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Acute toxicity of chlorpyrifos to the non-target organism *Cnesterodon decemmaculatus*

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Chlorpyrifos is the most used insecticide in Argentina. *Cnesterodon decemmaculatus* is a widely distributed, endemic fish from Neotropical America. It attains high densities in the shallow water assemblages of Argentina and Brazil. The aim of this study was to assess the acute toxicity of chlorpyrifos to *C. decemmaculatus*. The mean 96-h LC_{50} of three independent determinations was 105.3 (±3.1) μg/L. Sublethal effects were observed. Swimming behavioral changes at each chlorpyrifos exposure concentration were reported. *C. decemmaculatus* represents a good model for ecotoxicological risk assessment.

Keywords: acute toxicity; insecticide; fish; *Cnesterodon decemmaculatus*

Introduction

The "Pampa" is the main food production area of Argentina; it is an extensive plain with fertile soils and mild climate. Originally covered by grasslands, agriculture occupies at present 22.5 million ha (MAGyP 2013). Traditionally, farmers employed a mixed system of livestock and wheat/corn production. Soil use intensification has occurred in recent decades. The genetically modified soybean plant resistant to glyphosate was introduced into the Argentine market in 1996 and was fast adopted by the farmers. Soy cultivation in Argentina has steadily increased since then, from 6.7 to 20 million ha at present; corn cultivation increased from 4.2 to 6.1 million ha; and wheat decreased from 7.4 to 3 million ha in the same period (MAGyP 2013). Wheat and soy varieties with a short growing period allowed two harvests per year, wheat followed by soy. Livestock was moved to marginal areas or concentrated in feedlots. Pesticide consumption increased from 6 million kilograms in 1992 (Pengue 2000) to 36 million kilograms in 2010 (CASAFE 2012). Chlorpyrifos is the most heavily used insecticide (CASAFE 2012); it is applied 1–2 times per growing season at 144–960 g of active ingredient per hectare (Mugni 2009). Nearby South American countries developed similar trends. Soy is widespread in Brazil, Uruguay, Paraguay, and Bolivia; the crop is usually managed in the same way, applying large amounts of insecticides, often chlorpyrifos (Bindraban et al. 2009). Chlorpyrifos is a broad-spectrum organophosphate insecticide. Its toxic effects may include neurological, behavioral, and possibly reproductive effects (De Silva & Samayawardhena 2005). Repeated pesticide applications on crops represent a risk to adjacent surface waters (Mugni et al. 2012). Marino and Ronco (2005) detected chlorpyrifos in rivers running through the main agricultural districts in the Argentine Pampa.

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Jergentz et al. (2005) and Mugni et al. (2011) detected chlorpyrifos in the water, suspended matter and bottom sediments in first-order streams passing through soy cultivated plots.

Fish have often been used as ecotoxicological models for risk assessment and for testing insecticide toxicity to non-target fauna (De la Torre et al. 1997, 2002, 2007; Saha & Kaviraj 2008; Sharbidre et al. 2011; El-Amrani et al. 2012). *C. decemmaculatus* (Jenyns, 1842, Pisces, Cyprinodontiformes) is an endemic fish from Neotropical America, widely distributed in South American basins located in Argentina, Chile, Paraguay, Brazil, and Uruguay. *C. decemmaculatus* attains high densities and often represents the dominant fish in the shallow water assemblages of southern South America (Gómez et al. 1998).

The present contribution represents the first report of the acute toxicity of chlorpyrifos to *C. decemmaculatus*.

Materials and methods

Test organism

C. decemmaculatus belongs to the family Poeciliidae, a small, viviparous, micro-omnivorous, benthic–pelagic, non-migratory fish (maximum size: 25 and 45 mm for male and female, respectively). *C. decemmaculatus* ubiquity in different environments is related to its strong euritopic potential. Ranges of tolerance of *C. decemmaculatus* to many environmental parameters such as temperature, salinity and pH are comparatively large (Gómez et al. 1998). Specimens of *C. decemmaculatus* were originally collected from an uncontaminated stream located 25 km south of La Plata City and transported to the laboratory, where they were cultured for two weeks. Stream water and sediments were analyzed for chlorpyrifos, endosulfan, and cypermethrin, the most widely used insecticides in Argentina until 2013 when endosulfan was forbidden. No detectable concentrations were determined (detection limits were: 1 µg/kg in sediments; 0.01 µg/l chlorpyrifos and endosulfan, and 0.025 µg/L cypermethrin in water). They were kept in large plastic containers with stream water, which was gradually replaced with dechlorinated tap water to compensate for evaporation losses. *C. decemmaculatus* individuals were fed daily with Shulet® Carassius commercial fish food.

Toxicity tests

Procedures for *C. decemmaculatus* toxicity tests followed standardized protocols (USEPA 2002). Ten adult *C. decemmaculatus* individuals, 20–30 mm in length, were exposed to different chlorpyrifos concentrations in 1L dechlorinated tap water, placed in 3 L beakers. At the reported size fishes were older than 3 months. Three replicates of each tested concentration were assayed. Tests were performed without feeding throughout the exposition (96 h), as recommended for non-renewal fish expositions (USEPA 2002), in the natural photoperiod, at 22 ± 2 °C. Dissolved oxygen was 7.5 ± 0.2 mg/L, conductivity was 1285 ± 2 µS/cm. Mortality was recorded at 24, 48, 72, and 96 h of exposure. Dead individuals were removed immediately. As a validity criterion for the negative control, less than 10 % mortality was considered acceptable (USEPA 2002). Observations of swimming behavioral response of *C. decemmaculatus* to chlorpyrifos exposures at each assayed concentration were conducted every 24 h during the acute toxicity test. Preliminary tests allowed choosing an appropriate chlorpyrifos concentration range within which lethal effects could be tested. Three independent 96-h LC_{50} determinations were performed in June, July, and August 2012. Toxicity tests were performed using a certified chlorpyrifos standard (97 % active ingredient) from Accustandard. Chlolrpyrifos was added in a single dose at the beginning of the exposition period. A stock solution of 150 mg/L was prepared with analytical grade methanol (J.T. Baker). Different exposure concentrations were prepared by diluting the stock solution in dechlorinated tap water.

The dosing volume of the stock solution never exceeded 1.0 mL, the amount used for the highest exposure concentration. A set of control replicates was performed by adding methanol in the same amount as present in the highest exposure treatment.

Insecticide water analysis

Assayed chlorpyrifos concentrations were 135, 120, 100, 90, 70, 50, and 30 µg/L. Chlorpyrifos concentrations in replicates of the two highest doses were determined after 2 h of exposure; measured concentrations were 130 (± 4) and 120 (± 2) µg/L, respectively. Water samples taken from the tested concentrations were passed through C18 columns (Agilent, solid phase extraction (SPE)) and frozen until analysis. Extracts were eluted from C18 columns with 2 mL hexane, followed by 2 mL dichloromethane. The sample extracts were injected into a gas chromatograph with electron capture detection (GC-ECD) (Hewlett Packard, HP 6890), equipped with an HP1 column, 30 m length × 0.25 mm I.d. × 0.25 µm film thickness. N_2 was the carrier; ramp and detector temperatures were 190–250 and 320 °C, respectively.

Recovery from the C18 columns was tested by passing through a solution of known concentration. The C18 columns showed a 97 ± 5.0 % chlorpyrifos recovery.

Sample storage at −20 °C was also tested to assess the holding time of the tested pesticides. Solvents used for pesticide analysis were pesticide grade, J.T. Baker. The detection limit was 0.01 µg/L.

Data analysis and statistics

Mortality data obtained from the 96 h exposures to different chlorpyrifos concentrations were used to estimate the lethal concentration values and their corresponding 95 % confidence limits by means of the Probit model utilizing the EPA Probit Program Version 1.5 software.

Results and discussion

Results from the three independent chlorpyrifos lethal concentrations to *C. decemmaculatus* are shown in Table 1. The overall mean 96 h LC 50 was 105.3 ± 3.1 µg/L. No mortality was observed in the controls. An important aspect in determining the suitability of a test for routine use is reproducibility (Sucahyo et al. 2008). The low variability observed among independent assays is indicative of the high reproducibility attained in chlorpyrifos toxicity testing with *C. decemmaculatus*.

Table 2 summarizes lethal chlorpyrifos concentrations for several fish species reported in the literature. A wide range of lethal concentrations were reported. *C. decemmaculatus* appears to be among the most sensitive fish to chlorpyrifos. Only two fish species, *Oreocromis mossambicus* and *Gambusia yucatana*, showed lower LC_{50} values. The chlorpyrifos concentration lethal to *C. decemmaculatus* was lower than that reported for species of similar habits and ecology such as *Pimephales promelas* and

Table 1. Acute toxicity of chlorpyrifos to the fish *C. decemmaculatus*.

Point	June 2011		July 2011		August 2011	
	Conc. (μg/L)	95 % Conf. lim.	Conc. (μg/L)	95 % Conf. lim.	Conc. (μg/L)	95 % Conf. lim.
LC 1.00	33.6	19.0–44.0	25.34	12.6–36.1	37.8	25.0–47.9
LC 5.00	46.9	32.0–56.0	38.83	23.8–50.1	50.6	37.4–60.5
LC 10.00	56.0	42.0–65.3	48.75	33.3–60.0	59.1	46.3–68.7
LC 15.00	63.1	50.2–72.2	56.84	41.7–68.0	65.7	53.4–75.0
LC 50.00	104.7	92.1–128.1	108.79	94.5–130.4	102.4	92.0–114.7
LC 85.00	173.7	138.5–277.1	208.23	164.1–326.3	159.6	138.2–201.6
LC 90.00	195.8	151.6–334.6	242.80	185.0–409.9	177.3	150.7–232.6
LC 95.00	233.8	173.2–443.1	304.84	220.3–576.1	207.1	171.0–288.1
LC 99.00	326.1	221.5–752.3	467.13	304.7–1094.7	277.2	215.6–432.4
Slope ± SE	4.7 ± 0.9	2.9–6.5	3.7±1.3	2.4–4.9	5.4 ± 0.8	3.8–6.9
Intercept ± SE	−4.5 ± 1.7	−7.9 to −1.1	−2.5 ± 0.6	−5.0 to −0.01	−5.8 ± 1.6	−9.0 to −2.7

Notes: Reported lethal concentrations correspond to Probit estimations from mortality in each assayed exposure concentration (135, 120, 100, 90, 70, 50, and 30 μg/L) performed by triplicate.

Table 2. Lethal chlorpyrifos concentrations to different fish species.

Species	Exposure time (h)	Toxicity LC_{50} (μg/l)	References
Oreocromis mossambicus	96-h LC_{50}	26	Rao et al. (2003)
Gambusia yucatana	96-h LC_{50}	85	Rendón-von Osten et al. (2005)
Cnesterodon decemmaculatus	96-h LC_{50}	105	This paper
Pimephales promelas	96-h LC_{50}	140	Roex et al. (2002)
Gambusia affinis	96-h LC_{50}	150	Boone and Chambers (1996)
Oreochromis niloticus	96-h LC_{50}	154	Oruç (2010)
Cyprinus carpio	96-h LC_{50}	160	Halappa and David (2009)
Poecilia reticulata	96 h LC_{50}	176	Sharbidre et al. (2011)
Pimephales promelas	96 h LC_{50}	200	Belden and Lydy (2006)
Pimephales promelas	96-h LC_{50}	203	Tilak et al. (2004)
Rutilus rutilus	96-h LC_{50}	250	Tilak et al. (2004)
Ictalurus punctatus	96-h LC_{50}	280	Tilak et al. (2004)
Gambusia affinis	96-h LC_{50}	297	Kavitha and Rao (2008)
Gambusia affinis	96-h LC_{50}	298	Rao et al. (2005)
Oryzias latipes	48-h LC_{50}	300	Carlson et al. (1998)
Gambusia affinis	96-h LC_{50}	340	De Silva and Samayawardhena (2002)
Danio rerio	240-h LC_{50}	430	Kienle et al. (2009)
Gambusia affinis	96-h LC_{50}	520	Varó et al. (2000)
Anguilla anguilla	96 h LC_{50}	540	Ferrando et al. (1991)
Carassius auratus	96 h LC_{50}	542	Tilak et al. (2004)
Cyprinus carpio	96-h LC_{50}	582	Xing et al. (2012)
Pimephales promelas	96-h LC_{50}	806	Tilak et al. (2004)
Oreochromis niloticus	96-h LC_{50}	1570	Gül (2005)
Poecilia reticulata	96-h LC_{50}	1790	Selvi et al. (2005)
Clarias macrocephalus	96-h LC_{50}	33,000	Chawanrat et al. (2007)

Oryzias latipes; these species are similar in body size and share the same kind of habitat, comprised of muddy headwaters pools, creeks, small rivers, and vegetated marshes. *C. decemmaculatus* is widely distributed, sensitive to pollutants, easy to rear in the laboratory, taxonomically distinct and well described, and prominent in its ecological community, requisite conditions for species suitable for toxicity testing (Hickey 1989).

Only two of the fish species reported in Table 2 are present in South America, *Cyprinus carpio* and *Gambusia affinis*, and both are cosmopolitan species introduced from Asia and North America, respectively. The Argentine freshwater fish fauna comprises 461 species (Cussac et al. 2008), and the Neotropical fauna of South and Central America more than 2500 fish species (Lowe-Mc Connel 1975). The effects of widely used agrochemicals on the regional fauna remain largely unreported.

In the present study, the fishes exposed to the lowest tested chlorpyrifos concentration (30 μg/L) showed occasional episodes of sudden erratic swimming behavior. Fish exposed to 50 and 70 μg/L showed less activity: fishes stayed motionless at a certain location, generally at mid-water level for prolonged periods. Behavioral response started between 2 and 6 h after dosing. Loss of equilibrium became more frequent in the 90 μg/L concentration. The 100 μg/L concentration group stayed motionless close to the water surface and later fell to the bottom. Fish in the two highest concentrations of 120 and 135 μg/L showed all responses at high intensities: hanging vertically in water and "gulping for air," rapid gill movement, keeping the gills in open position for prolonged periods, erratic swimming, sudden swimming motion in a spiral fashion after long periods of motionlessness, and prolonged and motionless lying down on the aquarium bottom and suddenly starting to move.

Abnormal swimming behavior produced by chlorpyrifos exposure to different fish species has repeatedly been reported. Levin et al. (2004) observed a quantitative swimming behavioral impairment of zebrafish larva (*Danio rerio*) to chlorpyrifos exposures at concentrations higher than 10 μg/L. De Silva and Samayawardhena (2002) reported changes in swimming behavior and pathological changes in gill histology in *Poecilia reticulata* exposed to chlorpyrifos concentrations as low as 0.5, 1, and 2 μg/L. Sharbidre et al. (2011) reported swimming behavioral changes in *P. reticulata* exposed to chlorpyrifos concentrations of 1.7, 2.2, and 4.4 μg/L, and simultaneously detected oxidative stress induction in brain, liver, and gills. Rao et al. (2005) reported swimming impairment in *G. affinis* exposed to chlorpyrifos concentration of 60 μg/L (1/5 of the lethal concentration) and related the observed effect to acetyl cholinesterase inhibition in brain.

Several studies report chlorpyrifos concentrations in streams and rivers draining intensively cultivated basins of Argentina. Jergentz et al. (2005) reported pesticide concentrations in a first-order stream born in a soybean plot on a farm following common management practices. Chlorpyrifos was detected in the stream water coinciding with rain events following field application. Concentrations were in the range of 0.09–0.45 μg/L. Chlorpyrifos was also measured (0.07–0.3 μg/L) in the runoff events that followed applications (Jergentz et al. 2004). Marino and Ronco (2005) surveyed pesticide concentrations in streams and rivers in intensively cultivated areas of Buenos Aires, Argentina. High chlorpyrifos concentrations (0.2–10.8 μg/L) were detected in several samplings. Mugni et al. (2011) measured chlorpyrifos concentrations of 0.01–17 μg/L in a first-order stream running through a cultivated farm close to La Plata, Argentina.

Present evidence suggests that chlorpyrifos concentrations in Argentine rural basins lie below concentrations lethal to *C. decemmaculatus*. However, swimming impairment has been shown to occur at concentrations close to those reported in the agricultural streams, thus representing a risk for the competitive fitness of *C. decemmaculatus* in the natural environment. Swimming impairment of chlorpyrifos to other fish species has been reported at concentrations well below those measured in the Argentine streams.

Therefore, eventual sublethal effects on the regional fish assemblages seem plausible. Further studies are needed on this subject.

Conclusion

C. decemmaculatus is sensitive to chlorpyrifos exposure. Toxicity test results were highly reproducible. Swimming impairment was observed at low exposure concentrations, close to those reported in streams of Argentine agricultural basins. *C. decemmaculatus* represents an appropriate model for ecotoxicological studies. Being widely distributed and normally attaining high densities in shallow South America water bodies, *C. decemmaculatus* seems suitable for use as a sentinel organism for environmental impact assessment.

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