O. hatcheri* and 0, 5, 10, 15, 25 and 50 $\mu g\,L^{-1}$ for *J. multidentata*). Data from two experiments with duplicates were used together for fitting the logistic model equation using a non linear regression method. Units are $\mu g\,L^{-1}$ AZM.

Table 2
Brain and muscular esterase activities in *Odontesthes hatcheri* and *Jenynsia multidentata.*

	AZM ($\mu g\,L^{-1}$)	Brain		Muscle	
		AchE	CarbE	AchE	CarbE
	0	$0.196^{a} \pm 0.001$	$0.019^{a} \pm 0.002$	$0.498^{a} \pm 0.005$	$0.018^{a} \pm 0.001$
O.h.	0.1	$0.175^{b} \pm 0.005$	$0.017^{a} \pm 0.000$	$0.428^{b} \pm 0.002$	$0.018^{a} \pm 0.001$
	0.5	$0.101^{c} \pm 0.003$	$0.016^{a} \pm 0.002$	$0.539^{c} \pm 0.010$	$0.027^{b} \pm 0.000$
	0	$0.118^{a} \pm 0.046$	$0.093^{a} \pm 0.017$	$0.492^{a} \pm 0.148$	$0.041^{a} \pm 0.006$
J.m.	5	$0.099^{ab} \pm 0.032$	$0.108^{ab} \pm 0.011$	$0.424^{a} \pm 0.195$	$0.041^{a} \pm 0.011$
	10	$0.069^{b} \pm 0.022$	$0.118^{b} \pm 0.009$	$0.381^{a} \pm 0.185$	$0.052^{b} \pm 0.010$

Mean and standard deviation of esterase activities measured in supernatant of homogenized brain and muscle after acute exposure to AZM (n = 9 for *O. hatcheri* and n = 12 for *J. multidentata*). Different letters denote significant differences within treatments (ANOVA test, $p < 0.05$). Units are in UI mg protein^{-1}.

Table 3
Gill antioxidant and detoxifying enzyme activities in *Odontesthes hatcheri* and *Jenynsia multidentata.*

	AZM ($\mu g\,L^{-1}$)	GR	CAT	GST	CarbE
	0	$82.6^{a} \pm 6.8$	$5.38^{a} \pm 0.24$	$45.75^{a} \pm 3.28$	$70.7^{a} \pm 5.1$
O.h.	0.1	$58.3^{b} \pm 11.2$	$2.92^{b} \pm 0.16$	$15.29^{b} \pm 0.80$	$42.8^{b} \pm 0.9$
	0.5	$63.4^{ab} \pm 3.9$	$5.15^{a} \pm 0.29$	$12.22^{b} \pm 0.64$	$38.8^{b} \pm 1.7$
	0	$43.1^{a} \pm 4.8$	$2.39^{a} \pm 1.33$	$11.3^{a} \pm 1.9$	$122.0^{a} \pm 56.8$
J.m.	5	$45.2^{a} \pm 2.5$	$1.25^{a} \pm 0.32$	$11.2^{a} \pm 1.4$	$118.8^{a} \pm 55.5$
	10	$48.6^{a} \pm 3.8$	$0.88^{a} \pm 0.55$	$12.2^{a} \pm 0.9$	$143.3^{a} \pm 78.3$

Mean and standard deviation of antioxidant and detoxifying enzyme activities measured in supernatant of homogenized gills after acute exposure to AZM (n = 9 for *O. hatcheri* and n = 12 for *J. multidentata*). Different letters denote significant differences within treatments (ANOVA test, $p < 0.05$). Units are in mUI mg protein^{-1}.

Table 4
Oxidative stress and antioxidant capacity in *Odontesthes hatcheri* and *Jenynsia multidentata* gills.

	AZM ($\mu g\,L^{-1}$)	GSH	TBARS	ROS	TOSC
		(nmol mg protein^{-1})	(nmol mg protein^{-1})	(nmol H_2O_2 equivalents µg protein^{-1})	(nmol TROLOX equivalents mg protein^{-1})
	0	$25.7^{ab} \pm 1.4$	$24.4^{a} \pm 2.2$	$327^{a} \pm 70$	$337^{a} \pm 104$
O.h.	0.1	$28.8^{a} \pm 0.7$	$30.5^{b} \pm 0.9$	$560^{a} \pm 218$	$485^{a} \pm 116$
	0.5	$24.8^{b} \pm 2.1$	$26.4^{a} \pm 0.9$	$267^{a} \pm 25$	$322^{a} \pm 128$
	0	$36.4^{a} \pm 11.1$	$61.8^{a} \pm 37.5$	$54.9^{a} \pm 37.3$	$862^{a} \pm 251$
J.m.	5	$77.6^{b} \pm 10.2$	$56.4^{a} \pm 37.3$	$90.3^{a} \pm 14.2$	$301^{b} \pm 227$
	10	$72.0^{b} \pm 24.7$	$68.8^{a} \pm 39.6$	$100.4^{a} \pm 102.8$	$731^{a} \pm 31$

Mean and standard deviation of non-enzymatic oxidative stress and antioxidant response parameters measured in homogenized gills after acute exposure to AZM (n = 9 for *O. hatcheri* and n = 12 for *J. multidentata*). Different letters denote significant differences within treatments (ANOVA test, $p < 0.05$).

in the PCA plane showed a clear separation among them by PC2 (esterases), while 0.1 μgL^{-1} AZM treatment was split from control and 0.5 $\mu g\,L^{-1}$ AZM by PC1 (oxidative status and antioxidant response). For *J. multidentata*, control, 5 and 10 $\mu g\,L^{-1}$ AZM treatments were gradually segregated in the PC1 projection (Fig. 2C). In this case, nearly all the variables influenced the first component PC1 (74.5%), although GR, GST and the CarbE grouped separately from CAT, muscular and brain AchE in their response, differing from *O. hatcheri*. The projection in the second component (PC2 27%) segregated the intermediate treatment of 5 $\mu g\,L^{-1}$ AZM, mainly driven by gill oxidative stress biomarkers and GSH.

3.6. Environmental risk analysis

Risk assessment was performed taking into account local information regarding environmental concentrations of AZM and ecotoxicological data for species inhabiting water courses in Alto Valle (Table 5). The inclusion of AZM toxicity for *O. hatcheri* and *J. multidentata* to a previous data set (Anguiano et al., 2014) showed a very good fitting for a percentile species risk model (percent of endangered species = 0.846 × log[AZM] − 1.459; $R^2 = 0.957$). The toxicity endpoint LC50–96 h for *O. hatcheri* matched with a percentile probability of 32% of affected local species and 19% of potentially affected species in general. The LOEC value for *O. hatcheri* matched with a 28% probability of affected local species, close to the LC50 endpoint. For *J. multidentata*, LC50 was located in the percentile 45% of local species affected and the estimated LOEC was in 33% of affected species. Considering the risk assessment for aquatic species in general, *J. multidentata* LC50 matched a 30% of risk.

Risk quotient approach indicated probable acute effects for *O. hatcheri* in river and irrigation channels (environmental AZM concentrations 1.19 $\mu g\,L^{-1}$ and 1.77 $\mu g\,L^{-1}$ respectively, RQ > 0.1). The risk gradually increased for NOEC endpoint, which suggested highly

Table 5
Risk analysis for *Odontesthes hatcheri* and *Jenynsia multidentata* potential exposure to AZM in local environmental conditions.

Endpoint	Value (μg L^{-1})	Percentile		Maximum Environmental Concentrations[c]		
		All species[a]	Local species[b]	river EC	channel EC	drainage water
				1.19 μg L^{-1}	1.77 μg L^{-1}	22.48 μg L^{-1}
O. hatcheri				Risk Quotient[d]		
LC50	7.02	18.71	32.23	0.17	0.25	3.20
LC10	3.83	13.20	27.58	0.31	0.46	5.87
LC1	1.84	–	–	0.65	0.96	12.22
BMK EC50[e]	0.5	–	–	2.38	3.54	44.96
	0.1	–	–	11.90	17.70	224.80
J. multidentata						
LC50	29.86	31.89	44.74	0.04	0.06	0.75
LC10	8.01	19.91	33.30	0.15	0.22	2.81
LC1	1.91	–	–	0.62	0.93	11.77
BMK EC50[d]	10	–	–	0.12	0.18	2.25
	5	–	–	0.24	0.35	4.50

[a] Values estimated using equation from Tossi et al. (2009), risk probability analysis for aquatic species in general.
[b] Probability risk analysis for species inhabiting Alto Valle water courses, using data from Anguiano et al. (2014), Kristoff et al. (2006) and data supported in this work. Logistic model fitted: percentile = 0.846 × log ([AZM] – 1.459); R^2 = 0.957, n = 9.
[c] Maximal concentrations of AZM determined in superficial water from the respective sources; data from Tossi et al. (2009) and Loewy et al. (2011).
[d] Risk Quotient = AZM Environmental concentration/Endpoint concentration.
[e] Effective concentration tested giving at least a 50% of biomarker response.

probable deleterious effects (RQ close to 1). For drainage water (maximum environmental AZM concentration 22.5 μg L^{-1}), risk was unacceptable (RQ > 1 for the three endpoints) (Table 5). In turn, RQ were applied to those biomarkers presenting maximum effects in the PCA and response effects equal to or greater than 50%. Results showed critical RQ levels (all greatly exceeding 1) for brain AchE and muscular CarbE responding to 0.5 μg L^{-1} AZM, and gill CAT, GST, CarbE and ROS responding to 0.1 μg L^{-1} AZM. In contrast, *J. multidentata* showed minimal risk in river or channel water (RQ < 0.1) and critical risk in drainage water (RQ = 0.75). Risk for chronic effect endpoint NOEC ranged from probable in river or channels, to highly probable in drainage water (RQ = 12). Biomarkers in *J. multidentata* resulted in high risk warning RQ only for drainage water, being brain AchE and gill CAT and non enzymatic biomarkers those responding to 5 or 10 μgL^{-1} AZM (Table 5).

4. Discussion

We compared the toxicity of the OP insecticide AZM in two local fish that are potentially exposed to this class of agrochemicals. In this regard, we developed a static exposure experiment with controlled initial concentrations of AZM, which proved to be sublethal in the case of biomarkers studies. The observed effects are then expected to integrate the continuous uptake of the OP from the media and its metabolization in target and detoxifying organs. According to previous studies with radiolabelled OP, the toxicant is quickly absorbed from the media into the biotic compartment up to a steady state condition. Subsequently, OP concentration in the media smoothly decays with an apparent clearance constant common to all the compartments (Venturino et al., 2001b; Ferrari et al., 2002). According to these data and considering the similarity in the octanol-water partition coefficients of the OP (log Kow = 3.77 for AZM and 3.83 for the OP used in clearance studies), 49% of the initial AZM concentration would remain in the media after 96 h of exposure. In the acute toxicity assays, *O. hatcheri* showed a higher sensitivity towards AZM exposure compared to *J. multidentata*, with a LC50 four times lower. The homogeneity of *O. hatcheri* population response to the OP was also high (mortality slope = 3.3) compared with *J. multidentata*, where a greater variability was observed. This variability resulted in close effective concentrations for the estimated LOEC and in consequence in the risk facing eventual contamination episodes. Environmental concentrations in regional water courses in the Alto Valle have been found between 1 and 23 μg L^{-1} AZM in different sources (rivers, irrigation channels and drainages) (Loewy et al., 2011). The calculated acute RQ suggest moderate to high ecotoxicological effects in *O. hatcheri*, while no acute risk is apparent in *J. multidentata* in cleaner waters such as rivers. Exposure effects in *J. multidentata* are probable in drainages according to LOEC endpoint. The approach of using biomarkers responding to sublethal AZM concentrations as endpoints to calculate RQ proved very useful to warn about underlying molecular impacts. This approach demonstrates a high probability of deleterious effects at the biochemical-molecular levels in *O. hatcheri*, even when no apparent acute toxicity is appreciated. In turn, and coincidently with the lower sensitivity towards AZM in *J. multidentata*, the RQ calculated with the concentrations that trigger biomarkers responses indicate only a moderate probability of toxic effects at the biochemical level.

The ratios between the lethal concentrations (LC50) and sublethal concentrations of AZM triggering biomarker responses are about 6 in *J. multidentata*, but as high as 70 in *O. hatcheri*. These differences in the sensitivity on biochemical and molecular effectors suggest mechanistic and toxic-dynamical particularities in each species. The PCA performed in this work comprising both species supports the conclusion that there are differential species-related responses of biomarkers to AZM exposure. There are also noticeable differences in the basal biomarker levels between the two species (Tables 2–4). The fact that *O. hatcheri* has higher brain AchE specific activity but lower brain and muscle CarbE activity may be determinant of its comparatively higher sensitivity to the OP. The relationship between AchE inhibition and mortality is not clear, or at least it is not comparable among different fish species (Ferrari et al., 2004a,b, 2007). In *O. hatcheri*, the difference between the EC50 for AchE inhibition and the LC50 suggests that other mechanisms participate in AZM toxicity. In fact, *O. hatcheri* gills have higher basal levels of the antioxidant enzymes GR and CAT, but the primary detoxifying enzymes CarbE and GST acting as demethylase (Venturino et al., 2001a) are lower than the respective basal levels in *J. multidentata*. These facts reinforce the hypothesis of different molecular mechanisms in the detoxification vs. toxicity targeting of AZM in these two species.

The PCA approaches in each species point out the differential responses of biochemical parameters to AZM exposure. AchEs are the first clearly diverging biomarker: while in *J. multidentata* the classical inhibitory response is observed after OP exposure, an induction response probably due to de novo synthesis is evoked for

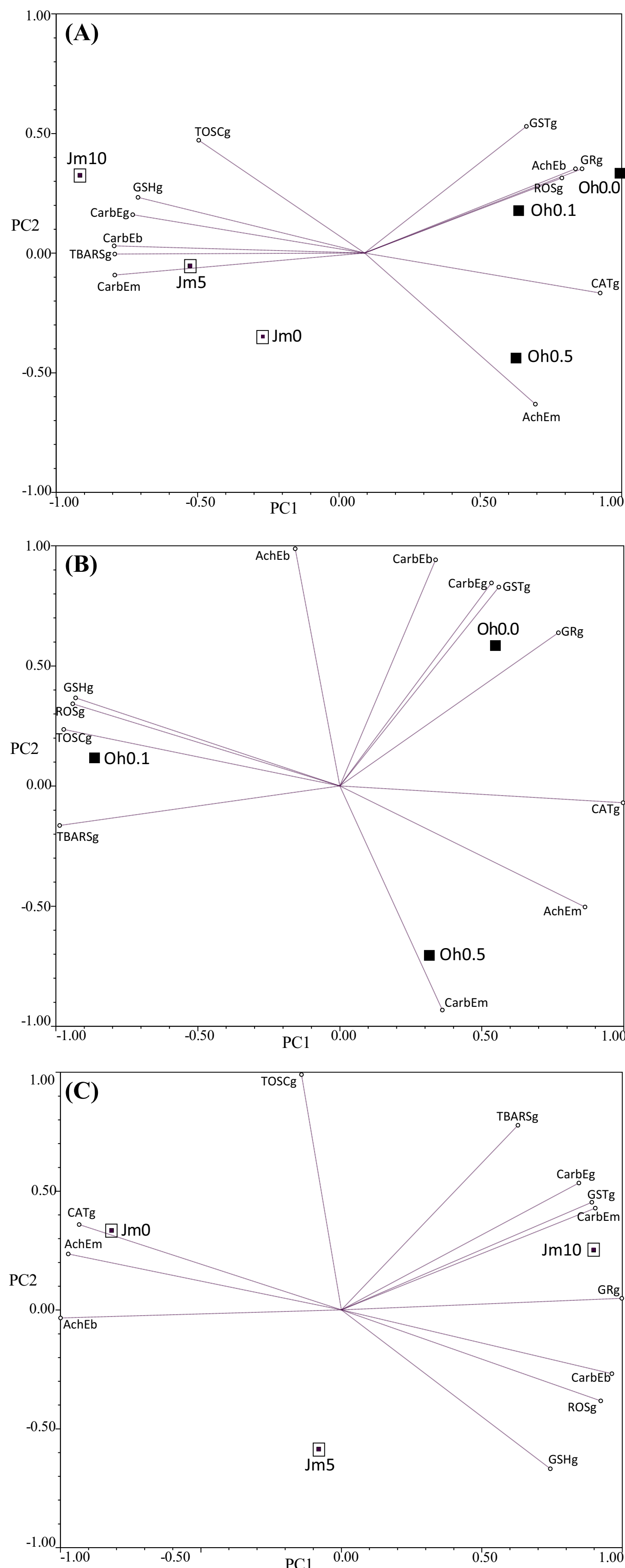

Fig. 2. (A) Principal Component Analyses (PCA) of enzymatic and non-enzymatic oxidative stress and antioxidant response parameters and esterase activities measured in brain (b), muscle (m) and gills (g) of *O. hatcheri* (O.h.) and *J. multidentata* (J.m.) from control and sublethal 96 h exposures for biochemical biomarkers assessment. AchE: acetylcholinesterase; CarbE: carboxylesterase; GST: glutathione-*S*-transferase; GR: glutathione reductase; CAT: catalase; GSH: reduced glutathione; ROS: reactive oxygen species; TBARS: thiobarbituric acid-reactive substances; TOSC: total oxyradical scavenging capacity. (B–C) PCA applied to *O. hatcheri* and *J. multidentata*, respectively, to identify the relative contribution of the different biomarkers to variation in response for each species.

muscular AchE in *O. hatcheri*. This kind of response has been previously reported for toad larvae exposed *in situ* to OP (Rosenbaum et al., 2012). Brain CarbE also shows a differential response to AZM, being increased in *J. multidentata* but not affected in *O. hatcheri*. However, in neither species, brain AchE activity could be protected from inactivation by AZM. In muscle, CarbE activity was induced in both species by the highest AZM concentrations, protecting muscular AchE. According to these differential responses, PCA shows an opposite response for AchEs respect to CarbEs in *J. multidentata*. In *O. hatcheri*, PCA does not follow this pattern, and brain AchE and CarbE respond in the same direction. The respective biomarkers responses in gills of each species also show relevant differences. The detoxifying enzymes GST and CarbE are highly sensitive to AZM in *O. hatcheri*. Both enzymes show opposite trends: while GST activity is induced 2-fold, CarbE shows a decrease acting as a buffering activity that protects AZM targets. Both biomarkers have relevant weight in the PC2 to explain variability. In turn, there is no significant response of any of the measured enzymes in *J. multidentata* gills. In contrast, exposure of *J. multidentata* to endosulfan is able to elicit changes in GST, GR, GSH peroxidase, CAT and lipid peroxidation in gills (Ballesteros et al., 2009). In *O. hatcheri* gills, there is a significant inhibition of GR and CAT activities, while ROS and TBARS are increased by exposure to 0.1 $\mu g\,L^{-1}$ AZM. Accordingly, both enzyme biomarkers explain variability in the same zone of PCA and opposite to oxidative stress biomarkers. These effects suggest that the antioxidant capacity is overcome by AZM exposure in *O. hatcheri* gills, causing an increase in ROS. This would in turn augment lipid peroxidation and inhibit enzymes with susceptible active sites, such as CAT and GR. Similarly, gills from *J. multidentata* exposed to AZM have an overproduction of ROS, which is noticeable in *ex vivo* gill preparations. It is important to note that GSH levels are augmented too, which would help to avoid oxidative damage in *J. multidentata* gills, keeping down the levels of free radicals (Bianco et al., 2013). It is possible that the 2X induced levels of GSH in *J. multidentata* gills were adequate to avoid lipid peroxidation or GR inactivation, in spite of increased levels of ROS.

When the biomarker responses are collectively analyzed in the context of PCA for each species, a clearly differentiated pattern is perceived. In *O. hatcheri*, the most important variability due to the response towards AZM exposures is achieved by the oxidative stress and antioxidant responses (first component, with 74.1% of the total variability). Detoxifying enzymes and AchEs lie mainly on the second component of variability and AchEs and CarbEs roughly run in the same sense of variation. *J. multidentata* in turn shows AchEs and detoxifying (CarbEs and GSTs) separated from each other, explaining 74.5% of the total variability in the first component. In this species, the antioxidant response and oxidative stress are distributed between the first and the second component (except for GR that lies completely in the first component). These differences point to main effects and different degrees of impact of the OP on molecular targets. They may be also related to the basal detoxifying activities and responsiveness in each species, as discussed above. The oxidative stress and antioxidant enzymes inhibition seem to be the main molecular impairments in *O. hatcheri* exposed to sublethal AZM concentrations, while the classical target AchE appears to be a secondary target.

We conclude that there are important differences in the biochemical targets and detoxifying activities that underlie the differential toxicity of the insecticide AZM in *O. hatcheri* and *J. multidentata*, as visualized in PCA approach. These two fish species are

potentially exposed to OP during the productive season in the Alto Valle region, Patagonia Argentina. Based on biomarkers analysis and environmental AZM concentration data, there are some scenarios within the irrigated valley basin that pose unacceptable risks to the Patagonian silverside.

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References

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123, http://dx.doi.org/10.1016/j.scitotenv.2008.11.038.

Anguiano, O.L., Castro, C., Venturino, A., Ferrari, A., 2014. Acute toxicity and biochemical effects of azinphos methyl in the amphipod Hyalella curvispina. Environ. Toxicol. 29, 1043–1053, http://dx.doi.org/10.1002/tox.21834.

Asakawa, T., Matsushita, S., 1979. Thiobarbituric acid test for detecting lipid peroxide. Lipids 14, 401–406, http://dx.doi.org/10.1007/BF02533425.

Ballesteros, M.L., Wunderlin, D.A., Bistoni, M.A., 2009. Oxidative stress responses in different organs of Jenynsia multidentata exposed to endosulfan. Ecotoxicol. Environ. Saf. 72, 199–205, http://dx.doi.org/10.1016/j.ecoenv.2008.01.008.

Ballesteros, M.L., Gonzalez, M., Wunderlin, D.A., Bistoni, M.A., Miglioranza, K.S.B., 2011. Uptake, tissue distribution and metabolism of the insecticide endosulfan in *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes). Environ. Pollut. 159, 1709–1714, http://dx.doi.org/10.1016/j.envpol.2011.02.037.

Bebe, F.N., Panemangalore, M., 2003. Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. J. Environ. Sci. Health., Part B 38, 349–363, http://dx.doi.org/10.1081/PFC-120019901.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140 http://uran.donetsk.ua/~masters/2012/fkita/pavlenko_a/library/article_9.pdf.

Bianco, K., Yusseppone, M.S., Otero, S., Luquet, C.M., Ríos de Molina, M.C., Kristoff, G., 2013. Cholinesterases and neurotoxicity as highly sensitive biomarkers for an organophosphate insecticide in a freshwater gastropod (*Chilina gibbosa*) with low sensitivity carboxylesterases. Aquat. Toxicol. 144–145, 26–35 http://dx.doi.org/10.1016/j.aquatox.2013.09.025.

Caballero de Castro, A., Rosenbaum, E.A., Pechen de D'Angelo, A.M., 1991. Effect of malathion on *Bufo arenarum* hensel development—I: esterase inhibition and recovery. Biochem. Pharmacol. 41, 491–495, http://dx.doi.org/10.1016/0006-2952(91)90619-G.

Crane, M., Newman, M.C., 2000. What level of effect is a no observed effect? Environ. Toxicol. Chem. 19, 516-–519, http://dx.doi.org/10.1002/etc.5620190234.

Ellman, G.L., Courtney, D.K., Andres Jr., V., Feathrstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinsterase activity. Biochem. Pharmacol. 7, 88–95, http://dx.doi.org/10.1016/0006-2952(61)90145-9.

Ferrari, A., Bubach, D., Ribeiro Guevara, S., Arribére, M.A., Venturino, A., Pechen de D'Angelo, A.M., 2002. Distribución de paratión y mercurio y biomarcadores de efecto en la exposición de *Cyprinus carpio* a concentraciones subletales. Acta Toxicol. Argentina 10, 5–10.

Ferrari, A., Anguiano, O.L., Soleño, J., Venturino, A., Pechen de D'Angelo, A.M., 2004a. Different susceptibility of two aquatic vertebrates (*Oncorhynchus mykiss* and *Bufo arenarum*) to azinphos methyl and carbaryl. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 139, 239–243, http://dx.doi.org/10.1016/j.cca.2004.11.006.

Ferrari, A., Venturino, A., Pechen de D'Angelo, A.M., 2004b. Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphosmethyl, parathion and carbaryl exposure in the goldfish (*Carassius auratus*). Ecotoxicol. Environ. Saf. 57, 420–425, http://dx.doi.org/10.1016/S0147-6513(02)00069-6.

Ferrari, A., Venturino, A., Pechen de D'Angelo, A.M., 2007. Muscular and brain cholinesterase sensitivities to azinphosmethyl and carbaryl in the juvenile rainbow trout *Oncorhynchus mykiss*. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 146, 308–313, http://dx.doi.org/10.1016/j.cbpc.2007.04.002.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87, 245–254 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1567830/.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139, 249/22/7130.full.pdf+html.

Hualde, J.P., Torres, W.D.C., Moreno, P., Ferrada, M., Demicheli, M.A., Molinari, L.J., Luquet, C.M., 2011. Growth and feeding of patagonian pejerrey Odontesthes hatcheri reared in net cages. Aquacult. Res. 42, 754–763, http://dx.doi.org/10.1111/j.1365-2109.2011.02827.

Jokanović, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166, 139–160, http://dx.doi.org/10.1016/S0300-483X(01)00463-2.

Kristoff, G., Verrengia Guerrero, N., Pechen de D'Angelo, A.M., Cochón, A.C., 2006. Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: *Biomphalaria glabrata* and *Lumbriculus variegatus*. Toxicology 222, 185–194, http://dx.doi.org/10.1016/j.tox.2006.02.018.

Lackner, R., 1998. Oxidative stress in fish by environmental pollutants. In: Braunbeck, T., Hinton, D.E., Streit, B. (Eds.), Fish Ecotoxicology. Birkhause Verlag, Basel.

Liendro, N., Ferrari, A., Mardirosian, M., Lascano, C.I., Venturino, A., 2015. Toxicity of the insecticide chlorpyrifos to the South American toad Rhinella arenarum at larval developmental stage. Environ. Toxicol. Pharmacol. 39, 525–535, http://dx.doi.org/10.1016/j.etap.2014.12.022.

Loewy, R.M., Monza, L.B., Kirs, V.E., Savini, M.C., 2011. Pesticide distribution in an agricultural environment in Argentina. J. Environ. Sci. Health Part B 46, 662–670, http://dx.doi.org/10.1080/03601234.2012.592051.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 http://devbio.wustl.edu/InfoSource/ISPDFs/Lowry%201951.pdf.

Pereira, L., Fernandes, M.N., Martinez, C.B., 2013. Hematological and biochemical alterations in the fish *Prochilodus lineatus* caused by the herbicide clomazone. Environ. Toxicol. Pharmacol. 36, 1–8, http://dx.doi.org/10.1016/j.etap.2013.02.019.

Rosenbaum, E.A., Duboscq, L., Soleño, J., Montagna, C.M., Ferrari, A., Venturino, A., 2012. Response of biomarkers in amphibian larvae to in situ exposures in a fruit-producing region in North Patagonia, Argentina. Environ. Toxicol. Chem. 31, 2311–2317, http://dx.doi.org/10.1002/etc.1950.

Schaedle, M., Bassham, J.A., 1977. Chloroplast glutathione reductase. Plant Physiol. 59, 1011–1012, http://dx.doi.org/10.1104/pp.59.5.1011.

Sharbidre, A.A., Metkari, V., Priyanka, P., 2011. Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, *Poecilia reticulata*. Pestic. Biochem. Physiol. 101, 132–141, http://dx.doi.org/10.1016/j.pestbp.2011.09.002.

Tossi, A.P., Pechen de D'Angelo, A.M., Savini, M.C., Loewy, R.M., 2009. Assessing pesticide hazards on surface water from the northern patagonian region Argentina. Acta Toxicol. Argentina 17, 1–7.

USEPA, Pesticides: Environmental effects. United States environmental Protection Agency, http://www.epa.gov/oppefed1/ecorisk.ders/toera.analysis.eco.htm#Ecotox.

Van der Oost, R., Beyer, J., Vermeulen, N.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149, http://dx.doi.org/10.1016/S1382-6689(02)00126-6.

Venturino, A., Anguiano, O.L., Gauna, L., Cocca, C., Bergoc, R.M., Pechen de D'Angelo, A.M., 2001a. Thiols and polyamines in the potentiation of malathion toxicity in larval stages of the toad Bufo arenarum. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 130, 191–198, http://dx.doi.org/10.1016/S1532-0456(01)00241-1.

Venturino, A., Gauna, L.E., Bergoc, R.M., Pechen de D'Angelo, A.M., 1992. Effect of exogenously applied polyamines on malathion toxicity in the toad Bufo arenarum Hensel. Arch. Environ. Contam. Toxicol. 22, 135–139, http://dx.doi.org/10.1007/BF00213312.

Venturino, A., Rovedatti, M.G., Gauna, L., Loewy, M., Pechen de D'Angelo, A.M., 2001b. Model study of factors influencing steady state clearance for lipophilic toxicants in aquatic microcosms. J. Biol. Syst. 9, 123–143.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137–161, http://dx.doi.org/10.1016/0166-445X(91)90033-6.