Acute Toxicity and Biochemical Effects of Azinphos Methyl in the Amphipod *Hyalella curvispina*

Olga Liliana Anguiano,[1,2] Claudia Castro,[3] Andrés Venturino,[1,4] Ana Ferrari[1,5]

[1]Grupo LIBIQUIMA, Instituto Multidisciplinario de Investigación y Desarrollo de la Patagonia Norte, IDEPA (CONICET-UNCo), Argentina

[2]Depto de Química, Facultad de Ingeniería, Universidad Nacional del Comahue, Neuquén Province, Argentina

[3]Facultad de Ciencias del Ambiente, Universidad Nacional del Comahue, Neuquén Province, Argentina

[4]Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, Río Negro Province, Argentina

[5]Facultad de Ciencias Médicas, Universidad Nacional del Comahue, Río Negro Province, Argentina

Received 4 July 2012; revised 31 October 2012; accepted 2 November 2012

ABSTRACT: We evaluated the acute toxicity and biochemical effects of the organophosphorus pesticide azinphos methyl (AM) in the amphipod *Hyalella curvispina* that inhabits ponds and irrigation channels of an intensive fruit-producing region in Rio Negro and Neuquén valley, North Patagonia, Argentina. The analysis by nonlinear regression of data from the 96 h-acute toxicity tests indicated the coexistence of two subpopulations of *H. curvispina* with different susceptibilities to AM. The 96 h-LC_{50} for the resistant subpopulation (166 $\pm$ 56 μg/L) was 216-fold higher than the 96h-LC_{50} value for the susceptible one (0.77 $\pm$ 1.33 μg/L).The two subpopulations could not be distinguished based on the biochemical measurements in control amphipods. Cholinesterase activity was significantly inhibited in AM-exposed amphipods in a concentration-dependent manner. The IC_{50} value obtained after 96 h of exposure (2.18 $\pm$ 1.95 μg/L) was significantly lower than the 48 h-IC_{50} value (29.6 $\pm$ 17.4 μg/L). Carboxylesterase activity was significantly inhibited after 48 h of exposure to 12.5 and 62.5 μg/L AM (inhibition, 51%). This enzyme was thus able to protect cholinesterase from inhibition at 12.5 μg/L AM. Reduced glutathione and catalase showed a significant increase after 24 h of exposure as an adaptive response to AM, whereas glutathione *S*-transferase activity was not significantly modified. The analysis of species sensitivity distribution showed that both subpopulations of *H. curvispina* were more tolerant to AM than most amphipod species, and that the susceptible subpopulation was more sensitive to AM than the other local aquatic species analyzed. The maximum concentration of AM in drainage water within the fruit-producing area reported by other studies would affect most of the amphipod species (99%) and also a 44% of local aquatic ones. The results obtained in this study point out the usefulness of including amphipods like *H. curvispina* in ecotoxicity studies and monitoring programs to perform pesticide risk assessments. © 2012 Wiley Periodicals, Inc. Environ Toxicol 29: 1043–1053, 2014.

Keywords: Crustacean; insecticides; organophosphorus; esterases; antioxidant responses; risk assessment

Correspondence to: A. Ferrari; e-mail: aferrari@conicet.gov.ar.

Contract grant sponsor: The Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICT-2007-00214.

Contract grant sponsor: The Universidad Nacional del Comahue 04 I 004.

Published online 28 November 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.21834

INTRODUCTION

The valley of Río Negro and Neuquén, North Patagonia Argentina, is a region of intensive fruit production. Irrigation channels deriving from the Limay, Neuquén, and Negro rivers in this farmland are important wetland habitats for aquatic invertebrate species. Within these areas, the spraying of pesticides has the potential to affect the associated wildlife. Organophosphorus pesticides (OP) have been intensively applied in this area to manage fruit pests; among these compounds, azinphos methyl (AM) was one of the most frequently applied as well as detected pesticides in both surface and underground water (Loewy et al., 2006, 2011; Anguiano and Pechen de D'Angelo, 2007). Although the concentrations of AM found in the field (0.1–22 μg/L) are generally below the lethal threshold for several aquatic organisms, the concentrations of some OP may exceed criteria for the protection of aquatic life and remain at concentrations of concern throughout the growing season from November to March (Loewy et al., 2006, 2011; Tossi et al., 2009).

The amphipod *Hyalella curvispina* (Amphipoda: Hyalellidae) is a macrocrustacean commonly found in lakes, ponds, and streams throughout Argentina and South America (Peralta, 2001). *Hyalella* species can be found swimming in the water, clinging to vegetation, or burrowing into sediment, being an important link in the aquatic food chain and a food source for fish and various invertebrates (Tarshis, 2000; Peralta, 2001). The species of the genus *Hyalella* are considered as good indicators of water contamination and have been included in bioassays because of their sensitivity to different xenobiotics such as insecticides and heavy metals (Tarshis, 2000; Moore et al., 2007; García et al., 2010). In addition, some recent ecotoxicological studies performed in South America have focused on the use of the species *H. curvispina* as a bioindicator of insecticide contamination (Jergentz et al., 2004; García et al., 2010; Mugni et al., 2011).

Cholinesterase (ChE) inhibition is considered as a specific and sensitive method for assessing the impact of OP on nontarget organisms, and it is widely used as a biomarker of exposure to this class of insecticides. Although most studies of aquatic OP contamination have focused on ChE evaluation in fish, some studies have been performed in different nontarget invertebrate species such as mollusks, both gastropods and bivalves, macrocrustacean (lobster, crabs, and crayfish) and oligochaetes (Fulton and Key, 2001; Kristoff et al., 2006; Vioque-Fernández et al., 2007). On the other hand, OP can also inhibit other B-esterases such as carboxylesterases (CabEs) (Jokanović, 2001). CabEs are enzymes that efficiently catalyze the hydrolysis of diverse compounds containing ester bonds to the respective free acids and alcohol. As these enzymes bind to many anticholinesterasic pesticides, they can reduce free concentrations of the OP, otherwise available to interact with ChE (Jokanović, 2001; Sogorb and Vilanova, 2002). Therefore, CabEs play a role in OP detoxification by acting as an alternative target and thus protecting ChE from inhibition (Jokanović, 2001).

Besides its anticholinesterasic action, AM as well as other OP can affect detoxifying and antioxidant enzymes such as glutathione *S*-transferase (GST) and catalase (CAT). In addition, reduced glutathione (GSH) levels have been modified in organisms exposed to this compound (Ferrari et al., 2007, 2009). Both, GSH and antioxidant enzymes, have been extensively evaluated in ecotoxicological studies performed with diverse aquatic species such as fish species, amphibian embryos, aquatic gastropods, and oligochaetes (Oruç and Üner, 2004; Ferrari et al., 2007, 2009; Kristoff et al., 2008). Most aquatic invertebrate species exhibit higher sensitivity to OP and carbamates when compared to vertebrate species; however, in Argentina, few studies on the effects of OP have been carried out on aquatic invertebrates (Jergentz et al., 2004; Anguiano et al., 2008; Kristoff et al., 2008).

We have previously reported the occurrence of different populations of amphipods with differential susceptibility to OP, obtained from fruit-producing and pristine areas, in a comparative study including other invertebrates as well (Anguiano et al., 2008). We could determine that an increased basal activity of the detoxifying enzyme CabE was in part responsible for the resistance to OP in these species. The objective of this study was to further evaluate the acute toxicity of AM to the amphipod *H. curvispina* that inhabits irrigation channels in the fruit-producing area of the Rio Negro and Neuquén valley and validate the presence of two subpopulations with different AM susceptibilities. In addition, we sought to examine AM acute effects on *H. curvispina* biochemical markers such as the esterases ChE and CabE, as primary targets of action of OP, as well as on antioxidant biomarkers to assess their potential use in monitoring programs of insecticide impacted areas. Furthermore, we compare the susceptibility of these adapted subpopulations with *H. curvispina* from pristine regions, other amphipod species, and some local aquatic species by means of sensitivity distribution analysis, to discuss the relevance of including *H. curvispina* in environmental monitoring studies at agricultural regions.

MATERIALS AND METHODS

Chemicals

High-purity-certified standard of AM, (*S*-(3,4-dihydro-4-oxobenzo[*d*]-[1,2,3]-triazin-3-ylmethyl) *O,O*-dimethyl phosphordithioate) (98.3%) was purchased from Chem Service, West Chester, PA. Acetylthiocholine iodide, reduced GSH, bovine serum albumin, 1,chloro,2,4-dinitrobenzene (CDNB), dithio-*bis*-nitrobenzoate (DTNB), Fast Garnet GBC salt, α-naphthyl

acetate, α-naphthol, Triton X-100, and 1,5-*bis*(4-allyldimethylammoniumphenyl)pentan-3 one dibromide (BW284C5) were purchased from Sigma Chemical, St. Louis, MO. All the reagents used were of analytical grade.

Toxicological Tests

H. curvispina amphipods were collected from a secondary irrigation channel at the Fernández Oro (F. Oro) district located in an intensive area of fruit production in the Río Negro and Neuquén valley, North Patagonia, Argentina. The amphipods were maintained under laboratory conditions at least for 2 weeks before the beginning of the assays (U.S. Environmental Protection Agency, 1996).

Groups of 10 adult *H. curvispina* amphipods (average fresh weight, 9.1 ± 3.2 mg) were exposed to a range of AM concentrations in 1 L of filtered and dechlorinated water with continuous aeration (U.S. Environmental Protection Agency, 1996). Pesticide solutions were prepared by dissolving pure AM in acetone and diluting the standard solution with an appropriate amount of charcoal-filtered dechlorinated tap water (dissolved oxygen: 8 ± 1 mg/L, pH 7.2–7.5, dissolved solids 80.4 ± 3.53 mg/L, conductivity 161 ± 6.3 μS/cm) keeping acetone at 0.01% according to the recommendation of USEPA (1996). The exact concentrations of the insecticides in the standard solutions were checked by gas chromatography with a nitrogen and phosphorous detector (GC-NPD). Controls with acetone were assayed in parallel. Eight different nominal concentrations of AM were assayed in triplicate in the 96-h acute toxicity tests: 0, 0.5, 2.5, 12.5, 62.5, 312, 1498, and 2496 μg/L. The amphipods were not fed during the tests, which were carried out in static conditions at a constant temperature of 18 ± 1°C and 12:12 (*L*:*D*) photoperiod. Five independent experiments were performed. Survival was monitored daily, mortality was registered, and dead animals were removed. Animals were considered to be dead if no movement of the pleopods was visible during a 20-s observation period using a stereoscopic microscope (Anguiano et al., 2008).

Mortality data were analyzed by a nonlinear regression procedure (Venturino et al., 1992). One-population and two-population sigmoidal models were fitted to data, including control mortality. Best model fittings were assessed by Akaike's criterion.

One-subpopulation sigmoidal model (including control survival) (Venturino et al., 1992):

$$\text{Mortality}\% = 100 - \frac{P\%(\text{control survival})}{1 + ([\text{AM}]/\text{LC50})^{n}}$$

Two-subpopulation sigmoidal model (including control survival):

$$\text{Mortality}\% = 100 - \frac{P_{\text{A}}\%(\text{control survival})}{1 + ([\text{AM}]/LC50_{A})^{nA}} - \frac{P_{\text{B}}\%(\text{control survival})}{1 + ([\text{AM}]/LC50_{B})^{nB}}$$

P_{A} and P_{B} are the proportions for both subpopulations, and LC50 is estimated from the regression for each; $n_{\text{A;B}}$ are the respective slopes of the sigmoidal concentration–mortality responses.

ChE activity was evaluated in amphipods surviving the 96-h acute toxicity tests. In addition, 48-h acute toxicity test was performed to evaluate ChE remnant activity after 48 h of exposure. For the determination of detoxifying and antioxidant enzymes/compounds, different tests were performed. Groups of 20 adult *H. curvispina* amphipods were exposed to 62.5 and 125 μg/L AM in 2 L of filtered/dechlorinated water with continuous aeration in triplicate and samples were collected at 24, 48, and 96 h of exposure.

Biochemical Determinations

Esterase Activity Determinations

ChE was measured according to the methods described by Ellman et al. (1961) with modifications. Amphipods from the 48 or the 96-h acute test were individually homogenized with a Teflon homogenization rod at 4°C in 0.1 M sodium phosphate buffer (pH = 8) containing 1% Triton X-100 and 0.04 M sucrose. Each sample was centrifuged 10 min at 10,000 × *g* at 4°C and the supernatant was used as an enzyme source. Reactions were performed at 25°C in 1 mL of 100 mM phosphate buffer, pH 8.0, containing 0.2 mM 5,5′-dithio-2-*bis*-nitrobenzoate (DTNB), 0.75 mM acetylthiocholine iodide, and 50 μL of amphipod 10,000 × *g* supernatant. Activity was continuously recorded at 412 nm using a UV/visible spectrophotometer (Shimadzu, Kyoto, Japan). Each supernatant was assayed in triplicate. Enzymatic activity was corrected for spontaneous hydrolysis of the substrate and was expressed as mIU mg protein^{-1}.

The concentration values that inhibited 50% of control ChE enzymatic activity (IC_{50}) after 48 and 96 h of exposure to AM were estimated by nonlinear regression (Ferrari et al., 2004a).

CabE activity was measured according to the procedure described by Dary et al. (1990). Amphipods were individually homogenized in 500 μL of 0.1 M sodium phosphate buffer, pH 6.5, with 0.5% Triton X-100. The homogenate was centrifuged at 10,000 × g and 4°C for 10 min and the resulting supernatant was used as an enzyme source. Reactions were performed in homogenization buffer with 10 μL of supernatant. The reaction was initiated by the addition of 2 mM α-naphthylacetate as a substrate and 0.002 mM BW284C5 (an acetylcholinesterase inhibitor). Color development was accomplished by the addition of Fast Garnet GBC salt. Absorbance was recorded at 550 nm using a

UV/visible spectrophotometer (Shimadzu, Kyoto, Japan). Absorbance values were transformed to micromoles of α-naphthol from a α-naphthol standard curve. Each supernatant was assayed in triplicate.

Antioxidant/Detoxifying Enzymes and GSH Levels

The amphipods were randomly collected and processed as indicated below for each enzyme/compound determination. Two independent assays were carried out and three replicate samples were prepared for each treatment and time of exposure.

GSH S-Transferase. Each sample consisted of three amphipods homogenized with a Teflon homogenization rod at 4°C in 500 μL of 66 mM sodium phosphate buffer (pH 7.0) containing 25 mM sucrose. The homogenate was centrifuged at 10,000 × *g* at 4°C during 30 min and the supernatant was used as an enzyme source. GST (EC 2.5.1.18) activity was assayed in a final volume of 1.0 mL of 100 mM phosphate buffer, pH 6.5, containing 0.5 mM CDNB dissolved in 1% v/v acetonitrile and 2.5 mM GSH as substrates. Baseline (nonenzymatic reaction) was continuously recorded at 340 nm, and 20 μL of the supernatant was added (Habig et al., 1974; modified by Anguiano et al., 2001). Each supernatant was assayed in triplicate .The changes in the absorbance were recorded and corrected for spontaneous reaction, and the moles of conjugated CDNB were calculated using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Catalase. Each sample consisted of two amphipods homogenized with a teflon homogenization rod at 4°C in 500 μL of 143 mM potassium phosphate buffer, pH 7.4, containing 6.3 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 × *g* at 4°C during 30 min and the supernatant was used as enzyme source. CAT (EC 1.11.1.6) activity was determined by recording the continuous decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm (modified from Beers and Sizer, 1952). The reaction was performed in 1 mL sodium phosphate buffer 50 mM, pH 7.0, containing 25 mM H_2O_2. The enzymatic activity determination was carried out at the linear range of response with respect to substrate concentration. The absorbance of the reaction mixture was strictly controlled to be 1.000. Baseline absorbance was controlled to be stable, and 20 μL of supernatant was added to initiate the catalyzed reaction. Each supernatant was assayed in triplicate. Specific activity was expressed as CAT units/mg protein (μmol of substrate reacted per min^{-1}, at 25 mM initial concentration) using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

GSH determination. Two adult amphipods were homogenized with a Teflon homogenization rod at 4°C in 500 μL of 143 mM potassium phosphate buffer, pH 7.4, containing 6.3 mM EDTA. The crude homogenates were mixed 1:1 v/v with 10% trichloroacetic acid and centrifuged at 10,000 × *g* for 10 min at 4°C. GSH was immediately measured as acid-soluble thiols (modified from Ellman, 1959; Ferrari et al., 2009) in 0.2 mL of supernatants, using 1 mL of 1.5 mM DTNB in 0.25 M sodium phosphate buffer, pH 8.0. The mixture was incubated during 20 min and the absorbance at 412 nm was measured. Acid-soluble thiols were quantified using a calibration curve with pure GSH as standard.

Protein Content Determination

Total protein content in each sample was determined according to the method of Lowry et al. (1951). Absorbance was measured at 750 nm and transformed into protein concentration from a bovine serum albumin standard curve.

Statistical Analysis

Mean ± standard error (SE) of biochemical data were calculated. Differences between treatments were assessed by factorial ANOVA for each biochemical parameter, considering time of exposure and AM concentration as independent factors. ChE and CabE activity data after 48- or 96-h exposure to AM were analyzed by one-way ANOVA. Fisher's least significant differences *post hoc* test was used to assess the statistical differences between control and exposed amphipods.

Risk Evaluation for *H. curvispina* and Comparison with Local Aquatic Species

To compare the toxicity of AM determined in this study for *H. curvispina* inhabiting F. Oro irrigation channels, analysis of species sensitivity distributions was performed (ECOFRAM, 1999; Montagna et al., 2011) using acute toxicity data (LC_{50} values) for aquatic freshwater organisms. Other amphipods (Nebeker and Gaufin, 1964; Sanders, 1969; Johnson and Finley, 1980; Mayer and Ellersieck, 1986; Ankley and Collard, 1995; Pantani et al., 1997; Anguiano et al., 2008) and local aquatic freshwater vertebrates evaluated in our previous studies (Ferrari et al., 2004a,b) were included in the probabilistic analysis. The species were ranked by decreasing sensitivity and the rank was transformed to percentile values: $[i/(n + 1)]$, where i is the species rank, and n is the total number of species listed (ECOFRAM, 1999). Probit regressions were performed on percentiles versus log-transformed LC_{50} values to obtain the regression lines. Maximal AM concentrations measured in surface and subsurface freshwater in the region by Tossi et al. (2009) and Loewy et al. (2011) were introduced in the equations to establish the percentage of species potentially affected. The AM concentration corresponding to the 10th percentile was also calculated from the probabilistic distribution for local aquatic species.

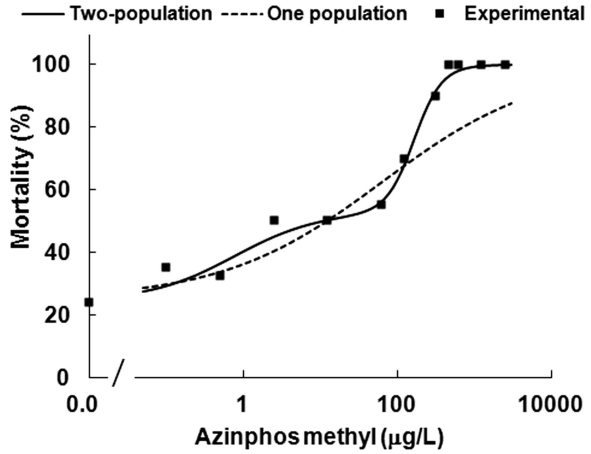

Fig. 1. Mortality of *H. curvispina* after 96 h of exposure to AM. Curves represent the nonlinear fitting of mortality data (sigmoidal) according to one- or two-population models. Squares represent mean experimental mortality data. Standard errors were <10% of mean values. One-population model standard deviations of residuals and AIC (Akaike's coefficient; Akaike, 1974) were 8.88 and 100.72, respectively. Two-population model standard deviation and AIC were 7.34 and 96.91, respectively.

RESULTS

Acute Toxicity and ChE Inhibition

The analysis by nonlinear regression of data obtained from the 96-h acute toxicity tests revealed the coexistence of two subpopulations of *H. curvispina* with different susceptibilities to the OP AM (Fig. 1). This was previously stated in the 48-h bioassays (Anguiano et al., 2008). The calculated LC_{50} values are listed in Table I. The most resistant subpopulation (B) showed a 96 h-LC_{50} value which was 216-fold higher than the susceptible one (subpopulation A). The continuous exposure to AM during 96 h increased toxicity by a factor of 2 with respect to the 48-h exposure test, as could be judged by the significant decrease in the LC_{50} value of the resistant subpopulation (43% of 48 h-LC_{50}). The susceptible subpopulation also showed an important decrease in the LC_{50} value after 96 h of exposure with respect to the 48-h value although the difference was not significant. Besides, the amphipods exposed to AM concentrations between 312 and 2400 µg/L showed visible signs of OP intoxication, that is erratic and fast locomotive movements at the beginning of exposure, followed by body contraction and immobility with intermittent movement of the pleopods at the end of the exposure time. Amphipods exposed to 2.5 µg/L AM or below did not show any behavioral differences when compared to control ones.

ChE activity was significantly inhibited in AM-exposed amphipods in a concentration-dependent manner [Fig. 2(A,B)]. The activity of *H.curvispina* ChE was significantly

TABLE I. Lethal concentration fifty (LC_{50}) and cholinesterase inhibitory concentration values (IC_{50}) in *H. curvispina* adults acutely exposed to AM[a]

	Time of Exposure	
Parameter	48 h	96 h[b]
Subpopulation A proportion (%)	41.3 ± 5.0	28.6 ± 14.2
LC_{50} A (µg/L)	**2.54 ± 0.52**[c]	**0.77 ± 1.33**
n A	3.7 ± 9.1	0.79 ± 0.92
Subpopulation B proportion (%)	58.8 ± 4.5	47.1 ± 13.4
LC_{50} B (µg/L)	**385.2 ± 42.0**[c]	**166.7 ± 56.0**[**]
n B	4.8 ± 2.2	2.42 ± 1.56
SD residuals	9.91	7.34
IC_{50} (µg/L) for ChE	**29.6 ± 17.4**	**2.18 ± 1.95**[*,d]

[a] LC_{50} and IC_{50} values are based on nominal concentrations. SD, standard deviation; *n*, curve concentration–mortality slope.

[b] Control mortality = 20%.

[c] data obtained from Anguiano et al. (2008).

[**] Significant differences between 48 and 96 h of exposure, $p = 0.0034$ by Student's *t*-test.

[*,d] Significantly lesser than 48 h-IC_{50} value, $p = 0.02$ (Student's *t*-test performed on log-transformed data).

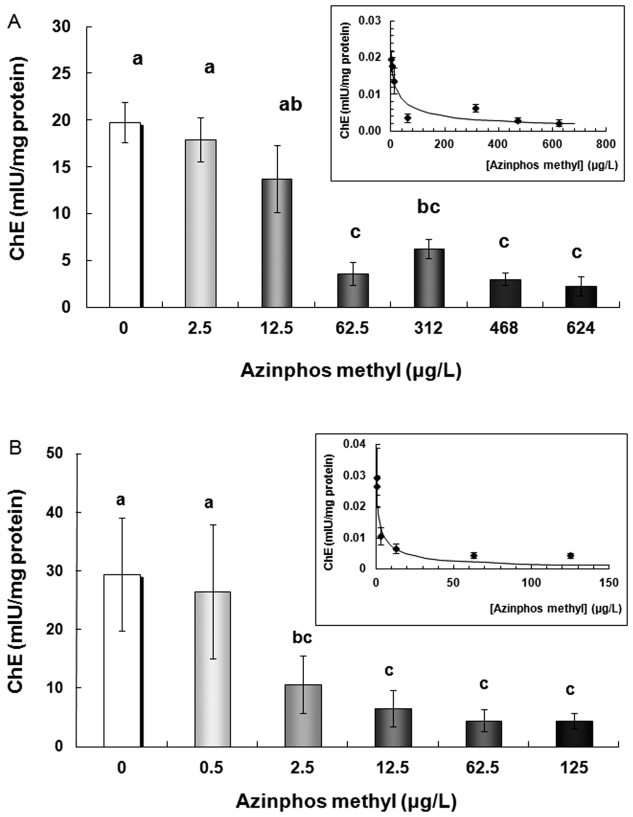

Fig. 2. ChE activity of *H. curvispina* amphipods exposed to different concentrations of AM. (A) 48 h of exposure. (B) 96 h of exposure. Data represent mean ± SE of six replicates. Different letters indicate significant differences ($p < 0.05$). Insets correspond to the nonlinear fitting of sigmoidal model to data.

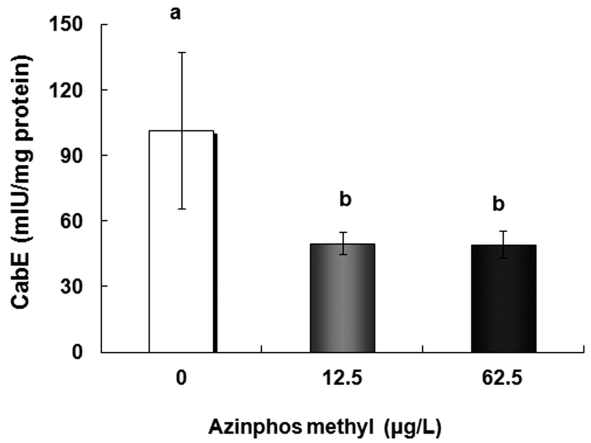

Fig. 3. CabE activity of *H. curvipina* exposed to AM during 48 h. Data represent mean ± SE of six replicates. Different letters indicate significant differences ($p < 0.05$).

inhibited (between 68 and 89%) after 48 h of exposure to concentrations of AM equal to or higher than 62.5 µg/L. By 96 h of exposure to AM, concentrations between 2.5 and 125 µg/L caused significant inhibition of ChE activity (64–85%). The IC_{50} values obtained after 48 and 96 h of AM exposure are listed in Table I. There was a noticeable and significant 10-fold decrease in the IC_{50} value after 96 h of exposure when compared with the 48-h value ($p = 0.02$ determined by Student's *t*-test performed on log-transformed data).

Detoxifying/Antioxidant Enzymes Activity and GSH Content

CabE activity was significantly inhibited after 48 h of exposure to 12.5 and 62.5 µg/L of AM (Fig. 3). The percentage of inhibition, approximately 51%, was similar for both concentrations tested.

The exposure to 62.5 and 125 µg/L of AM during 24, 48, 72, and 96 h did not cause significant changes in GST activity compared with the corresponding control values obtained at each time of exposure (Factorial ANOVA, interaction time × treatment not significant) [Fig. 4(A)].

On the other hand, CAT activity was significantly increased after 24 and 48 h of exposure to 125 µg/L AM (109 and 257% vs. control, respectively), whereas it returned to control values after 72 and 96 h of exposure [Fig. 4(B)]. The lowest concentration assayed, 62.5 µg/L AM, did not cause significant changes in CAT activity with respect to control values.

Similarly, GSH content was significantly increased in *H. curvispina* exposed to 125 µg/L AM during 24 and 48 h (68 and 119%, respectively). In turn, the concentration of 62.5 µg/L AM caused a significant increase in GSH content after 24 h (43%), whereas it caused a decrease of 28% after 72 h of exposure [Fig. 4(C)]. At the end of the assay (96-h exposure), no differences between treatments could be observed.

Risk Assessment

The species sensitivity distribution analysis clearly showed two groups of organisms differing in their susceptibility to AM [Fig. 5(A,B)]. Most of the amphipod species were highly susceptible and their logarithmic LC_{50} values for

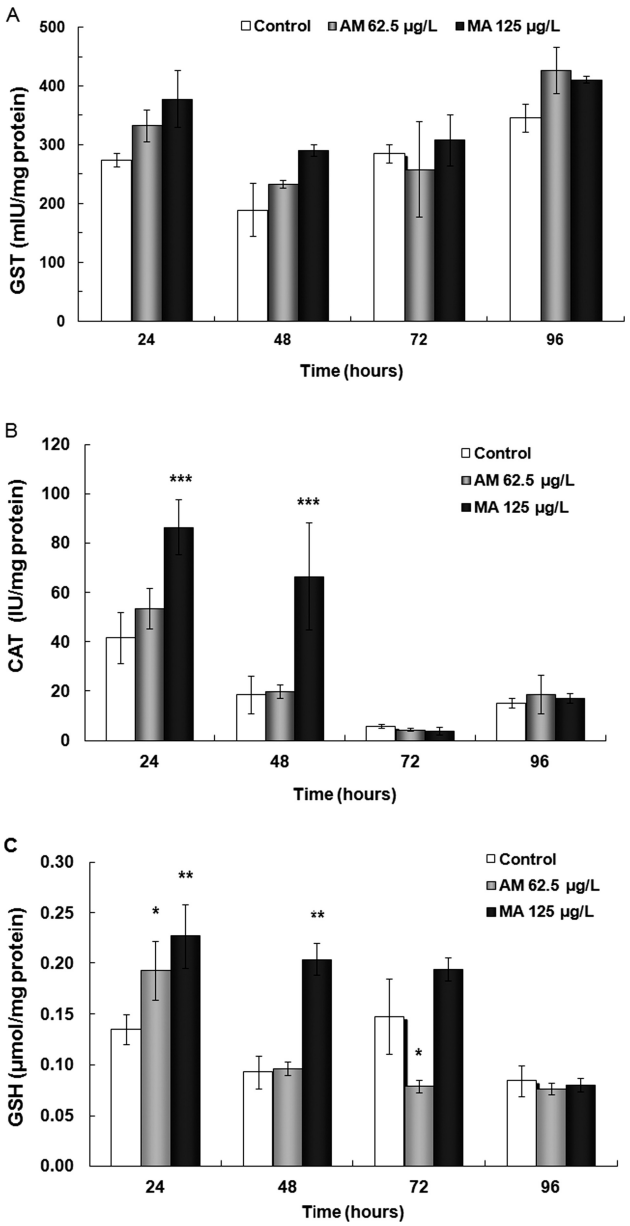

Fig. 4. Antioxidant/detoxifying enzymes activity and glutathione (GSH) levels of *H. curvispina* exposed to AM from 24 to 96 h. (A) GST, (B) CAT, and (C) GSH content. Data represent mean ± SE of six replicates. Asterisks indicate significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

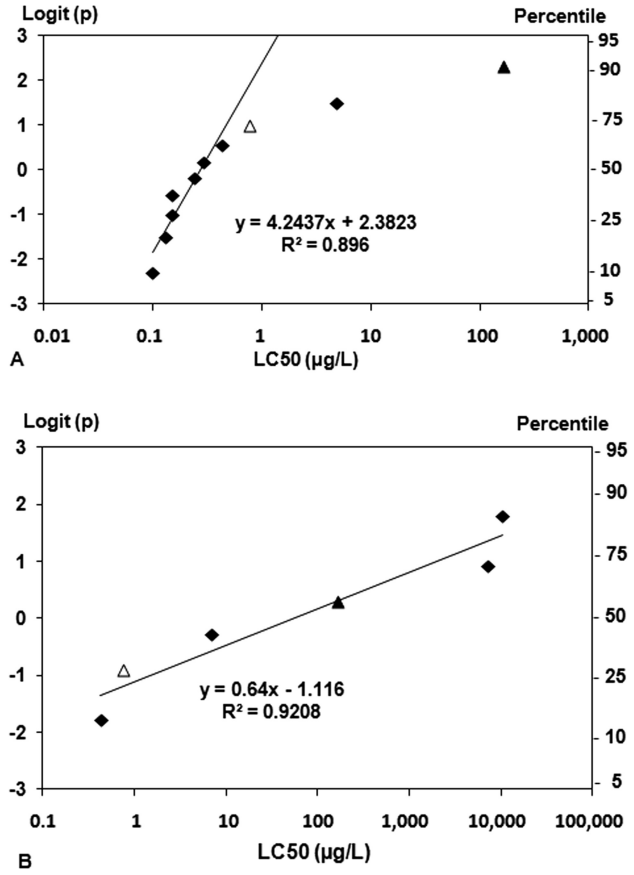

Fig. 5. Distribution of AM acute toxicity values for freshwater aquatic organisms. Probit transformation of percentile rank was fitted as a function of log-transformed LC_{50} for (A) amphipod species listed in Table II; (B) other aquatic organisms including local ones, with LC_{50}s determined at our laboratory (Ferrari et al., 2004a,b). The susceptible (open triangle) and resistant (dark triangle) subpopulations of *H. curvispina* from irrigation channels of F. Oro are shown in both species sensitivity distributions, but were included for model fitting in the group of local aquatic organisms. Logit(p) $= \ln(p/(100 - p))$; p = percentile.

AM adopted a normal distribution as indicated by the probit fitting. The exceptions were *Echinogammarus tibaldii* and both subpopulations of *H. curvispina* collected in irrigation channels of the fruit-producing area (F. Oro). Both subpopulations of *H. curvispina* as well as *H. curvispina* from the pristine site were included in the second group of local freshwater organisms, which displayed lower sensitivity to AM [Fig. 5(B)]. The maximum concentration of AM reported in subsuperficial drainage waters in the Rio Negro valley, 22.48 μg/L (Loewy et al., 2011), was introduced in the sensitivity distribution models fitted to amphipod species and to the aquatic organisms evaluated in our previous studies. As a result, 99.9% of amphipod species would be potentially affected when exposed to the maximal concentration of AM detected, whereas 43.8% of local aquatic organisms, previously studied in our laboratory, would be affected [Fig. 5(B)]. The maximum concentrations of AM detected in irrigation channels—1.77 μg/L—and in the Rio Negro river—1.19 μg/L (Tossi et al., 2009), would affect 96.9 and 93.7% of amphipod species, and 27.7 and 25.6% of local aquatic organisms, respectively. The concentration of AM which would affect the 10th percentile of local aquatic species was calculated to be 0.028 μg/L AM from the fitted distribution equation.

DISCUSSION

The acute toxicity data obtained after 96 h of exposure to the insecticide AM confirm the coexistence of two subpopulations of *H. curvispina* which differs in their susceptibility to this insecticide in the irrigated valley of Rio Negro, North Patagonia Argentina. As we previously described, the frequent application of this insecticide in the Río Negro valley and the dynamic nature of the irrigation channels within this region seems to be the cause of the appearance of two subpopulations with different susceptibilities to this toxicant (Anguiano et al., 2008). Amphipods, as well as other aquatic invertebrates, are subjected to a high-selection pressure owing to the pesticides that reach the channels during the productive season. Those organisms that are able to survive pesticide exposure may refuge in the persistent ponds during autumn and winter. Once irrigation is reestablished, new individuals coming from unexposed areas are introduced in these channels (Anguiano et al., 2008). It is interesting to note that the susceptible subpopulation of *H. curvispina* presented a LC_{50} value close to that of other amphipod species [Fig. 5(A)] and to *H. curvispina* from a pristine area. On the other hand, the high value of LC_{50} obtained for the resistant subpopulation of *H. curvispina* from F. Oro suggests that these organisms have suffered an important selection pressure by AM. Compared to other amphipod species, *H. curvipina* from F. Oro presents less susceptibility to AM, as indicated by the corresponding LC_{50} values listed in Table II and demonstrated by the sensitivity distribution curve shown in Figure 5(A). Only the amphipod *E. tibaldii* showed a greater tolerance to AM when compared with the susceptible subpopulation of *H. curvispina* from F. Oro (Table II and Fig. 5(A)). Nevertheless, the maximum environmental concentrations of AM detected in irrigation channels and in the Río Negro river let us infer that most of the amphipod species would be threatened, and that a percentage of local aquatic species could also be affected. Thus, AM levels pose an acute hazard to the sensitive subpopulation of *H. curvispina*, both in the irrigation system and in the Rio Negro river, despite its high flow (866.05 m^3/s). The intensive application of AM, regarding the frequency (every 15 days during the productive season November–March) and amount employed (0.7 kg of the active ingredient per hectare) (Anguiano and

TABLE II. Comparative acute toxicity of the insecticide AM to different amphipod species

Species	ESTADIO	TIME of Exposure (h)	Chemical Description	LC_{50} (μg/L) (LC 95%)
G. fasciatus	Adult	96	88–100% purity	0.15 (0.11–0.20)[a]
G. fasciatus	Adult	48	Technical	0.16 (0.08–0.32)[b]
G. fasciatus	Adult	96	Technical	0.10 (0.07–0.14)[b]
G. italicus	♂ Adult	96	99% Purity	0.24 (0.22–0.27)[c]
E. tibaldii	Adult	96	99% Purity	4.8 (4.3–5.5)[c]
G. lacustris	2-Month old	48	Technical	0.25 (0.19–0.35)[d]
G. lacustris	2-Month old	96	Technical	0.15 (0.11–0.20)[d]
G. lacustris	NR	96	Emulsifiable concentrate	0.13[e]
H. azteca	Juvenile (7–14 days old)	96	95–99% Purity	0.29 (0.20–0.42)[f]
H. curvispina	Adult (subpopulation A)	48	98.3% Purity	2.54 (2.02–3.06)[g]
	Adult (subpopulation B)			385.2 (343.2–427.2)[g]
H. curvispina	Adult (subpopulation A)	96	98.3% Purity	0.77 (0–2.1)[h]
	Adult (subpopulation B)			166.7 (110.7–222.7)[h]

The bioassays were performed under static conditions. NR, not reported. Subpopulation A: subpopulation of *H. curvispina* from F. Oro more susceptible to AM. Subpopulation B: subpopulation of *H. curvispina* from F. Oro more tolerant to AM.

[a] Johnson and Finley (1980).
[b] Mayer and Ellersieck (1986).
[c] Pantani et al. (1997).
[d] Sanders (1969).
[e] Nebeker and Gaufin (1964).
[f] Ankley and Collard (1995).
[g] Anguiano et al. (2008).
[h] This study (Table I).

Pechen de D'Angelo, 2007) enables AM to reach surface waters of the Rio Negro and Neuquén valley. Other studies indicated that *Hyalella* species were affected by pesticide runoff in water bodies within farming zones. Moore et al. (2007) reported that *H. azteca* growth was impaired after exposure to sediment and surface waters contaminated with herbicides and insecticides owing to runoff from adjacent agricultural areas. Jergentz et al. (2004) observed high mortality of *H. curvispina* during *in situ* assays performed in small agricultural streams in the main soybean area of Argentina, owing to chlorpyrifos and cypermethrin contamination.

ChE of *H. curvispina* showed moderate sensitivity to AM; the enzyme was significantly inhibited in a concentration-dependent manner. The inhibition achieved was also dependent on the time of exposure, as demonstrated by the IC_{50} values obtained after 48 and 96 h of exposure. The 48 h-IC_{50} value for ChE inhibition was 13.6 times higher than the corresponding IC_{50} value for 96 h. Similarly, Xuereb et al. (2009) reported that ChE inhibition in the amphipod *Gammarus fossarum* caused by the OP chlorpyrifos was dependent on both concentration and time of exposure. Besides, the IC_{50} values obtained after 48 and 96 h of exposure were 11.7 and 2.8 times higher than the corresponding LC_{50} values for the susceptible subpopulation and 13 and 76 times lower than the LC_{50} of the tolerant one. ChE activity in unexposed individuals could not be separated in different groups and thus we could not assess different levels of activity in each subpopulation. Besides, ChE inhibition curves did not reflect a pattern which could have suggested the presence of different subpopulations in terms of enzymatic activity, that is, a biphasic response. This could be owing to the fact that the surviving amphipods in which ChE were determined probably belonged to the AM-resistant subpopulation. The results obtained indicate that ChE activity could be useful as an early biomarker mainly for the tolerant subpopulation. Jemec et al. (2010) have stated that biochemical markers in some cases are not more sensitive than the whole organisms' response. Moreover, in the invertebrate *Helicoverpa zea* exposed to OP, no correlation between mortality and ChE inhibition was observed (Massa et al., 2008). In invertebrates, the relationship between ChE inhibition and mortality is not clear owing to differences among species and anticholinesterasic compounds (Fulton and Key, 2001; Xuereb et al., 2009).

CabE was also significantly inhibited by both 12.5 and 62.5 μg/L AM after 48 h of exposure. The degree of inhibition was similar for both concentrations (51%), and it would correspond to the response of the resistant subpopulation as no individuals from the sensitive subpopulation are expected to survive those levels of exposure. At the concentration of 12.5 μg/L AM, ChE was not affected, whereas CabE resulted in 51% of inhibition. This suggests a greater sensitivity of the detoxifying enzyme compared to ChE, together with a good protection of ChE by sequestration of the insecticide (Sogorb and Vilanova, 2002). This was also reported for other aquatic species (Barata et al., 2004; Ferrari et al., 2011). However, ChE activity inhibition by AM could not be prevented by CabE sequestration at the highest concentration tested (62.5 μg/L AM).

ChE inhibition has been associated with oxidative stress generation (Milatovic et al., 2006). In addition, antioxidant and detoxifying enzymes have been reported to be affected by anticholinesterasic compounds in aquatic invertebrate species (Barata et al., 2007; Kristoff et al., 2008). In this study, we observed a protective response of *H. curvispina* amphipods exposed to AM during the first 24 and 48 h, characterized by a significant increase in CAT activity and GSH content with respect to control values. CAT is one of the most relevant antioxidant enzymes, and its activity has been reported to increase owing to OP exposure in different organisms (Bianchini and Monserrat, 2007; Coelho et al., 2011). Besides, Kim et al. (2010) have recently observed in the microcrustacean *Daphnia magna* a significant increase of CAT activity along with an increase in CAT mRNA expression owing to pro-oxidative stressors exposure. Similarly, in digestive gland of mussels CAT transcription was induced because of exposure to the OP chlorpyrifos (Canesi et al., 2011). Thus, the increase of CAT activity observed in this study could result from induction of enzyme expression.

GSH is the main cellular antioxidant and the ability to sustain high levels of this tripeptide has been associated with an increase in pesticide tolerance in two species of marine bivalves (Peña-Lopis et al., 2002). GST activity was also reported to increase as a result of OP exposure owing to its role in the detoxification process of these compounds (Jokanović, 2001). Furthermore, the increase of GST activity has been associated with pesticide tolerance in amphibian embryos (Anguiano et al., 2001). In spite of this fact, AM exposure did not cause any significant change in the activity of this detoxifying enzyme in this study, in accordance with the reports for other invertebrate species (Jemec et al., 2007; Kristoff et al., 2008; Massa et al., 2008). Besides, the amphipods were unable to sustain the antioxidant protective response, as CAT activity and GSH levels returned to control values at 72 and 96 h of exposure. A similar dual behavior of CAT activity and/or GSH levels with respect to the duration of exposure to xenobiotics was also observed in other aquatic organisms (Pinho et al., 2005; Ferrari et al., 2007). Pinho et al. (2005) reported a significant increase of CAT activity in crabs exposed during 2 days to microcystin, whereas the enzymatic activity decreased below control values after 1 week of exposure. Similarly, juvenile *Oncorhynchus mykiss* exposed 24 h to the insecticide carbaryl showed an increase in CAT activity and GSH levels, whereas both parameters were significantly decreased after 96 h of exposure (Ferrari et al., 2007).

CONCLUSIONS

In conclusion, acute toxicity tests confirm the presence of two subpopulations of the amphipod *H. curvispina* with different susceptibilities to the insecticide AM in the irrigation channels of insecticide-treated area of Rio Negro valley, North Patagonia, Argentina. The probabilistic analysis of species sensitivity distribution for AM including the toxicity data obtained in this study reveals that previously reported concentrations of AM in drainage, irrigation channels, and in the Río Negro river pose an acute risk for the susceptible subpopulation of *H. curvispina*. In addition, *H. curvispina* esterases could be used as biomarkers of AM exposure/response, mainly for the most tolerant subpopulation. Besides, AM was able to transiently increase CAT and GSH, indicating that an antioxidant response is elicited by this compound in the exposed amphipods. Those responses should not be considered good biomarkers by themselves but could be useful if evaluated together with esterase activities, as suggested previously for other species (Lionetto et al., 2003; Barata et al., 2007; Rosenbaum et al., 2012). The results obtained in this study point out the usefulness of including *in situ*-collected species like *H. curvispina* amphipods in monitoring programs to perform pesticide hazard assessment in agricultural sites.

Ferrari, A and Venturino A are staff researchers of CONICET. The authors thank Dr. Cecilia Inés Lascano for her critical reading of the manuscript.

REFERENCES

Akaike H. 1974. A new look at the statistical model identification. IEEE Trans Autom Control 19:716–723.

Anguiano OL, Caballero A, Pechen de D'Angelo AM. 2001. The role of glutathion conjugation in the regulation of early toad embryo's tolerance to pesticides. Comp Biochem Physiol 128:35–43.

Anguiano OL, Pechen de D'Angelo AM. 2007. Provincia de Río Negro y Provincia de Neuquén. In: Ministerio de Salud, Secretaría de Ambiente y Desarrollo Sustentable: AAMMA, editors. La problemática de los agroquímicos y sus envases, la incidencia en la salud de los trabajadores, la población expuesta y el ambiente. Ministerio de Salud de la Nación, OPS: AAMMA, Buenos Aires, pp181–201.

Anguiano OL, Ferrari A, Soleño J, Martínez MC, Venturino A, Pechen de D'Angelo AM, Montagna CM. 2008. Enhanced esterase activity and resistance to azinphosmethyl in target and nontarget organisms. Environ Toxicol Chem 27:2117–2123.

Barata C, Solayan A, Porte C. 2004. Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. Aquat Toxicol 66:125–139.

Barata C, Damasio J, López MA, Kuster M, López de Alda M, Barceló D, Riva MC, Raldúa D. 2007. Combined use of biomarkers and in situ bioassays in *Daphnia magna* to monitor environmental hazards of pesticides in the field. Environ Toxicol Chem 26:370–379.

Beers RF, Sizer IW. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195:133–140.

Bianchini A, Monserrat JM. 2007. Effects of methyl parathion on *Chasmagnathus granulatus* hepatopancreas: Protective role of sesamol. Ecotoxicol Environ Saf 67:100–108.

Canesi L, Negri A, Barmo C, Banni M, Gallo G, Viarengo A, Dondero F. 2011. The organophosphate chlorpyrifos interferes with the responses to 17β-estradiol in the digestive gland of the marine mussel *Mytilus galloprovincialis*. PLoS 6:e19803.

Coelho S, Oliveira R, Pereira S, Musso C, Domingues I, Bhujel RC, Soares AM, Nogueira AJ. 2011. Assessing lethal and sublethal effects of trichlorfon on different trophic levels. Aquat Toxicol 103:191–198.

Dary O, Georghiou GP, Parsons E, Pasteur N. 1990. Microplate adaptation of Gomori's assay for quantitative determination of general esterase activity in single insects. J Econ Entomol 83:2187–2192.

ECOFRAM. 1999. Risk Assessment Methods Report of the Aquatic Workgroup. U.S. Environmental Protection Agency, Office of Pesticide Programs, Ecological Committee on Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Washington, DC.

Ellman BL. 1959. Tissue sulfhydryl group. Arch Biochem Biophys 82:70–77.

Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95.

Ferrari A, Anguiano OL, Soleño J, Venturino A, Pechen de D'Angelo AM. 2004a. Different susceptibility of two aquatic vertebrates (*Oncorhynchus mykiss* and *Bufo arenarum*) to azinphos methyl and carbaryl. Comp Biochem Physiol C Toxicol Pharmacol. 9:239–243.

Ferrari A, Venturino A, de D'Angelo AM. 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.

Ferrari A, Venturino A, Pechen de D'Angelo AM. 2007. Effects of carbaryl and azinphos methyl on juvenile rainbow trout (*Oncorhynchus mykiss*) detoxifying enzymes. Pest Biochem Physiol 88:134–142.

Ferrari A, Lascano CI, Anguiano OL, D'Angelo AM, Venturino A. 2009. Antioxidant responses to azinphos methyl and carbaryl during the embryonic development of the toad *Rhinella (Bufo) arenarum* Hensel. Aquat Toxicol 93:37–44.

Ferrari A, Lascano C, Pechen de D'Angelo AM, Venturino A. 2011. Effects of azinphos methyl and carbaryl on *Rhinella arenarum* larvae esterases and antioxidant enzymes. Comp Biochem Physiol C Toxicol Pharmacol 3:34–39.

Fulton MH, Key PB. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem 20: 37–45.

García ME, Rodrígues Capítulo A, Ferrari L. 2010. Age-related differential sensitivity to cadmium in *Hyalella curvispina* (Amphipoda) and implications in ecotoxicity studies. Ecotoxicol Environ Saf 73:771–778.

Habig WH, Pabst MJ, Jacoby WB. 1974. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139.

Jemec A, Drobne D, Tisler T, Trebse P, Ros M, Sepcić K. 2007. The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test. Comp Biochem Physiol C Toxicol Pharmacol 144:303–309.

Jemec A, Drobne D, Tisler T, Sepcic K. 2010. Biochemical biomarkers in environmental studies-lessons learnt from enzymes catalase, glutathione S-transferase and cholinesterase in two crustacean species. Environ Sci Pollut Res Int 17:571–581.

Jergentz S, Pessacq P, Mugni H, Bonetto C, Schulz R. 2004. Linking in situ bioassays and population dynamics of macroinvertebrates to assess agricultural contamination in streams of the Argentina pampa. Ecotoxicol Environ Saf 59:133–141.

Johnson WW, Finley MT. 1980. Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates. Resource Publ. 137. Fish Wild. Serv., USDI, Washington, DC.

Jokanović M. 2001. Biotransformation of organophosphorus compounds. Toxicology 166:139–160.

Kim J, Kim S, An K W, Choi C Y, Lee S, Choi K. 2010. Molecular cloning of *Daphnia magna* catalase and its biomarker potential against oxidative stresses. Comp Biochem Physiol 152: 263–269.

Kristoff G, Guerrero NV, de D'Angelo AM, Cochón AC. 2006. Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: *Biomphalaria glabrata* and *Lumbriculus variegatus*. Toxicology 222:185–194.

Kristoff G, Verrengia Guerrero NR, Cochón AC. 2008. Effects of azinphos-methyl exposure on enzymatic and non-enzymatic antioxidant defenses in *Biomphalaria glabrata* and *Lumbriculus variegatus*. Chemosphere 72:1333–1339.

Lionetto MG, Caricato R, Giordano ME, Pascariello MF, Marinosci L, Schettino T. 2003. Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. Mar Pollut Bull 46:324–330.

Loewy RM, Carvajal LG, Novelli M, Pechen de D'Angelo AM. 2006. Azinphos methyl residues in shallow groundwater from the fruit production region of northern Patagonia, Argentina. J Environ Sci Health B 41:869–881.

Loewy RM, Monza LB, Kirs VE, Savini MC. 2011. Pesticide distribution in an agricultural environment in Argentina. J Environ Sci Health B 46:662–670.

Lowry OH, Rosebroung NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Massa R, Blevins S, Chao SL. 2008. Role of acetylcholinesterase and glutathione S-transferase following exposure to nicosulfuron and diazinon in *Helicoverpa zea*. Ecotoxicol Environ Saf 71:230–235.

Mayer FLJ, Ellersieck MR. 1986. Manual of acute toxicity: Interpretation and data base for 410 chemicals and 66 species of freshwater animals. U. S. Department of Interior Bur Sports Fish Wildlife Tech.

Milatovic D, Gupta RC, Aschner M. 2006. Anticholinesterase toxicity and oxidative stress. Sci World J 6:295–310.

Montagna CM, Pechen de D'Angelo AM, Venturino A. 2011. Toxicology of the Herbicide Acrolein: Risk Assessment in

Aquatic Environments. In: Kortejkamp A, editor. Herbicides and environment. Rijeka, Croatia: Intech p 405–420.

Moore MT, Lizotte RE Jr, Knight SS, Smith S Jr, Cooper CM. 2007. Assessment of pesticide contamination in three Mississippi Delta oxbow lakes using *Hyalella azteca*. Chemosphere 67:2184–2191.

Mugni H, Ronco A, Bonetto C. 2011. Insecticide toxicity to *Hyalella curvispina* in runoff and stream water within a soybean farm (Buenos Aires, Argentina). Ecotoxicol Environ Saf 74:350–354.

Nebeker AV, Gaufin AR. 1964. Bioassays to determine pesticide toxicity to the amphipod crustacean, *Gammarus lacustris*. Environ Toxicol Chem 8:705–718.

Oruç EÖ, Üner N. 2004. Tissue specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comp Biochem Physiol C 137:43–51.

Pantani C, Pannunzio M, De Cristofaro M, Novelli AA, Salvatori M. 1997. Comparative acute toxicity of some pesticides, metals, and surfactants to *Gammarus italicus* Goedm. and *Echinogammarus tibaldii* Pink. and Stock (Crustacea: Amphipoda). Bull Environ Contam Toxicol 59:963–967.

Peña-Llopis S, Ferrando MD, Peña JB. 2002. Impaired glutathione redox status is associated with decreased survival in two organophosphate-poisoned marine bivalves. Chemosphere 47:485–497.

Peralta MA. 2001. Crustacea Eumalacostraca. Guía para la determinación de los artrópodos bentónicos sudamericanos. Tucumán, Argentina: Universidad Nacional de Tucumán.

Pinho GLL, Moura da Rosa C, Maciel FE, Bianchini A, Yunesc JS, Proença LAO, Monserrat JM. 2005. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol Environ Saf 61:353–360.

Rosenbaum EA, Duboscq L, Soleño J, Montagna CM, 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.

Sanders HO. 1969. Toxicity of pesticides to the crustacean *Gammarus lacustris*. U.S. Department of Interior Bur Sports Fish Wildlife Tech. Paper 25.

Sogorb MA, Vilanova E. 2002. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol Lett 128:215–228.

Tarshis S. 2000. Crustaceans can tell us to clean up our act. Agricultural Research 48:9.

Tossi AP, Pechen de D'Angelo AM, Savini MC, Loewy RM. 2009. Assessing pesticide hazards on surface water from the northern patagonian región Argentina. Acta Toxicol Argent 17:1–7.

U.S. Environmental Protection Agency. 1996. Ecological effects test Guidelines: OPPTS 850.1020 Gammarid acute toxicity test. EPA/712/C-96/130. Prevention, Pesticides and Toxic Substances, Washington, DC.

Venturino A, Gauna LE, Bergoc RM, Pechen de D'Angelo AM. 1992. Effect of exogenously applied polyamines on malathion toxicity in the toad *Bufo arenarum* Hensel. Arch Environ Contam Toxicol 22:135–139.

Vioque-Fernández A, de Almeida EA, López-Barea J. 2007. Esterases as pesticide biomarkers in crayfish (*Procambarus clarkii*, Crustacea): Tissue distribution, sensitivity to model compounds and recovery from inactivation. Comp Biochem Physiol C Toxicol Pharmacol 145:404–412.

Xuereb B, Lefèvre E, Garric J, Geffard O. 2009. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): Linking AChE inhibition and behavioural alteration. Aquat Toxicol 94:114–122.