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Acute Toxicity of Endosulfan to the Non-target Organisms *Hyalella curvispina* and *Cnesterodon decemmaculatus*

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Abstract Pesticide consumption in Argentina has steadily increased over the last two decades, while one of these compounds, namely endosulfan, is commonly found in environmental samples. Also the fish *Cnesterodon decemmaculatus* and the amphipod *Hyalella curvispina* are widely distributed in agricultural areas of southern South America. The aim of the present contribution was therefore to determine the acute toxicity of endosulfan to both organisms, and compare it with species sensitivity distributions (SSD) and measured field concentrations. The 48 h-LC_{50} (with 95 % confidence limits) were 1.8 (1.6–2.1) µg/L for *C. decemmaculatus* and 16.4 (15.1–17.7) µg/L for *H. curvispina*. *C. decemmaculatus* was more sensitive than 74 % of fish based on the SSD. Endosulfan concentrations in stream water reported in the literature were often higher than the *C. decemmaculatus* LC_{50}. It may hence be concluded that *C. decemmaculatus* is a suitable sentinel organism for ecotoxicological risk assessment in South America.

Keywords Acute toxicity · Insecticide · Amphipod · Fish

The usage of soil was intensified during the recent decades in Argentina. Traditionally, farmers employed a mixed system of livestock and wheat/corn production. However, after the introduction of genetically modified glyphosate-resistant soybean plant into the market in 1996, the farmers steadily increased its cultivation to attain at present roughly half the total production and cultivated area (MAGyP 2013). Genetically modified soy represents 95 % of the total soy production in Argentina (Bindraban et al. 2009). Today, wheat and soy varieties with a short growing period allow two harvests per year, wheat followed by soy. Livestock has been moved to marginal areas or concentrated in feedlots. At the same time, pesticide consumption increased from 6 million kilograms in 1992 (Pengue 2000) to 32 million kilograms in 2012 (CASAFE 2013). Also nearby South American countries show similar trends. Soy, for instance, is widespread in Brazil, Uruguay, Paraguay and Bolivia, and the crop is usually managed in the same way (Bindraban et al. 2009). Repeated pesticide applications represent a risk to adjacent surface waters due to their transfer via stray drift or run-off (Schulz 2004). Jergentz et al. (2005) and Mugni et al. (2011) detected toxicity pulses and measured endosulfan in water and suspended matter in first order streams passing through soy cultivated plots. Endosulfan was one of the most heavily used pesticides in Argentina (CASAFE 2013). Although recently banned (SENASA 2013), it still appears regularly in environmental samples (Ballesteros et al. 2014) representing a potential risk for environmental health.

In this context, crustaceans and fish have often been used as an ecotoxicological model for risk assessment and for testing pesticide toxicity to non-target fauna. Standard protocols have been developed for acute toxicity water tests (USEPA 2000, 2002; APHA 1998). Because of their wide distribution in the northern hemisphere, the crustaceans *Daphnia magna, Ceriodaphnia* sp., *Gammarus* sp., *Hyalella azteca*, and the fishes *Oncorhynchus mykiss*, *Pimephales promelas* and *Cyprinus carpio* have often been

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used in aquatic toxicity testing. Several of these organisms are, however, absent in South America. The freshwater amphipod *Hyalella curvispina* and the fish *Cnesterodon decemmaculatus* (Jenyns, 1842), a Cyprinodontiformes of the Poeciliidae family, have wide distributions and are often dominant in the communities of shallow environments in southern South America (García et al. 2011; Gómez et al. 1998). Moreover, their distribution overlaps with the agricultural areas (Bindraban et al. 2009).

The aim of this study was therefore to determine the acute toxicity of endosulfan to the amphipod *H. curvispina* and the fish *C. decemmaculatus*, and to compare it with the SSD of fishes and crustaceans. Endosulfan's acute toxicity to *H. curvispina* and *C. decemmaculatus* was additionally compared with reported stream water concentrations providing an insight for the environmental risk assessment of this compound.

Materials and Methods

Toxicity tests were performed using a certified endosulfan standard (98 % active ingredient) from Accustandard. A stock solution of 176 mg/L was prepared with analytical grade methanol (J. T. Baker). The endosulfan concentration of the stock solution was analytically determined and was 95 % of the nominal concentration.

Exposure solutions for *H. curvispina* were prepared by spiking the endosulfan stock solution into moderately hard synthetic water (APHA 1998) to attain nominal assayed concentrations of 2, 10, 15, 20, 25 and 30 µg/L. Endosulfan concentrations in the replicates of the lowest doses were determined 2 h after spiking; measured concentration was 1.7 ± 0.14 µg/L. The exposure solutions for *C. decemmaculatus* were prepared by spiking the endosulfan stock solution into dechlorinated tap water to attain nominal assayed concentrations of 0.5, 0.75, 1.5, 2, 3 and 4 µg/L. Endosulfan concentrations in replicates of the two highest doses were also determined 2 h after spiking; measured concentrations were 2.6 ± 0.3 and 3.4 ± 0.4 µg/L, respectively. In order to verify that methanol did not cause toxicity at the assayed concentrations a set of three control replicates was performed adding methanol in the same amount as present in the highest exposure treatment. No mortality was observed in the controls.

Recovery from the C18 columns was tested by passing through a solution of known endosulfan concentration. The C18 columns showed a 95 % ± 3 % endosulfan recovery. Sample storage at −20°C was also tested to assess the holding time of the tested pesticides: After 2 weeks of storage, which reflects the storage time of the columns, 82 % ± 15 % of the endosulfan was recovered.

For the quantification of endosulfan in the exposure medium, it was passed through C18 columns [Agilent, solid phase extraction; SPE, 6 mL volume, 1 g of Octadecyl (C-18) sorbent]; the columns were air-dried for 30 min and frozen until analysis. Extracts were eluted from C18 columns with 2 mL hexane followed by 2 mL dichloromethane.

Pesticide analysis was conducted using an Hewlett Packard, HP 6890 equipped with dual split–splitless inlets, dual-capillary columns, an Agilent ChemStation, and 2.0 µL injection volume were used for pesticide analyses. The Hewlett Packard, HP 6890 GC was equipped with micro electron-capture detectors (µECDs). For endosulfan analysis, the analytical column was a HP 1 (19091Z-433) cross-linked methyl siloxane capillary column, 30 m × 0.25 mm inner diameter × 0.25 µm film thickness. Column oven temperatures were: inlet at 190°C for 4 min; ramp at 190–250°C and hold for 20 min. The retention time was 8.1 and 9.0 min for endosulfan I and II, respectively. The carrier gas used was ultrahigh-purity (UHP) N_2 at 34 mL/min and the inlet temperature at 250°C. The µECD temperature was 320°C, with a constant makeup gas flow of 60 mL/min UHP nitrogen. All solvents used were of analytical quality (J. T. Baker). The detection limit was 0.01 µg/L.

Specimens of *H. curvispina* and *C. decemmaculatus* were collected from an uncontaminated stream located 25 km south of La Plata City (35°08′ 00.66″S 57°23′ 54.55″W) and reared in the laboratory. Environmental parameters measured at the sampling site were as follows; temperature 6–25°C, dissolved oxygen 5.4–10.3 mg/L and conductivity 234–461 µS/cm. Nutrients measured in the laboratory were: ammonia 5–37 µg $N–NH_4^+$/L, nitrate 77–187 µg $N–NO_3^-$/L and soluble reactive phosphorus (SRP) 19–119 µg P/L. The organisms were kept in glass aquariums with stream water, which was gradually replaced by unchlorinated tap water to compensate for evaporation losses. The locally abundant macrophyte *Lemna* sp. was placed on the surface of the water since *H. curvispina* fed on the periphytic community associated with its rhizosphere. Moreover, the food was supplemented by a mixture of fresh lettuce leaves and dried algae ad libitum twice a week.

Cnesterodon decemmaculatus is a small, viviparous, micro-omnivorous, benthic–pelagic, non-migratory fish (Gómez et al. 1998). Individuals were fed daily ad libitum with Shulet® Carassius commercial fish food. The animals were cared for in accordance with SENASA (Argentinean National Service for Sanitary and Quality of Agriculture and Food) guidelines 617/2002 for biological testing.

The *H. curvispina* 48 h-LC_{50} determination was repeated on three occasions throughout a 6 month period, from September 2011 to March 2012. Procedures for *H. curvispina* toxicity tests were similar to standardized protocols for *H. azteca* (USEPA 2000). Briefly, *H. curvispina* individuals of 5–10 mm in length were exposed to different endosulfan concentrations diluted in 100 mL of the exposure solution contained in 250 mL glass beakers. Three replicates containing ten individuals each were tested with each assayed concentration. Tests were performed without feeding, at 22 ± 2°C, and natural photoperiod, ranging from 13–11 h light/dark in September to 11–13 h light/dark in March. Mortality was recorded after 24 and 48 h exposure and dead individuals were removed. As a validity criterion for the negative control, <10 % mortality was considered acceptable.

The *C. decemmaculatus* 96 h-LC_{50} determination was repeated on three occasions during a 4 month period, from February to June 2011. Toxicity tests followed standardized protocols for the fathead minnow, *Pimephales promelas,* from USEPA (2002). Ten *C. decemmaculatus* individuals, 20–30 mm in length, were exposed to different endosulfan concentrations diluted in 1 L exposure solution placed in 3 L beakers. Three replicates of each test concentration were realized. Tests were performed without feeding, at 22 ± 2°C, and natural photoperiod, ranging from 13 to 11 h light/dark in February, to 10–14 h light/dark in June; dissolved oxygen was 7.5 ± 0.2 mg/L, conductivity 1285 ± 0.8 µS/cm. Mortality was recorded at 24, 48, 72 and 96 h of exposure and dead individuals were removed. As a validity criterion for the negative control, <10 % mortality was considered acceptable (USEPA 2002).

Mortality data obtained from 48 h endosulfan exposures were used to estimate lethal concentrations for *H. curvispina* and *C. decemmaculatus*. The 96 h lethal concentration for *C. decemmaculatus* was also estimated. LC_{50}s values and their corresponding 95 % confidence limits were calculated by fitting a two-parameter log-logistic model. Slope and LC_{50} (inflection point of the exposure–response curve) of each fitted curve were compared. All statistical analysis was performed using the drc (Analysis of Dose–Response Curves) package in R (Ritz and Streibig 2005). Significance level for all the applied tests was 0.05.

To compare the sensitivity of *C. decemmaculatus* and *H. curvispina* to other fishes and crustaceans, we calculated the species sensitivity distributions (SSDs) for endosulfan with the USEPA SSD Generator software (USEPA 2015a) using acute 48 h toxicity data for freshwater crustaceans and fishes in the USEPA ECOTOX database (USEPA 2015b; http://cfpub.epa.gov/ecotox/).

Results and Discussion

The results of endosulfan toxicity tests with *H. curvispina* and *C. decemmaculatus* are shown in Fig. 1. Estimated 48 h-LC_{50} and 95 % confident limits to *C. decemmaculatus* and *H. curvispina* were 1.8 (1.6–2.1) and 16.4 (15.1–17.7) µg/L, respectively. No mortality was observed in the controls. No significant differences were observed between slopes, but LC_{50} were significantly different ($p < 0.0001$), indicating higher endosulfan toxicity to *C. decemmaculatus*. Estimated 96 h-LC_{50} to *C. decemmaculatus* was 1.7 (1.6–1.9) µg/L.

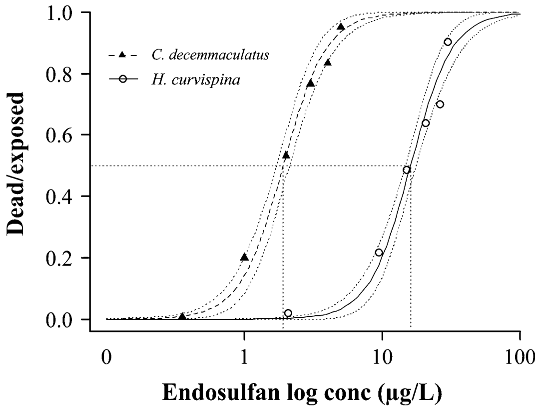

Fig. 1 Exposure-response curve (48 h) and 95 % confidence limits for endosulfan to the fish *C. decemmaculatus* and the amphipod *H. curvispina. Dotted lines* represent estimated LC_{50}s

The SSDs of fishes and crustaceans for endosulfan are shown in Figs. 2 and 3. *C. decemmaculatus* sensitivity is relatively high, while that of *H. curvispina* is relatively low. The SSD for endosulfan, based on data for 27 fish species indicates that *C. decemmaculatus* is more sensitive than 74 % of the reported species. More sensitive organisms tested include *Mugil cephalus*, *Mugil curema*, *Leiostomus xanthurus*, *Monopterus albus*, *Carassius auratus and Orizyas latipes.* From these, only *Mugil* spp., are common in South America. They occur in shallow and brackish waters and are euryhaline ranging from hypersaline lagoons to freshwaters (González Castro et al. 2006). Thus, they don't represent common components in inland streams draining agricultural areas.

The SSD for endosulfan based on data for 30 crustacean species indicates that *H. curvispina* is less sensitive than 57 % of species. However *H. curvispina* is highly sensitive to other insecticides, such as cypermethrin (Mugni et al. 2013). Overall, present results are consistent with previously reported comparatively lower LC_{50}s of fishes to endosulfan than that of many invertebrate species.

Reported endosulfan concentrations in the literature cover a wide range. Mugni et al. (2011) reported a high concentration (38 µg/L) in coincidence with the first rain after application in a first-order stream running through a cultivated farm. Di Marzio et al. (2010) surveyed 13 small first and second order streams in cultivated areas in Buenos Aires and Cordoba Provinces, Argentina. The sampling was performed in coincidence with a period of insecticide application. Endosulfan was detected in all studied streams,

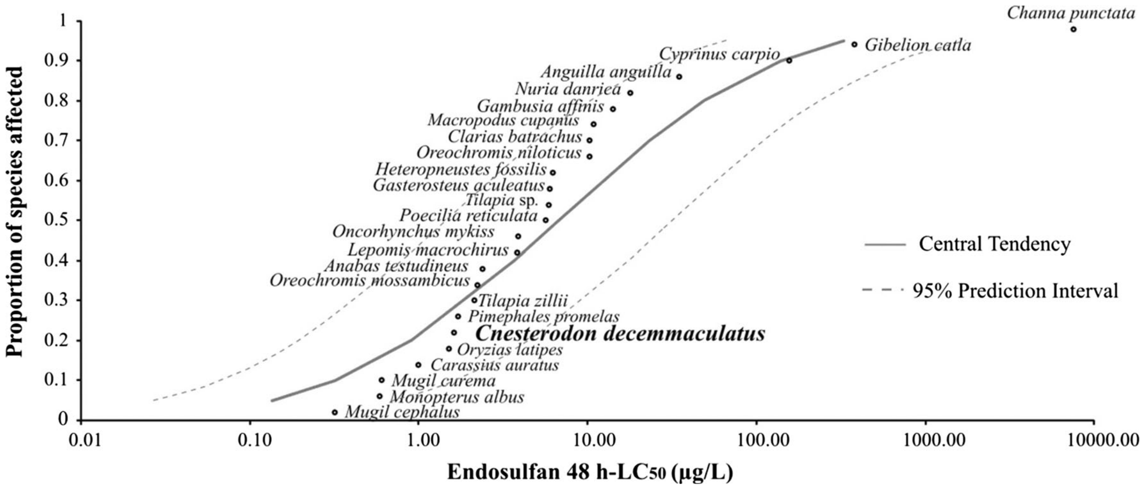

Fig. 2 Sensitivity of *C. decemmaculatus* compared to other fishes, calculated by species sensitivity distributions (SSDs) for endosulfan using acute 48 h toxicity data

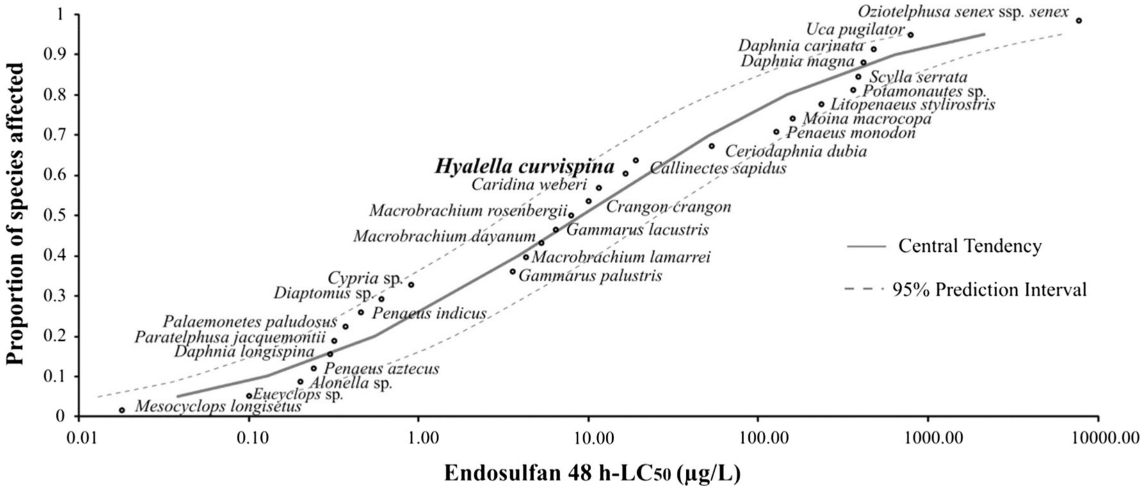

Fig. 3 Sensitivity of *H. curvispina* compared to other crustaceans, calculated by species sensitivity distributions (SSDs) for endosulfan using acute 48 h toxicity data

ranging from 0.8 to 20 µg/L; half of them were higher than the LC_{50} of *C. decemmaculatus*. Schulz (2001) reported water concentrations of 8.5–12.3 µg/L in small streams tributaries of the Lourens River draining a fruit orchard area in South Africa. High values were also reported in farm ditches in British Columbia, Canada, up to 13.4 µg/L (Wan et al. 1995) and in the Gwydir River, southeastern Australia, up to 4 µg/L (Muschal 1998).

Ecological risk can be estimated by using the Hazard Quotient (HQ) approach (Giesy et al. 2000). The HQ is defined as the ratio between the measured environmental concentration and the toxicity reference value (Giesy et al. 2000). If the HQ values exceed 1.0, harmful effects cannot be excluded. If the HQ is <1.0, harmful effects are not likely to occur. The estimated HQ values using reported endosulfan concentrations in the Pampasic streams and the *C. decemmaculatus* LC_{50} estimated in the present work ranged 0.4–21, being higher than 1 in half of the studied streams. The estimated HQ for *H. curvispina* ranged 0.05–2.3. Please note that sublethal effects are expected at much lower concentrations than the LC_{50} suggesting higher risks over the long run. All reported endosulfan concentrations in the studied streams were higher than the *C. decemmaculatus* LC_1 (0.3 µg/L), a concentration pointing incipient mortality, higher than those expected to cause sublethal effects (Hanken and Stark 1998; Newman and Unger 2002). If the HQ values are calculated using the LC_1, the estimated values became remarkably high: 2.3–126. Harmful effects are therefore expected, suggesting endosulfan toxicity to *C. decemmaculatus* in the Argentine Pampasic streams.

Irrespective of the risk estimations, *C. decemmaculatus* is highly sensitive to endosulfan. At the same time this species is widely distributed and often attains high densities in shallow South America water bodies. Therefore, *C. decemmaculatus* seems suitable for use as a sentinel organism for environmental impact assessment of endosulfan.

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