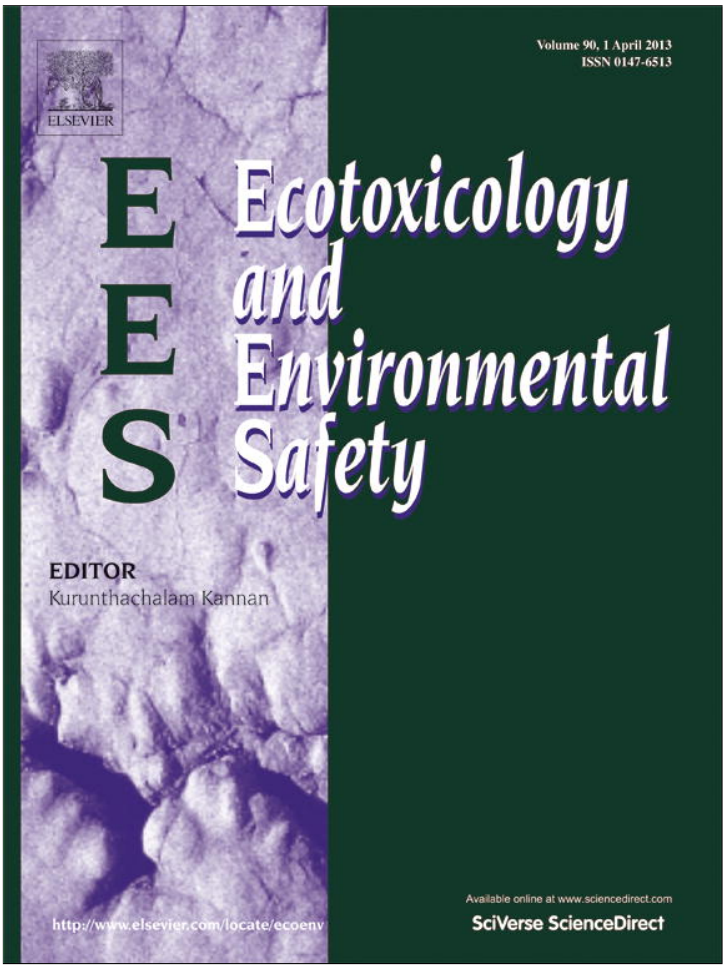

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Different effects of subchronic exposure to low concentrations of the organophosphate insecticide chlorpyrifos in a freshwater gastropod

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ABSTRACT

Chlorpyrifos is an organophosphate insecticide used for pest control on a number of food crops in many parts of the world. In recent years, there has been an important decrease in the number of organisms of *Planorbarius corneus*. Since the presence of pesticides in the water can be one of the reasons for this decrease, it is very important to study the effect of subchronic exposure to environmental concentrations of pesticides on these organisms. The aim of the present work was to investigate different effects of the subchronic exposure to low concentrations of the organophosphate chlorpyrifos in *P. corneus* and the possibility to use these as biomarkers. To this end, we have exposed the organisms to 0.4 and 5 $\mu g\,L^{-1}$ of chlorpyrifos for 14 days and recorded the number of egg masses, the number of eggs per mass, the number of eggs without embryo, the time for hatching, and the % of hatching and survival. We have also determined the activities of cholinesterases, carboxylesterases and glutathione S-transferase in whole organism soft tissue and in the gonads. A 14 days exposure to 0.4 $\mu g\,L^{-1}$ caused an increase in the number of egg masses without eggs and a decrease in carboxylesterases measured with *p*-nitrophenyl butyrate. However the exposure to 5 $\mu g\,L^{-1}$ also caused an increase in the time for hatching, a decrease in the % of hatching and survival and also inhibition of cholinesterases and carboxylesterases with *p*-nitrophenyl acetate and butyrate. In contrast, the glutathione S-transferase has not been modified with the tested concentrations. We concluded that when *P. corneus* exposed to chlorpyrifos for 14 days, the CES determined with *p*-nitrophenyl butyrate proved to be the most sensitive biomarker. However, exposure to environmental concentrations showed a decrease in the reproduction ability which could cause a decrease in the number of organisms of this species.

1. Introduction

Chlorpyrifos is an organophosphate (OP) insecticide widely used in a variety of food crops to control a great number of insects and is frequently detected in surface waters around the world (Palma et al., 2009). The values of chlorpyrifos concentrations estimated for surface waters reported by the Environmental Protection Agency range between 0.026 and 0.4 $\mu g\,L^{-1}$ (EPA USEPA, 2006). However, both direct and indirect applications can cause higher chlorpyrifos concentrations in small streams and wetlands adjacent to agricultural fields than those estimated by EPA (3.7–700 $\mu g\,L^{-1}$) as reported by Moore et al. (2002) and Wood and Starck (2002). Chlorpyrifos has a relatively persistent nature compared to other organophosphorus insecticides, with a half life in water ranging from 29 to 74 days (Racke, 1993; EPA USEPA, 2006; Palma et al., 2009).

OPs are thought to exert their toxicity by binding to acethylcholinesterase, which hydrolyzes the neurotransmitter acetylcholine, inhibiting the action of this enzyme. This causes the accumulation of acetylcholine in synapses and, consequently, an overstimulation of neurotransmission followed by depression or paralysis and eventual death. Cholinesterases (ChEs) are a family of enzymes that hydrolyze choline esters and belong to the B esterases groups: esterases that are inhibited by OPs (Sanchez-Hernandez, 2007). In aquatic invertebrates, it has been reported that ChEs differ in many aspects from either vertebrate acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) (Bocquené et al., 1997; Sanchez-Hernandez, 2007). The ChEs of aquatic invertebrates generally show a preference for acetylthiocholine (AcSCh) as substrate, however, there are some species that hydrolyze propionylthiocoline (PrSCh) faster than

Abbreviations: CES, carboxylesterases; ChE, cholinesterase; CI_{50}, concentration that produce 50% of inhibition; DTNB, 5,5-dithio-2-bis-nitrobenzoate; G, gonads; GST, glutation S-transferase; OP, organophosphate insecticide; *p*-NPA, *p*-nitrophenyl acetate; *p*-NPB, *p*-nitrophenyl butyrate; T, whole organism soft tissue.

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AcSCh (Basack et al., 1998; Hannam et al., 2008; Mora et al., 1999; Talesa et al., 1990; Varó et al., 2002). Assessment of ChE inhibition in wildlife population has been proposed as a general method for detecting environmental contamination from OPs, particularly since many of these chemicals have relatively short half lives in the aquatic environment and a rapid metabolism in biota (Gagnaire et al., 2008; Lacorte et al., 1995; WHO, 1986). In contrast, after being exposed to Ops, ChE recovery in organisms is very slow. Therefore, enzymatic inhibition can be detected although there is no longer pesticide in the water (Escartin and Porte, 1996; Ferrari et al., 2004; Kristoff et al., 2006, 2011, 2012; Kumar et al., 2010; Rodríguez, 2009). This may offer an advantage in monitoring OPs over the use of chemical analysis alone (Arufe et al., 2007).

Carboxylesterases (CES) are another type of B-esterases. These enzymes catalyze the hydrolysis of a wide range of exogenous and endogenous esters and are assumed to play a protective role in anticholinesterase intoxication by removing a significant amount of pesticide by two main mechanisms: the detoxification by hydrolysis of ester bonds in some of these pesticides and by providing alternative sites of OP binding (Jokanovic, 2001; Sanchez-Hernandez, 2007).

Glutathione S-transferase (GST) belongs to a phase II family of detoxifying enzymes. Mainly by the action of this enzyme, glutathione (GSH) can form conjugates with a wide variety of electrophilic compounds. This conjugation is essential for the detoxification of xenobiotics but also for maintaining the normal physiological metabolism (Strange et al., 2000). For this reason, GST activity can be used as a biomarker of effect.

OPs can produce other adverse effects in exposed organisms. Therefore, other parameters such as oxidative stress, hematological, immunological, genotoxic and reproductive parameters can be studied to give information of the biological effects of pesticides on the test species and to be used as biomarkers.

Chlorpyrifos has been used in different *in vivo* toxicity tests in aquatic invertebrates. It has been reported that chlorpyrifos decreases ChE activity of *Artemia salina, Artemia parthenogenetica, Biomphalaria glabrata*, *Corbicula fluminea*, *Daphnia magna*, *Gammarus pulex*, *Lamellidens marginalis*, *Lumbriculus variegatus*, *Paratya australiensis*, *Planorbarius corneus*, *Potamopyrgus antipodarum*, and *Procambarus clarkii* (Amanullah et al., 2010; Barata et al., 2004; Cacciatore et al., 2011; Cooper and Bidwell, 2006; Gagnaire et al., 2008; Kumar et al., 2010; Rodríguez, 2009; Varó et al., 2002; Vioque-Fernández et al., 2007; Xuereb et al., 2007) and CES activity of *B. glabrata, L. variegatus, P. clarkii* and *P. corneus in vivo* (Cacciatore et al., 2011; Rodríguez, 2009; Vioque-Fernández et al., 2007). However, less is known about the chronic effects of low concentrations of pesticides on more ecologically relevant endpoints such as growth and reproduction (Roex et al., 2003). Some authors have reported toxic effects due to chlorpyrifos on reproduction, survival and embryonic development in vertebrates (De Silva and Samayawardhena, 2005; Farag et al., 2010) and in invertebrates species (Jager et al., 2007; Li-Xia et al., 2009; Palma et al., 2009; Varó et al., 2006; Zaliznick and Nugegoda, 2006). In the case of crustacean, Palma et al. (2009) reported in *Daphnia magna* a reduction in the number of offspring produced per male and abnormalities including arrested eggs; Zaliznick and Nugegoda (2006) have studied the effect of chlorpyrifos on the next two generations of *Daphnia carinata* reporting that the pesticide affected survival and fecundity of animals in the first generation while in the second one, a longer time of hatching was observed. Varó et al. (2006) have studied the effect of chlorpyrifos on capsulated and decapsulated cysts of *Artemia* sp. showing that the pesticide caused a decrease in hatching and survival. However, little is known about the chlorpyrifos effect on the gastropods reproduction. Although gastropods in the case of Ops, are not the most sensitive group of organisms (Van Wijngaarden et al., 2005), freshwater gastropods represent about 20% of recorded mollusk extinctions (Strong et al., 2008).

P. corneus is a freshwater hermaphroditic gastropod that is distributed all over the world (Jopp, 2006). In previous works of our laboratory, we have characterized the activities of B-esterases and we have also reported that the exposure for 48 h to chlorpyrifos inhibits ChE and CES of whole organism soft tissue (Cacciatore et al., 2011, 2012). It has been reported that *P. corneus* population declines constantly (Jopp, 2006; Wiese, 2005) so it is very important to study different effects of the subchronic or chronic exposure to low concentrations of contaminants. In particular, the alterations on reproduction which have an important ecological significance because the survival of the species is determined by the success in giving birth to new individuals.

Chronic and subchronic exposures are relative terms especially in relation to the species. They are often designed according to the expected lifespans of the species involved (Barile, 2008). Generally, a chronic exposure implies an exposure of at least 10% of the lifetime. An acute exposure corresponds to until 96 h while the subchronic exposure involves a duration between the acute and the chronic exposure (Barile, 2008). In laboratory conditions, it has been registered that *P. corneus* can live until 2 years (Collins Baker, 1945), so a 14 days exposure should be considered a subchronic one.

The aim of the present work was to investigate different toxic effects of subchronic exposure to low concentrations of the OP chlorpyrifos, in order to find the most senstitive biomarker. To this end, we (1) determined ChE, CES and GST activities in whole organism soft tissue and in the gonads after 14 days of exposure. We chose to work on gonads to study the possible link of biochemical responses to the effects on the reproduction. Also, enzyme sensitivity to the pesticide was compared in whole organisms soft tissue and in the gonads (2) studied the effect of the pesticide on different parameters of reproduction in adult snails and egg masses exposed by recording the number of egg masses, the number of egg masses without eggs, the number of eggs per egg mass, the number of non-embryonated eggs per mass, the time for hatching, the percentage of hatching and the percentage of survival of the offspring.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AcSCh), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl butyrate (*p*-NPB), 5,5̄-dithio-2-bis-nitrobenzoate (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione and chlorpyrifos were purchased from Sigma-Aldrich of Argentina S.A. All other chemicals used were of analytical reagent grade.

2.2. Organisms

Adult *P. corneus* snails were purchased from Discus Morón S.R.L., Buenos Aires, Argentina. Afterwards the snails were reared in our laboratory in an aerated glass aquaria (17–20 L), at a temperature of 22 ± 2 °C, and under a 14:10 (L:D) h artificial photoperiod regime. For all the experiments, adult snails of similar size (12 ± 2 mm) were used.

2.3. Bioassays

To perform the bioassays, we used 1 L glass vessels, containing 800 mL for each solution (dechlorinated water, 0.001% of acetone in dechlorinated water, 0.4 μg L^{-1} of chlorpyrifos in dechlorinated water and 5 μg L^{-1} of chlorpyrifos in dechlorinated water). During the bioassay animals were fed once a week. No mortality was observed either in the control groups or in the treated groups. All the bioassays were performed at 22 ± 2 °C under a photoperiod of 14:10 (L:D) h. The following physico–chemical parameters were recorded: total hardness = 67 ± 3 mg $CaCO_3$ L^{-1}; alkalinity = 29 ± 2 mg $CaCO_3$ L^{-1}; pH 7.0 ± 0.2 and

conductivity=250 ± 17 μS. The stock solution of the insecticide was prepared in acetone. Chlorpyrifos concentration was tested by HPLC with UV detector (detection wavelength: 230 nm). Each concentration of chlorpyrifos was obtained by diluting the stock solution with dechlorinated water. To avoid pesticide degradation, the bioassays were performed by changing the solution of chlorpyrifos every day (Cacciatore et al., 2011; Gagnaire et al., 2008; Kumar et al., 2010). The constancy of the chlorpyrifos concentration in the aqueous solution during 24 h has been previously demonstrated in stability studies conducted in our laboratory (unpublished results of Cacciatore). The concentration of acetone in the bioassays was 0.001%, less than 0.002% as recommended by Hutchinson et al. (2006) in the case of reproduction studies with aquatic organisms. The concentrations used were 5 μg L^{-1} of chlorpyrifos (Sub 2), chosen because it produces 50% of inhibition of ChE after 48 h of exposure and 0.4 μg L^{-1} of chlorpyrifos (Sub 1) which does not cause inhibition of ChE after 48 h of exposure in the whole organism soft tissue (Cacciatore et al., 2011).

Reproduction bioassays were performed in the following way: four containers of six organisms in 800 mL of the test solutions were used for each group (water control, solvent control, Sub 1 and Sub 2). On day (- 4), all snails were placed in containers with dechlorinated water for 4 days. Then, on day (0) all the egg masses laid in the last 4 days were withdrawn and the solutions were changed for 14 days as follows: subchronic exposure groups in containers with 0.4 and 5 μg L^{-1} chlorpyrifos, respectively, and in 0.001% acetone as the solvent control in accordance to Kristoff et al. (2011).

Bioassays for ChE, CES and GST were performed in the following way: Two containers with 18 snails were prepared for each group: water control, solvent control, Sub 1 (0.4 μg L^{-1} of chlorpyrifos) and Sub 2 (5 μg L^{-1} of chlorpyrifos). After 14 days, six snails were homogenized separately to obtain six whole organism soft tissue homogenates. To obtain six gonads homogenates, 4–7 gonads were used for each homogenate. Adult snails used for the reproduction bioassays, after 14 days, were also used for the enzymatic determination when needed to achieve the amount of gonads required for the six gonads homogenates.

2.4. Homogenates

Animals were placed on ice for 6–8 min. The shells were carefully removed and the soft tissue was isolated at 0 °C. The body soft tissues were washed in distilled water, placed on filter paper to drain extra fluids, and weighed. For the homogenates from the whole organism soft tissue, we used one snail per homogenate, whereas for each gonad homogenate (G), we needed 4–7 gonads. Tissues were homogenized in 10 V of 20 mM Tris/HCl buffer, pH 7.5, plus 0.5 mM EDTA and homogenates were centrifuged at 11,000 × *g* for 20 min at 4 °C. The resulting supernatants were immediately used as enzymatic source.

2.5. Cholinesterase activity (ChE)

ChE activity was measured in the supernatant fraction, in 100 mM phosphate buffer, pH 8, 0.2 mM DTNB, acetylthiocholine iodide (AcSCh) 1.5 mM, according to the method of Ellman et al. (1961). AcSCh was used as substrate because it is the preferred substrate for ChE in this species (Cacciatore et al., 2012). The concentration of the substrate chosen allows us to work at the V_{max} of the enzyme. Activity was recorded continuously at 412 nm. Rates were corrected for spontaneous hydrolysis of the substrate and non-specific reduction of the chromogen by tissue extracts and specific activity was expressed as nmoles of product produced per min per mg protein.

2.6. Carboxylesterase activity (CES)

Hydrolysis of *p*-NPA and *p*-NPB by CES was determined, according to Ferrari et al. (2007). Reactions were performed in 2.5 mL 100 mM phosphate buffer pH 8.0 containing 5% acetone and 1 mM *p*-NPA or *p*-NPB. The concentration of the substrates chosen allows us to work at the V_{max} of the enzyme. Activity was continuously recorded at 400 nm. Specific activity was calculated using the molar extinction coefficient for *p*-nitrophenol (18.6 mM^{-1} cm^{-1}). Specific activity was expressed as nmol of *p*-nitrophenol produced per min per mg protein.

2.7. Glutation S-transferase activity (GST)

GST activity was assayed by the method of Habig et al. (1974). The reaction mixture contained 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM GSH, 10 μl of enzyme and 100 mM phosphate buffer pH 6.5 in a final volume of 3.0 mL. The formation of CDNB-GSH conjugate was evaluated by monitoring the increase in absorbance at 340 nm. Results are expressed as μmol of conjugate produced per min per mg protein.

2.8. Protein content

Protein content was determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.9. Parameters of reproduction and survival of the offspring

Along those 14 days, for each group (water control, solvent control, Sub 1 and Sub 2) the number of egg masses was registered and each egg mass was transferred to individual containers. For each egg mass, the number of eggs per egg mass, the number of non-embryonated eggs per egg mass, the number of egg masses without eggs, the time for hatching and the hatchings per egg mass were observed with a magnifying glass. At the moment of hatching, the pesticide (in the groups with pesticide) and the acetone (solvent control) were removed and replaced by dechlorinated water according to Kristoff et al. (2011). The offspring was fed with lettuce and living snails were counted after 1 month. The survival percentage was calculated for each egg-mass.

2.10. Data analysis

Results were expressed as mean ± SD. We used six replicates for each group to determine ChE, CES and GST and four replicates of six snails to register the number of egg masses for each group. Also, for the other parameters of reproduction all the egg masses for each group were used (approximately 70 egg masses). Data were analyzed by one-way ANOVA. Where overall *F* statistic was significant pair wise comparisons among treatment groups performed by Tukey HSD post test using VassarStats (http://vassarstats.net). The level of significance used was 0.05.

3. Results

3.1. Cholinesterase activity

Fig. 1 shows ChE activity in the whole organism soft tissue and in the gonads. No statiscally significant differences were observed between the control with water and the control with 0.001% acetone ($P > 0.05$). Therefore, only the results of the water control are shown. The exposure to 0.4 μg L^{-1} caused no significant inhibition of ChE in total homogenate and gonads ($P > 0.05$) while the exposure to 5 μg L^{-1} (CI_{50} of ChE at 48 h) produced a inhibition of approximately 90% after 14 days of exposure in total homogenate and gonads.

3.2. Carboxylesterase activity

With regard to the activity of CES, Fig. 2 shows the activity using *p*-NPA as substrate (A) and using *p*-NPB (B).

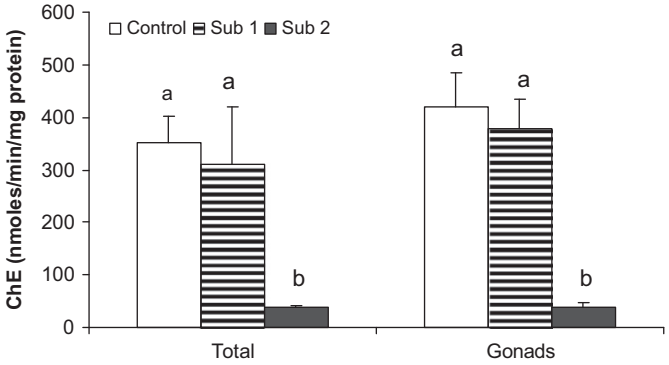

Fig. 1. Cholinesterase (ChE) activity in adult *P. corneus* exposed to Sub 1 (0.4 μg L^{-1}) and Sub 2 (5 μg L^{-1} of chlorpyrifos) for 14 days in whole organism soft tissue and gonads. The determination was carried out with ASCh as substrate, using Ellman's method. The data represent the mean ± SD of at least six snails, and six pools of 4–7 gonads. For each region, total and gonads, the data with different letters are statistically different ($P < 0.05$). No statistically significant differences were observed between water control and solvent control groups ($P > 0.05$). Therefore, only the results of the water control are shown.

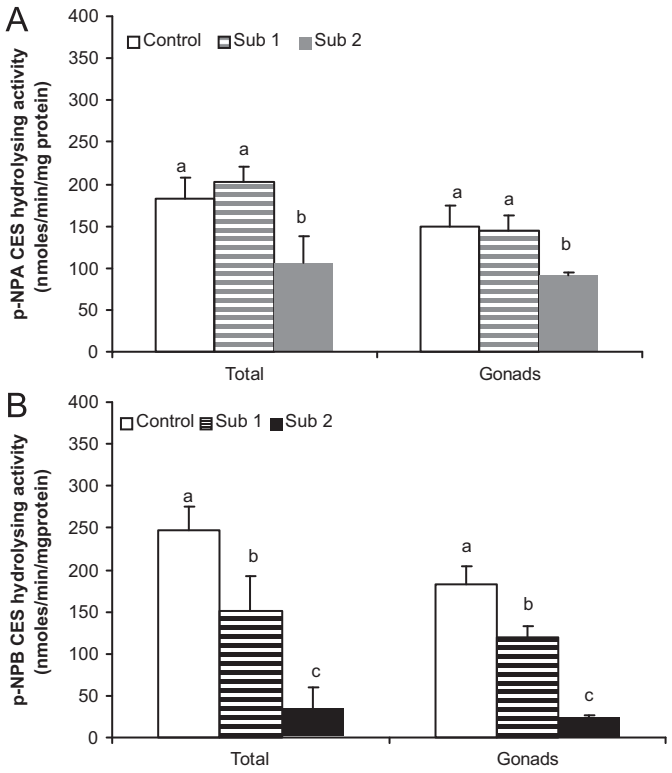

Fig. 2. Carboxylesterase (CES) activity using *p*-NFA (A) and *p*-NFB (B) as substrates in adult *P. corneus* exposed to Sub 1 (0.4 μg L^{-1}) and Sub 2 (5 μg L^{-1} of chlorpyrifos) in whole organism soft tissue and in gonads. The data represents the mean ± SD of at least six snails, and six pools of 4–7 gonads. For each region, the data with different letters are statistically different ($P < 0.05$). No statistically significant differences were observed between water control and solvent control groups ($P > 0.05$). Therefore, only the results of the water control are shown.

In the snails exposed to 0.4 μg L^{-1} we only observed inhibition of CES measured with *p*-NPB in total homogenate and in gonads (inhibition of 39 and 33%, respectively) while those exposed to 5 μg L^{-1} showed inhibition of CES with both substrates, 40% with *p*-NPA and 85% with *p*-NPB in T and G.

3.3. Glutation S-transferase activity (GST)

No statistically significant differences ($P > 0.05$) were observed in GST activity with the concentrations of chlorpyrifos tested respect to the control as shown in Fig. 3.

3.4. Number of egg masses, number of eggs per mass, number of egg masses without eggs and number of non-embrionated eggs per mass

Table 1 shows the results for the different groups regarding the total number of egg masses, the number of eggs per mass, the number of non-embryonated eggs per mass and the number of egg masses without eggs. The exposure to 0.4 μg L^{-1} and 5 μg L^{-1} of chlorpyrifos for 14 days did not cause statistically significant differences in most of these parameters respect to the control ($P > 0.05$), except for the number of egg masses without eggs which increased significantly.

3.5. Number of masses without hatching, time for hatching, percentage of hatching and of survival

Exposure to 0.4 μg L^{-1} of chlorpyrifos for 14 days did not cause statistically significant differences in these parameters respect to the control ($P > 0.05$). 5 μg L^{-1} caused an increase in the time for hatching and in the number of egg masses without hatching and an important decrease in the % of hatching and survival as shown in Table 2.

Fig. 4 shows different photographs of egg masses of the control group (A) and of Sub 2 group (B, C and D) at the same days of development. Photograph (B) shows that embryo growth was stopped at very early stages, photograph (C) shows bigger embryos which are dead and an amorphous egg mass can be seen on photograph (D).

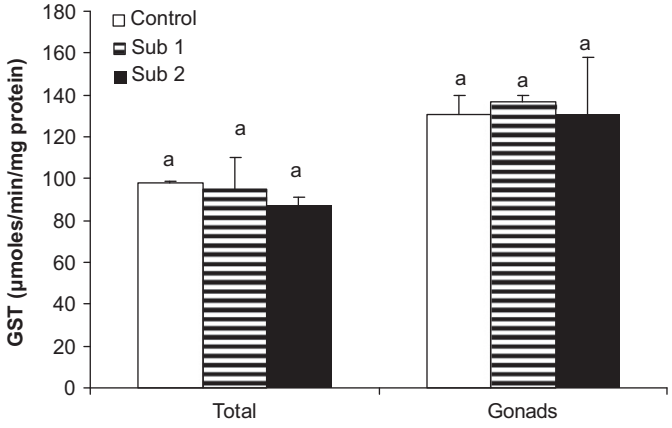

Fig. 3. Glutation S-transferase (GST) activity in adult *P. corneus* exposed to Sub 1 (0.4 μg L^{-1}) and Sub 2 (5 μg L^{-1} of chlorpyrifos) in whole organism soft tissue and in gonads. The data represents the mean ± SD of at least six snails, and six pools of 4–7 gonads. For each region, the data with different letters are statistically different ($P < 0.001$). No statistically significant differences were observed between water control and solvent control groups ($P > 0.05$). Therefore, only the results of the water control are shown.

Table 1
Number of egg masses, number of eggs per mass, number of non-embryonated eggs per mass and number of egg masses without eggs.

Groups	No. of egg masses (in 14 days)	No. of eggs/mass	No. of non-embryonated eggs/mass	No. of egg masses without eggs
Control	20 ± 6	13 ± 5	4 ± 4	0.5 ± 0.5
Sub 1	26 ± 7	11 ± 4	3 ± 2	7 ± 2*
Sub 2	22 ± 5	10 ± 6	3 ± 3	12 ± 5*

Data corresponds to the mean ± SD. For data of number of egg masses we used four replicates of six snails per container for each group. For the other parameters of reproduction we used all the egg masses per group. Sub 1, subchronic treatment with 0.4 μg/L and Sub 2, subchronic treatment with 5 μg/L. Data with * are statistically different ($P < 0.05$) to the control. No statistically significant differences were observed between water control and solvent control groups ($P > 0.05$). Therefore, only the results of the controls in acetone are shown.

Table 2
Number of egg masses without hatching, time for hatching, percentage of hatching and percentage of survival per mass after 1 month.

Groups	No. of egg masses without hatching	Time for hatching (days)	% of hatching	% survival (1 month)
Control	0.5 ± 0.6	10 ± 2	75 ± 15	72 ± 16
Sub 1	0.5 ± 0.6	10 ± 2	90 ± 11	68 ± 20
Sub 2	3 ± 1.6*	17 ± 6*	39 ± 18*	12 ± 8*

Data corresponds to the mean ± SD of all the egg masses for each group. Sub 1, subchronic treatment with 0.4 μg/L and Sub 2, subchronic treatment with 5 μg/L. Data with * are statistically different ($P < 0.05$) to the control. No statistically significant differences were observed between water control and solvent control groups ($P > 0.05$). Therefore, only the results of the controls in acetone are shown.

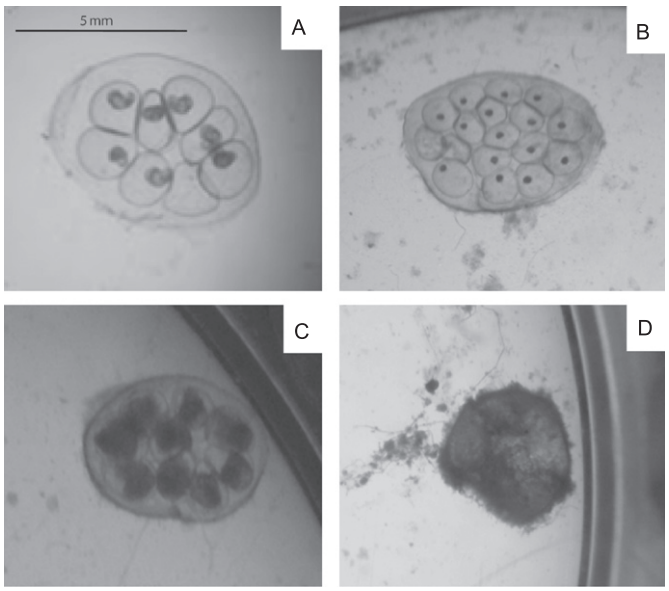

Fig. 4. Photographs of egg masses: (A): control and (B), (C) and (D) belong to egg masses of the group Sub 2 exposed to 5 $\mu g\,L^{-1}$ of chlorpyrifos. The optical magnification utilized was 30×. The egg masses measures correspond to 5.5 ± 0.8 mm long and 4.6 ± 0.8 mm width (mean ± SD of 20 egg masses).

4. Discussion

Exposure of organisms to contaminants may cause multiple toxic effects. The study of different effects in an exposed organism is essential for the understanding of the different biological responses and the mechanisms of toxicity of xenobiotics. Also, it is necessary to find the most sensitive biomarker in a bioindicator organism exposed to a particular toxic (Van der Oost et al., 2003).

As cholinesterases are the primary target of organosphosphates and carbamates insecticides, their inhibition has been used as a specific biomarker of exposure and effect for these insecticides (Edwards and Fisher, 1991; Timbrell, 2000; Walker et al., 2001). However, some authors have found other parameters that could be used to complete a toxicity study or that they may be more sensitive than ChEs in aquatic organisms (Ferrari et al., 2007; Kristoff et al., 2008, 2011). In this work, we have observed that a 14 days exposure to 0.4 $\mu g\,L^{-1}$ chlorpyrifos produced only a decrease in CES activity measured with *p*-NPB and an increase in the number of egg masses without eggs while no inhibition of ChE was observed. Similarly, Kristoff et al. (2011) have also described that ChE was not significantly inhibited during a 14 days exposure of the gastropod *B. glabrata* to an environmental concentration of the OP azinphos-methyl; instead, it led to a smaller number of egg masses.

Some authors have postulated that carboxylesterases are more sensitive than cholinesterases to inhibition by exposure to an OP insecticide (Kristoff et al., 2012; Vioque-Fernández et al., 2007). However in *P. corneus* exposed to chlorpyrifos, such results were only obtained by using *p*-NPB as substrate after a 14 days exposure. In contrast, after a 48 h exposure, ChE inhibition was either similar or greater than CES inhibition depending on the substrate (Cacciatore et al., 2011).

ChE inhibition and alterations in the reproduction parameters were observed with 5 $\mu g\,L^{-1}$ of chlorpyrifos. The main effects were on the hatching and survival as it has been observed by other authors in invertebrate species. For example, in the gastropod *B. glabrata* exposed for 14 days to 2.5 $mg\,L^{-1}$ or 5 $mg\,L^{-1}$ of azinphos-methyl, the authors reported a decrease in the number of hatchings per mass and non-living offspring after 1 month, respectively. However, in this case, azinphos-methyl concentrations were very high (Kristoff et al., 2011). In the present work and in previous works (Kristoff, 2010; Kristoff et al., 2011), the aim of our group was to study the effect that the pesticide had on reproduction from adult snails until the hatching of the offspring, exposing both the adult snails and egg masses. While doing this, we respected the times for hatching for each group simulating an environmental subchronic exposure. However, it would be interesting for future investigations to study the pesticide effect both on adult snails and egg masses separately in order to elucidate whether the effect of the pesticide is on the adult snail or upon the egg masses. Also, the offspring exposure to the pesticide could be evaluated in future works.

Concerning GST, we could appreciate that when snails were exposed to the chosen concentrations of chlorpyrifos, the activity was not modified. According to this result, Kristoff et al. (2008) and Domingues et al. (2007) showed no effects on GST in *B. glabrata* and *L. variegatus* exposed to azinphos-methyl for 2 days and in 4th instar larvae of two chironomid species exposed for 2 days to the OP dimethoate, respectively. In these cases, the conjugation of the OP with the glutathione mediated by GST would not be acting as a mechanism of detoxification of the pesticides.

In accordance with the obtained results, we could suggest that the most sensitive biomarker in adults snails of *P. corneus* exposed to chlorpyrifos for 14 days was CES activity measured with *p*-NPB. Regarding the results obtained in total homogenate and gonads, we did not observe differences in the responses of the B-esterases in both tissues; therefore, the use of the whole organism soft tissue is recommended because it requires a smaller number of snails.

The reproduction alterations can be one of the causes of the decline in the number of specimens of a species (van der Oost et al., 2003). Some authors have described that chlorpyrifos causes toxic effects on the reproduction of other aquatic invertebrates. In agreement with our results, Zaliznick and Nugegoda (2006) and Varó et al. (2006) reported a decrease in hatching and survival in adult organisms of *D. carinata* and cysts of *Artemia* sp., respectively. However, in these works, biochemical responses were not studied. Only a few amounts of works can be found that show comparisons between the effect in reproduction and biochemical responses to anticholinesterase pesticides exposure. Tripathi and Singh (2004) and Kristoff et al. (2011) compared ChE inhibition with reproductive alterations in the freshwater snails *L. acuminate* and *B. glabrata* exposed to carbaryl and azinphos-methyl, respectively. In both cases, authors found ChE inhibition greater than 45% and no eggs hatched. With a lower inhibition of ChE, they found a decrease in the number of egg masses and in the offspring. In the present work, when there was no inhibition of ChE in gonads, no significant alterations in reproduction were observed. Moreover, the high inhibition of ChE sustained over time, was accompanied by severe alterations in the reproduction. On the other hand, some authors have investigated possible relationships between CES and the reproduction of organisms. Mikhailov et al. (1997) found in a mollusc a carboxylesterase whose expression increased in the reproductive stage. Other authors have reported the presence of a carboxylesterase in the seminal fluid of the *Drosophila* insect which is involved in sperm protection and activation (Korochkin, 1980; Kubli, 1992). These authors have described that these enzymes are over-expressed in their respective male reproductive systems and that polypeptides with CES activity may play a role in the detoxifying process in male reproductive systems (Korochkin, 1980; Kubli, 1992; Mikhailov et al., 1997). However, new studies should be performed to achieve better understanding of the relationship between CES and reproduction.

Acute laboratory toxicity data for various groups of aquatic organisms exposed to Ops, showed that arthropods and a lesser

extend of fish, are the most sensitive groups, followed by algae and non-arthropods (Van Wijngaarden et al., 2005). In particular, representatives of crustaceans, insects and fish can be considered particularly sensitive to chlorpyrifos (Barron and Woodburn, 1995; Van Wijngaarden et al., 1993). With the chlorpyrifos concentrations that were used in this work, more severe effects may be caused in these groups of aquatic organisms. It should also be taken into account that subchronic and chronic exposures to low Ops concentrations can produce more severe effects in gastropods than acute exposures, mainly related to reproduction (Kristoff et al., 2011).

With regards to *P. corneus*, it has been reported that this species declines constantly (Jopp, 2006). For this reason, it is very important to investigate the effect of different contaminants on the reproduction of this species. Disturbances in the reproductive performance of *P. corneus* after subchronic and chronic exposures to sewage treatment work (STW) effluents containing natural hormones, synthetics steroids, pesticides, surfactants and plasticizers have been reported (Clarke et al., 2009). In this study, we have confirmed that the subchronic exposure to chlorpyrifos is able to cause alterations in the reproduction of *P. corneus* and in the survival of the offspring. Unpublished results of our group show that the subchronic exposure to environmental concentrations of azinphos-methyl also causes a decrease on hatching and survival of *P. corneus*. These suggest that OPs may contribute to the decline in the number of organisms of this species.

5. Conclusion

B-esterases and reproductive parameters can be used as effect biomarkers of the aquatic contamination with chlorpyrifos. In *P. corneus* exposed for 14 days to the insecticide, the carboxylesterases determined with *p*-NFB proved to be the most sensitive biomarker. However, exposure to environmental concentrations showed a decrease in the reproduction ability suggesting that the presence of pesticide in the water could be one of the reasons for which many freshwater mollusc species have become threatened or extinct in the past years.

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References

Amanullah, B., Stalin, A., Prabu, P., Dhanapal, S., 2010. Analisis of AChE and LDH in mollusc, *Lamellidens marginalis* after exposure to chlorpirifos. J. Environ. Biol. 31, 417–419.

Arufe, M.I., Arellano, J.M., García, L., Albendín, G., Sarasquete, C., 2007. Cholinesterase activity in gilthead seabream (*Sparus aurata*) larvae: characterization and sensitivity to the organophosphate azinphosmethyl. Aquat. Toxicol. 84, 328–336.

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

Barile, F.A., 2008. Principles of Toxicology Testing. Taylor and Francis Group, CRC Press, USA.

Barron, M.G., Woodburn, K.B., 1995. Ecotoxicology of chlorpyrifos. Rev. Environ. Contam. Toxicol. 144, 1–93.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J., Kesten, E.M., 1998. Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorus pesticides. Bull. Environ. Contam. Toxicol. 61, 569–576.

Bocquené, G., Roig, A., Fournier, D., 1997. Cholinesterases from the common oyster (*Crassostrea gigas*). Evidence for the presence of a soluble acetylcholinesterase insentive to organophosphate and carbamate inhibitors. FEBS Lett. 407, 261–266.

Cacciatore, L.C., Kristoff, G., Verrengia Guerrero, N., Cochón, A., 2012. Binary mixtures of azinphos-methyl oxon and chlorpyrifos oxon produce *in vitro* synergistic cholinesterase inhibition in *Planorbarius corneus*. Chemosphere 88, 450–458.

Cacciatore, L.C., Villar, M.R., Kristoff, G., Verrengia Guerrero, N.R., Cochón, A., 2011. Inhibición *in vivo* de la actividad colinesterasa en tejidos blandos de un gastrópodo de agua dulce por el carbaril y mezclas binarias del mismo con metilazinfos o clorpirifos. Acta Toxicol. Argent. (19), 98–99.

Clarke, N., Routledge, E.J., Garner, A., Casey, d., Benstead, R., Walker, D., Watermann, B., Gnass, K., Thomsen, A., Jobling, S., 2009. Exposure to treated sewage effluent disrupts reproduction and development in the seasonally breeding ramshorn snail (Subclass: Pulmonata, *Planorbarius corneus*). Environ. Sci. Technol. 43, 2092–2098.

Collins Baker, F., 1945. The Molluscan Family Planorbidae. The University of Illinois Press, Urbana, United States of America.

Cooper, N.L., Bidwell, J.R., 2006. Cholinesterase inhibition and impacts on behavior of the Asian clam, *Corbicula fluminea*, after exposure to an organophosphate insecticide. Aquat. Toxicol. 76, 258–267.

De Silva, P.M.C.S., Samayawardhena, L.A., 2005. Effects of chlorpyrifos on reproductive performances of guppy (*Poecilia reticulata*). Chemosphere 58, 1293–1299.

Domingues, I., Guilhermino, L., Soares, A.M.V.M., Nogueira, A.J.A., 2007. Assessing dimethoate contamination in temperate and tropical climates: potencial use of biomarkers in bioassays with two chironomid species. Chemosphere (69), 145–154.

Edwards, C.A., Fisher, S.W., 1991. The use of cholinesterase measurements in assessing the impacts of pesticides on terrestrial and aquatic invertebrates. In: Mineau, P. (Ed.), Cholinesterase Inhibiting Insecticides: Their Impact on Wildlife and the Environment. Elsevier, Amsterdam, Holand, pp. 255–275.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

E.P.A. USEPA: Interim Registration Eligibility Decision for Chlorpyrifos, 2006.

Escartin, E., Porte, C., 1996. Bioaccumulation, metabolism, and biochemical effects of the organphosphorus pesticide fenitrothion in *Procambarus clarkii*. Environ. Toxicol. Chem. 15, 915–920.

Farag, A.T., Radwan, A.H., Sorour, F., El-Okazy, A., El-Agamy, E.S., El-Sebal, A.E.K., 2010. Chlorpyrifos induced reproductive toxicity in male mice. Reprod. Toxicol. 29, 80–85.

Ferrari, A., Venturino, A., Pechen, D.̇., Angelo, A.M., 2004. Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphos methyl, parathion and carbaryl exposure in the goldfish (*Carassius auratus*). Ecotoxicol. Environ. Saf. 57, 420–425.

Ferrari, A., Venturino, A., Pechen, D.̇., Angelo, A.M., 2007. Effects of carbaryl and azinphos methyl on juveniles rainbow trout (*Oncorhynchus mykiss*) detoxifying enzymes. Pestic. Biochem. Phys. 88, 134–142.

Gagnaire, B., Geffard, O., Xuereb, B., Margoum, C., Garric, J., 2008. Cholinesterase activities as potential biomarkers: characterization in two freshwater snails, *Potamopyrgus antipodarum* (Mollusca, Hydrobiidae, Smith 1889) and *Valvata piscinalis* (Mollusca, Valvatidae, Muller 1774). Chemosphere 71, 553–560.

Habig, W.H., Pabst, M.J., Kakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hannam, M.L., Hagger, J.A., Jones, M.B., Galloway, T.S., 2008. Characterisation of esterases as potential biomarkers of pesticide exposure in the lugworm *Arenicola marina* (Annelida: Polychaeta). Environ. Pollut. 152, 342–350.

Hutchinson, T.H., Shillabeer, N., Winter, M.J., Pickford, D.B., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. Aquat. Toxicology 76, 69–92.

Jager, T., Crommentuijn, T., van Gestel, C.A.M., Kooijman, S.A.L.M., 2007. Chronic exposure to chlorpyrifos reveals two modes of action in the springtail *Folsomia candida*. Environ. Pollut. 145, 452–458.

Jokanovic, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166, 139–160.

Jopp, F., 2006. Comparative studies on the dispersal of the Great Ramshorn (*Planorbarius corneus* L.): a modelling approach. Limnologica 36, 17–25.

Korochkin, L.I., 1980. Genetic regulation of isozyme patterns in Drosophila during development. In: Market, C.L. (Ed.), Isozymes: Current Tropics in Medical and Biological Research, 304. Alan R. Liss, New York, pp. 159–202.

Kristoff, G., 2010. Estudio comparativo de biomarcadores en los invertebrados acuáticos *Biomphalaria glabrata* y *Lumbriculus variegatus* expuestos a pesticidas de relevancia ambiental. Tesis de Doctorado, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

Kristoff, G., Cacciatore, L.C., Verrengia Guerrero, N.R., Cochón, A.C., 2011. Effects of the organophosphate insecticide azinphos-methyl on the reproduction and cholinesterase activity of *Biomphalaria glabrata*. Chemosphere 84, 585–591.

Kristoff, G., Chiny Barrionuevo, D., Cacciatore, L.C., Verrengia Guerrero, N.R., Cochón, A., 2012. *In vivo* studies on inhibition and recuperation of B-esterase activity in *Biomphalaria glabrata* exposed to azinphos-methyl: analysis of enzyme, substrate and tissue dependence. Aquat. Toxicol. 112-113, 19–26.

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

Kristoff, G., Verrengia Guerrero, N.R., Pechén 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.

Kubli, E., 1992. The sex peptide. Bio Essays 14, 779–784.

Kumar, A., Doan, H., Barnes, M., Chapman, J.C., Kookana, R.S., 2010. Response and recovery of acethylcholinesterase activity in freshwater shrimp *Paratya*

australiensis (Decapoda: Atyidae) exposed to selected anti-cholinesterase insecticides. Ecotoxicol. Environ. Saf, 1503–1510.
Lacorte, S., Lartiges, S.B., Garrigues, P., Barcelo, D., 1995. Degradation of organophosphorus pesticides and their transformation products in estuarine waters. Environ. Sci. Technol. 29, 431–438.
Li-Xia, K., Yi-Long, X., Chun Wang, Z., Li-Li, D., 2009. Effects of three organophosphorus pesticides on population, growth and sexual reproduction of rotifer *Brachionus calyciflorus Pallas*. Acta Entomol. Sinica. 29, 182–185.
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.
Mikhailov, A.I., Torrado, M., Korochkin, L.I., Kopantzeva, M.A., Méndez, J., 1997. Male predominant carboxylesterase expression in the reproductive system of molluscs and insects: immunochemical and biochemical similarity between *Mytilus* male associated polypeptide (MAP) and Drosophila sex-specific esterase S. Comp. Biochem. Physiol. 118, 197–208.
Moore, M.T., Schulz, R., Cooper, C.M., Smith Jr., S., Rodgers Jr., J.H., 2002. Mitigation of chlorpyrifos run off using constructed wetlands. Chemosphere 46, 827–835.
Mora, P., Michel, X., Narbonne, J.-F., 1999. Cholinesterase activity as potencial biomarker in two bivalves. Environ. Toxicol. Pharmacol. 7, 253–260.
Palma, P., Palma, V.L., Fernández, R.M., Bohn, A., Soares, A.M.V.M., Barbosa, I.R., 2009. Embryo-toxic effects of environmental concentrations of chlorpyrifos in the crustacean *Daphnia magna*. Ecotoxicol. Environ. Saf (72), 1714–1718.
Racke, 1993. Environmental fate of chlorpyrifos. Rev. Environ. Contam. Toxicol. 131, 1–150.
Rodríguez, J., 2009. Efecto del insecticida clorpirifos sobre las actividades de esterasas-B en *Biomphalaria glabrata* y *Lumbriculus variegatus*. Tesis de licenciatura en Ciencias Biológicas. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.
Roex, E.W.M., Keijzus, R., Van Gestel, C.A.M., 2003. Acetylcholinesterase inhibition and increased food consumption rale in zebrafish, *Danio rerio* after chronic exposure to parathion. Aquat. Toxicol. 64, 451–460.
Sanchez-Hernandez, J.C., 2007. Ecotoxicological perspectives of B-esterases in the assessment of pesticide contamination. In: Plattenberg, R.H. (Ed.), Environmental Pollution: New Research. Nova Science Publishers, Inc., N.Y., USA, pp. 1–45.
Strange, R.C., Jones, P.W., Fryer, A.A., 2000. Glutathione S-transferase: genetics and role in toxicology. Toxicol. Lett. 112/113, 357–363.
Strong, E.E., Gargominy, O., Ponder, W.F., Bouchet, P., 2008. Global diversity of gastropods (Gastropoda; Mollusca) in freshwater. Hydrobiologia 595, 149–166.
Talesa, V., Contenti, S., Mangiabene, C., Pascolini, R., Rosi, G., Principato, G.B., 1990. Propionylcholinesterase from *Murex brandaris*: comparison with other invertebrate cholinesterases. Comp. Biochem. Physiol. 96, 39–43.
Timbrell, J.A., 2000. Principles of Biochemical Toxicology. Taylor and Francis, London.
Tripathi, P.K., Singh, A., 2004. Carbaryl induced alterations in the reproduction and metabolism of the freshwater snail *Lymnaea acuminata*. Pest. Biochem. Physiol. 79, 1–9.
Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.
Van Wijngaarden, R.P.A., Brock, T.C.M., Van den Brink, P.J., 2005. Threshold levels for effects of insecticides in freshwater ecosystems: a review. Ecotoxicology 14, 355–380.
Van Wijngaarden, R., Leeuwangh, P., Lucassen, W.G.H., Romijn, K., Ronday, R., Van der Velde, R., Willigenburg, W., 1993. Acute toxicity of chlorpyrifos to fish, a newt and aquatic invertebrates. Bull. Environ. Contam. Toxicol. 51, 716–723.
Varó, I., Amat, F., Navarro, J.C., Barreda, M., Pitarch, E., Serrano, R., 2006. Assessment of the efficacy of *Artemia* sp. (Crustacea) cysts chorion as barrier to chlorpyrifos (organophosphorus pesticide) exposure. Effect on hatching and survival. Sci. Total Environ. 366, 148–153.
Varó, I., Navarro, J.C., Amat, F., Guilhermino, L., 2002. Characterization of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. Chemosphere 48, 563–569.
Vioque-Fernández, A., Alves de Almeida, E., 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. 145, 404–412.
Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 2001. Principles of Ecotoxicology. Taylor and Francis, London.
WHO, 1986. Organophosphorus insecticides: a General introduction. Environmental Health Criteria, 63. WHO, Geneva.
Wiese, V., 2005. Nonmarine mollusca of Schleswig-Holstein (northernmost Germany). (February 2005). MOLLBASE-National Database of German Land-and Freswater-Mollusks.01.06.2005. 〈http://www.mollbase.org/sh/〉.
Wood, B., Starck, J.D., 2002. Acute toxicity of drainage ditch water from Washington State cranberry-growing region to *Daphnia pulex* in laboratory bioassays. Ecotoxicol. Environ. Saf. 53, 273–280.
Xuereb, B., Noury, P., Felten, V., Garric, J., Geffard, O., 2007. Cholinesterase activity in *Gammarus pulex* (Crustacea Amphipoda): characterization and effects of chlorpyrifos. Toxicology 236, 178–189.
Zaliznick, L., Nugegoda, D., 2006. Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata*. Ecotoxicol. Environ. Saf. 64, 207–214.