Bull Environ Contam Toxicol (2014) 92:625–630
DOI 10.1007/s00128-014-1239-0

Effects of Chlorpyrifos and Endosulfan on Different Life Stages of the Freshwater Burrowing Crab *Zilchiopsis collastinensis* P.: Protective Role of Chorion

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Received: 13 August 2013 / Accepted: 24 February 2014 / Published online: 4 March 2014

Abstract The lethality (LC_{50}) of endosulfan and chlorpyrifos is higher in adults (1990 and 156.78 μg/L respectively) than in embryos (95380 and 1242.54 μg/L respectively) of the crab *Zilchiopsis collastinensis*. The thickened embryonic coat of the freshwater species might isolate the embryo inside the egg, reducing the toxicity. Sublethal concentrations of chlorpyrifos and endosulfan caused an increase in hatching time and a decrease in effective hatching ($p < 0.05$), and only the control crabs survived until the first molt cycle. The effects of long-term exposure should be evaluated in the offspring in addition to the acute toxicity.

Keywords Biocides · Lethality · Effects in embryos

Pesticides such as endosulfan and chlorpyrifos are widely used in agricultural activities to minimize the damage produced by pests. Endosulfan is an organochlorine insecticide that inhibits neuronal function by blocking the GABA-gated chloride channels of the nervous systems (Murray et al. 1993). Chlorpyrifos is an organophosphorus insecticide that acts upon biota by the inactivation of acetylcholinesterase (AChE), which is responsible for terminating nerve impulse transmission at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine (Ach). The inactivation of AChE causes an accumulation of Ach, which eventually blocks the transmission of nerve impulses (Murty and Ramani 1992). The pesticides migrate after rain events, mainly through run-off, and reach aquatic ecosystems. The biota in the contaminated aquatic ecosystems is periodically exposed to pesticide inputs.

The burrowing crabs of the *Zilchiopsis* genera are freshwater species widely distributed in rivers and ponds. They play a key role in matter and energy exchange between terrestrial and aquatic environments with an intermediate position in the food webs. Like other freshwater organisms, these species have developed several adaptations to invade continental environments. Because the developing embryos were sensitive to osmotic stress, the eggs are covered with a thickened chorion, which isolates the embryo. The primitive pelagic larval phase is suppressed, and embryos hatch as juveniles. The incubation period increases, and females invest relatively high levels of maternal resources in each egg by hatching a small number of large eggs and holding the young in the abdomen for several days after the young crabs hatch (Ruppert and Barnes 1994). The reproductive period of the crab *Zilchiopsis collastinensis* occurs in spring and summer, which coincides with the highest pesticide application period. Despite the importance of this crab in aquatic ecosystems, there are few records concerning the effects of pesticides in this species, and, to the best of our knowledge, there are no records of the effects produced by pesticides in the embryonic phase. The objectives of this work were to determinate the acute toxicity of endosulfan and

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chlorpyrifos in adult female crabs and embryos of *Z. collastinensis* and the effects produced in the incubation period, hatching and survival of the offspring exposed to these insecticides.

Materials and Methods

Zilchiopsis collastinensis individuals were collected on the Paraná River floodplain (31°30′S, 60°41′W; Santa Fe, Argentina) away from cities and crop areas. Adult female crabs used for the acute toxicity tests were collected during winter before the reproduction period. The median lethal concentration assays were performed based on the standardized 96-h toxicity test (USEPA, 2002). The mean (±SD) carapace width of the crabs used was 47.45 (±2.08) mm. The largest individuals were less than 1.5 times larger than the smallest individual. Due to the antagonistic behavior observed in this species, the crabs were isolated in clear plastic perforated chambers and placed in groups of 4 individuals per replicate in 60 L aquariums filled with 30 L of dechlorinated water (control) or multiple concentrations of the pesticides (Table 1). Five replicates of each concentration were used (n = 20 individuals per concentration). The assays were performed at 25 ± 1°C with a 12:12 light/darkness photoperiod. The solutions were renewed at 24 h exposure intervals. Before the solution renewal, the dead organisms were counted and removed. The criterion of death was the absence of movement after stimulation and cheliped laxity. Crabs were not fed during the assays.

Ovigerous females were collected in late spring (November–December) and transported to the laboratory, where they were maintained until the embryos reached the 5° stage according to Lund et al. (2000) with eye pigmentation and a noticeable heartbeat. Once in this stage, the eggs were softly removed from the female abdomen with a brush and placed in two-compartment Petri dishes filled with 10 mL of dechlorinated water (control) or water containing the pesticides (Table 1). Ten embryos were placed in each dish compartment, and four replicates of each concentration were used (n = 40 embryos per concentration). Petri dishes were placed in a rocker shaker at 40 rpm inside a 25 ± 1°C incubator with a 24 h dark cycle. The solutions were renewed at 24 h exposure intervals. Before the solution renewal, the embryos were observed with a stereoscopic microscope, and the dead organisms were counted and removed. The criterion of death was the absence of heartbeat.

Effects of endosulfan and chlorpyrifos on embryo hatching time, effective hatching and survival after hatching were observed. Five sublethal concentrations, which were close to the adult toxicity test concentrations, were used. Forty embryos in the 5° stage were isolated in plastic 7 chamber, 6 mL plates filled with 5 mL of dechlorinated water (control) or water with the pesticides (Table 2). After an exposure of 96 h, a group of 20 embryos was separated and allowed to hatch in dechlorinated water, and the other group of 20 embryos was continuously exposed. The dechlorinated water and the solutions were renewed daily. Both treatments, including the controls, were performed

Table 1 Chlorpyrifos and endosulfan concentrations, both nominal and measured, used in CL_{50} tests

Adults			Embryos		
Concentration	Nominal	Measured (mean ± SD)	Concentration	Nominal	Measured (mean ± SD)
Chlorpyrifos (μg/L)					
C1	32	18.9 (2.68)	C1	360	388.4 (15.65)
C2	64	53.1 (3.67)	C2	720	509.4 (188.37)
C3	128	117.1 (13.58)	C3	1440	1528.69 (32.14)
C4	256	276.4 (10.82)	C4	2880	2127.12 (178.14)
C5	512	361 (133.97)	C5	5760	5977.52 (698.50)
C6	1024	1090 (21.71)	C6	11520	9710.9 (409.21)
			C7	23040	19312 (1272.65)
Endosulfan (μg/L)					
C1	875	902 (36)	C1	8000	10300 (1101)
C2	1250	1295 (101)	C2	16000	17300 (363)
C3	1780	2076 (79)	C3	32000	29900 (1039)
C4	2540	2681 (421)	C4	64000	73500 (3636)
C5	3620	3126 (608)	C5	128000	120800 (5106)
			C6	256000	259700 (30815)
			C7	512000	440800 (7375)

Table 2 Endosulfan and chlorpyrifos sublethal concentrations used in embryo hatching time, effective hatching and survival after hatching tests

	Endosulfan		Chlorpyrifos	
	Nominal	Measured (mean ± SD)	Nominal	Measured (mean ± SD)
Embryo sublethal concentrations (μg/L)				
SC0	0	<LD	0	<LD
SC1	500	629 (81)	45	26.2 (3.95)
SC2	1000	1165 (102)	90	74.7 (4.76)
SC3	2000	2158 (176)	180	164.3 (19.32)
SC4	4000	4465 (1082)	360	388.4 (15.65)
SC5	8000	10254 (1101)	720	509.4 (188.37)

LD: Endosulfan = 0.004 μg/L; Chlorpyrifos = 0.012 μg/L

until the animals died or entered the first molt cycle after hatching. The embryos and hatched crabs were observed every day before the water or solution renewal. After hatching, small plant pieces (*Ceratophillum demersum*) were offered to juvenile crabs because in previous works, we observed that survival increased with the addition of vegetal support (unpublished data).

The pesticide products tested included Zebra® (Red Surcos, Argentina), a commercial product containing 35 % endosulfan, and Clorpi® (Red Surcos, Argentina), a commercial product containing 48 % of chlorpyrifos. All the solutions were prepared the day they were used. Pesticide concentrations were measured by gas chromatography–electronic capture detection (GC–ECD), following the method 508 of USEPA (1989). Pesticide concentrations were measured by duplicate at initial time during the 96 h assays following a methodology which is permanently tested. After the acute exposure, pesticide concentrations were measured at day 7 and day 10 in embryo sublethal toxicity tests. As we always used the same methodology, we assume that the concentrations were similar in all days. It was suggested that a constant pesticide concentration in test solution could be kept during the exposure by the method of daily renewal (Li et al. 2006).

The concentration ranges used in the acute toxicity tests were determined after several range finding tests. A probit analysis was used to estimate the LC_{50} and the 95 % confidence limits, using measured concentrations, with Abbot's correction for control mortality. The differences in the LC_{50} were considered to be significant when the higher LC_{50}/lower LC_{50} ratio exceeded the critical value. Kruskal–Wallis tests followed by Dunn's method were performed to determinate the significant effects of each insecticide on the embryo hatching time. The pesticide effects on the hatched embryos were determined using the Chi square method, where the control group data was used as expected value and compared with the observed values in the different concentrations ($p < 0.05$) (Zar 1996).

Results and Discussion

The water quality did not significantly vary during the tests because it was renewed daily. The temperature, dissolved oxygen, pH and conductivity were 25 ± 1°C, 6.37 ± 2.28 mg/L, 7.12 ± 1.22 and 1228.68 ± 21.42 μS/cm, respectively.

The results of the 96-h endosulfan and chlorpyrifos toxicity tests showed that adult crabs were more sensitive to pesticides than embryos (Fig. 1). The embryo LC_{50} was significantly higher than the adult LC_{50} for both pesticides ($p < 0.05$). Additionally, crabs were more resistant to endosulfan than chlorpyrifos ($p < 0.05$).

According to the LC_{50} values, *Z. collastinensis* appears to be resistant to pesticides. Compared with other decapod crustaceans, such as prawn and shrimps, crabs appear to be generally more resistant to pesticides (Table 3). The evolutionary reduction of the abdomen in crabs decreased the exposed surface and might reduce the dermal intake (Ruppert and Barnes 1994).

The higher resistance of embryos to pesticides could be partially explained by the evolutionary adaptations that crustaceans have developed to invade continental environments. The eggs of freshwater species have a thickened embryonic coat to avoid the osmotic stress caused by this hypoosmotic environment (Glas et al. 1997; Key et al. 2003). The lower permeability of the coat reduces the contact with pesticides and increases the survival of the more isolated embryo. Additionally, because the modes of action of endosulfan and chlorpyrifos are generally disruptive to central nervous system activity, the lower embryo toxicity could be explained by the immaturity of the embryonic nervous system (Key et al. 2003).

The hatching time of embryos in the 5° stage was increased when they were exposed to the highest concentrations of endosulfan and chlorpyrifos for 96 h and continuous exposures (Fig. 2). An increase in the embryo hatching time was also observed in the grass shrimp *Palaemonetes pugio* after exposure to several insecticides including chlorpyrifos, endosulfan and fipronil; in the blue crab *Callinectes sapidus* after exposure to fenvalerate, and in the chinese mitten crab *Eriocher sinensis* after exposure to chlorpyrifos (Wirth et al. 2001; Key et al. 2003; Li et al. 2006). Nevertheless, there was not an increase in the incubation time after the exposure to the highest chlorpyrifos concentrations. Reductions in incubation time were observed in crabs exposed to atrazine (Li et al. 2006).

The delay in hatching might be related to the effects of the pesticides in the crabs. Although the chorion might

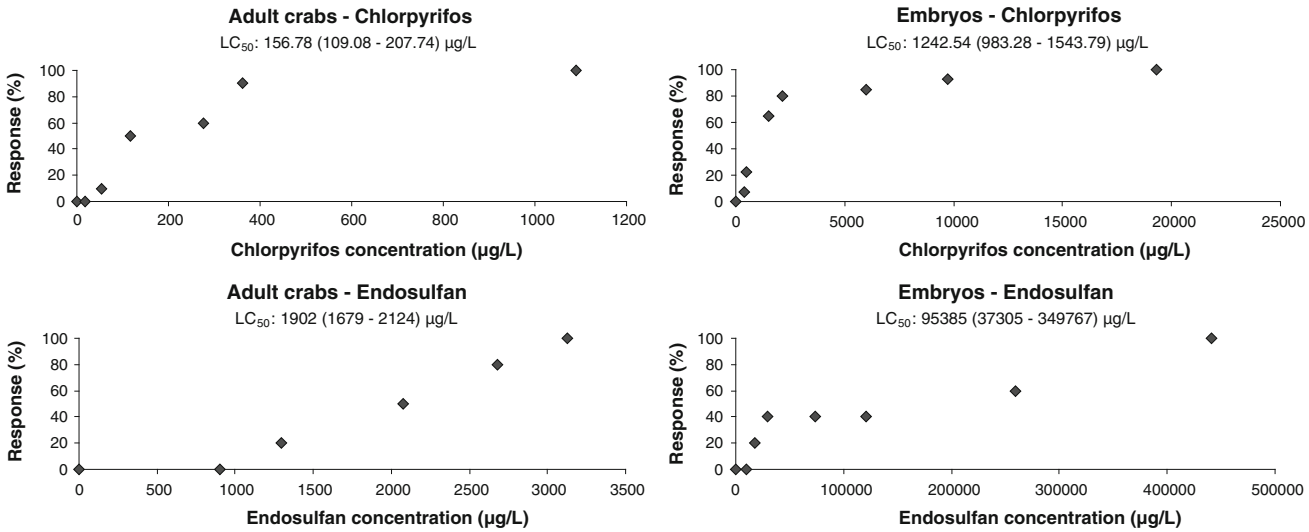

Fig. 1 Concentration—response curves of adults and embryos exposed to endosulfan and chlorpyrifos. LC_{50} values (95 % confidence limits) are included

Table 3 Median lethal concentration of several decapod crustaceans exposed to endosulfan and chlorpyrifos. Crabs are generally more resistant than prawns to those insecticides

	LC_{50} values		Endosulfan references	Chlorpyrifos references
	Endosulfan (µg l^{-1})	Chlorpyrifos (µg l^{-1})		
Prawns				
Cardina laevis	1.02 (0.87–1.19)		Sucahyo et al. (2008)	
Macrobrachium rosenbergii	0.2 (0.04–0.38)		Lombardi et al. (2001)	
Palaemon macrodactylus	17.1		Wirth et al. (2001)	
Palaemonetes pugio	0.32 (0.24–0.41)	0.37 (0.30–0.44)	Key et al. (2003)	Key and Fulton (1993)
Palaemonetes argentinus	6.144 (4.417–8.69)	0.49 ± 0.255	Montagna and Collins (2007)	
Crabs				
Oziotelphusa senex senex	18620		Rajeswari et al. (1988)	
Eriocher sinensis		143.05 (126.1–162.6)		Li et al. (2006)
Barytelphusa guerini		38.81 (37.09–40.46)		Srivastava et al. (2013)
Spiralothelphusa hydrodroma		120 (81–249)		Senthilkumar et al. (2007)
Trichodactylus borellianus	1860 ± 78	45.53 (25.28–67.75)	Montagna (2010)	
Zulchiopsis collastinensis	1990 (1680–2120)	155. 72 (107.18–207.72)		

partially isolate the embryo, some pesticide concentrations are able to enter the eggs and affect them. Lund et al. (2000) observed AChE inhibition in the embryos of *P. pugio* exposed to chlorpyrifos and malathion. Because the embryos must perform a series of movements to allow hatching, the effects of the pesticide on the movement coordination, caused by AChE inhibition or effects on the GABA receptors, could cause difficulties in embryo hatching and increase the incubation period.

The exposure to both pesticides also caused a decrease in effective hatching (i.e., crabs that survived and successfully hatched), especially in the higher pesticide concentrations and the continuously exposed group ($p < 0.05$) (Fig. 3). The crabs that died in the hatching process exhibited thin or broken chorions, which allowed the pesticides to contact the developing crabs without any barrier. In the groups acutely exposed to pesticides and then transferred to clean water the effective hatching was higher than in the permanently exposed, especially in the lowest concentrations. This data suggest a rapid recovery after the pesticide exposure, which is not observable in the highest concentrations, where the effects might not be reversible. The exposed crabs that accomplished hatching, both exposed permanently and exposed for 96 h, were not

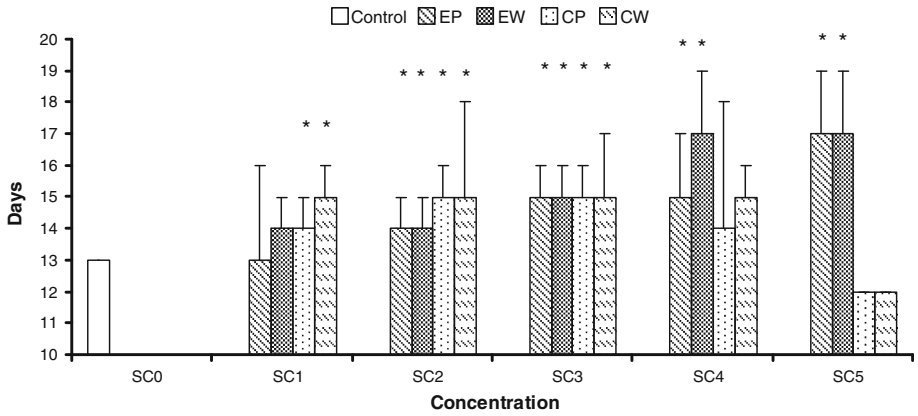

Fig. 2 Incubation time (median + interquartile range) for *Z. collastinensis* embryos permanently exposed to endosulfan and chlorpyrifos (EP and CP respectively) and exposed 96 h to endosulfan and chlorpyrifos and then transferred to clean water (EW and CW respectively). *Significantly different to the control (SC_0) ($p < 0.05$)

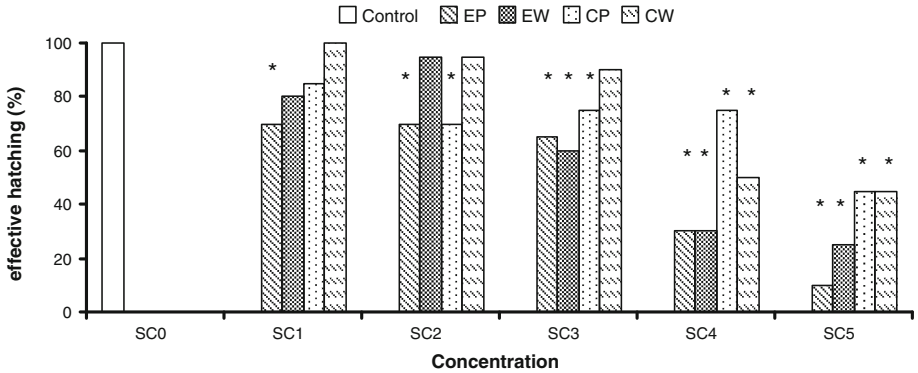

Fig. 3 Effective hatching (%) of *Z. collastinensis* embryos permanently exposed to endosulfan and chlorpyrifos (EP and CP respectively) and exposed 96 h to endosulfan and chlorpyrifos and then transferred to clean water (EW and CW respectively). *Significantly different to the control (SC0) ($p < 0.05$)

able to walk or eat, and all the juveniles exposed to both pesticides survived after 10 days. The control crabs were able to walk and feed, and they survived until the first molt cycle.

Although the chorion might act as a barrier, and the embryos survived, longer exposures than 96 h should be observed in future evaluations. The effects on the embryos at lower pesticide concentrations should also be evaluated.

Acknowledgments This study was supported by the grant CAI+D PI 69-352 (Universidad Nacional del Litoral).

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