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Short communication

Effects of chlorothalonil on development and growth of amphibian embryos and larvae

Shuangying Yu [a], Mike R. Wages [a], George P. Cobb [b], Jonathan D. Maul [a,*]

[a] Department of Environmental Toxicology, The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX 79416, USA
[b] Department of Environmental Science, Baylor University, Waco, TX 76798, USA

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ABSTRACT

Chlorothalonil is a broad spectrum fungicide widely used in agricultural and urban environments, yet little is known regarding its effects on amphibians. We examined effects of chlorothalonil on growth, malformations, and mortality in embryos and larvae of *Xenopus laevis* and *Spea multiplicata*, and assessed variation in sensitivity among aquatic organisms using a species sensitivity distribution (SSD). Chlorothalonil induced gut malformations in *X. laevis* embryos and inhibited growth. Tail degeneration was observed in larvae of both species and reduced tail length to total length ratios occurred at environmentally relevant concentrations (5.9 and 11.0 μg/L). The mechanism of tail degeneration is unclear, but alteration in the expression of genes involved in tail resorption is a hypothesized mechanism. Larval amphibians were more sensitive than invertebrates and fish. Based on our results and the range of reported environmental concentrations, chlorothalonil may pose a risk to larval amphibians in certain habitats and scenarios.

1. Introduction

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is the most commonly used fungicide in the U.S. (USEPA, 2011) and is widely used in agriculture to protect crops (USEPA, 1999). Chlorothalonil may be introduced to aquatic habitats by direct application, spray drift, and runoff, which may pose a risk to aquatic organisms. Chlorothalonil concentrations in runoff ranged from 50 to130 μg/L after two days of rainfall (Potter et al., 2001) and can be as high as 500 μg/L during the first runoff event (Wilson et al., 2010). Average body residue in Pacific treefrog (*Hyla regilla*) tadpoles collected from the Kaweah River basin, CA ranged from 33.3 to 47.7 ng/g wet weight (Datta et al., 1998).

Chlorothalonil is highly toxic to fish and invertebrates (USEPA, 1999). The 24–96 h LC50s ranged from 12 to 195 μg/L for freshwater invertebrates and 16 to 76 μg/L for freshwater fish (van Wezel and van Vlaardingen, 2004). Chlorothalonil is toxic to tunicate blood cells at 1 μM (Cima et al., 2008) and fish phagocytes at 250 μg/L (Baier-Anderson and Anderson, 1998). Chronic exposure at 2.0 μg/L can decrease hematocrit and cause gill damage in fish (Davies, 1987).

However, studies that address chlorothalonil toxicity to amphibians are limited. A 48-h LC50 of 160 μg/L was reported for Japanese common toad *Bufo bufo japonicus* tadpoles (Hashimoto and Nishiuchi, 1981). Recently, adverse effects on survival, liver tissue and immune cells, and corticosterone concentrations in anurans have been reported at chlorothalonil concentrations between 0.0164 and 164 μg/L (McMahon et al., 2011). Taken together, these studies indicate that chlorothalonil may have a significant impact on amphibians and toxicity data are needed. The objectives of this study were to: (1) examine effects of chlorothalonil on development and growth in two anuran species, African clawed frogs (*Xenopus laevis*) and Mexican spadefoot toads (*Spea multiplicata*), and (2) compare sensitivity of larval amphibians and other aquatic organisms to chlorothalonil.

2. Materials and methods

2.1. Experiment 1: embryo and larval Xenopus laevis 96-h acute toxicity tests

Breeding, housing, and field collection methods for *X. laevis* and *S. multiplicata* have been described in the supplemental materials. Toxicity tests were conducted following standardized FETAX methods (ASTM, 2004) with slight modifications. Embryos were not dejellied to achieve environmental realism and because egg jelly may protect embryos from ambient contaminants (Edginton et al., 2007).

Embryos were collected from three clutches, mixed, and examined for viability and developmental stage. Twenty NF stage 8–11 embryos (Nieuwkoop and Faber, 1975) were randomly assigned to each of 50 ml glass jars containing 40 ml FETAX solution spiked with chlorothalonil. Concentrations used were solvent control (acetone), 7.8, 13.0, 21.6, 36.0, and 60.0 μg/L (see Supplemental materials for rationale for choosing these concentrations). There were five replicates for each treatment.

* Corresponding author.
E-mail address: jonathan.maul@ttu.edu (J.D. Maul).

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In the larval experiment, NF stage 46 larvae from three clutches were mixed and 10 larvae were randomly placed in 1 L glass jars containing 900 ml FETAX solution. Treatments included solvent control, 10.2, 12.8, 16.0, 20.0, and 25.0 μg/L, and there were five replicates for each treatment. To compare growth and other morphological measurement before and after exposure, 18 larvae were randomly collected from the same clutches prior to the exposure. These were used to quantify initial size of larvae. Larvae were fed approximately an hour before water change with a diet described in Koss and Wakeford (2000).

Experiment 1 was conducted under static renewal conditions with daily water change. All animals were maintained on a 12:12, light:dark cycle at temperatures ranging from 22 to 24 °C. Water quality was measured every other day. Embryos and larvae were checked daily for mortality (i.e., no response to prodding). At the end of the exposure, larvae were euthanized and preserved in formalin. Each individual was examined for abnormalities under a microscope. Images of each larvae were taken and total length (head to tail) (TL), body length (BL), and snout-to-vent length (SVL) were measured using Image J (version 1.44P, Wayne Rasband, National Institutes of Health, USA). Larvae exposed to chlorothalonil showed reduced tail length; therefore, tail length was also examined and calculated from TL and BL (Tail = TL − BL). We used the tail to TL ratio to minimize the effect of growth rate on absolute tail length measurements and compared the ratio among treatments. Initial larval size measurements prior to the exposure were included in comparisons to demonstrate growth in controls during the experimental period.

2.2. Experiment 2: verification experiments for Xenopus laevis

To verify the results of Experiment 1, partial toxicity tests with three replicates per treatment were conducted for embryos and larvae with the same procedures and conditions as previous experiments. However, embryos from only two clutches were used due to lack of breeding or low quality eggs in other clutches. Three clutches were used for the larval experiment. Thirty-one larvae were euthanized and preserved in formalin before the exposure and used to determine initial size measurements. Larvae were fed daily 2 mg of Sera® Micro per experimental unit.

2.3. Experiment 3: larval Spea multiplicata 96-h acute toxicity test

Larvae (Gosner stage 25) from five clutches were mixed and were randomly assigned to each treatment. Five larvae were placed in 500 ml glass jars with 400 ml laboratory water and six replicates were used for each treatment. A 96-h static non-renewal toxicity test was performed. Treatments included solvent control, 3.2, 5.4, 9.0, 15.0, and 25.0 μg/L. Fifteen larvae were collected for initial size measurements. Larvae were not fed during the exposure. Water quality was measured at the end of the exposure. Morphological measurements were similar to *X. laevis*.

2.4. Comparison of chlorothalonil toxicity to amphibians, fish, and invertebrates

A species sensitivity distribution (SSD) curve was created for fish, invertebrates, and amphibians to compare sensitivity among taxa and evaluate the risk chlorothalonil poses to aquatic organisms. The SSD curve was fit with the log-probit distribution using the U.S. EPA Species Sensitivity Distribution Generator (SSD_Generator_V1.xlt). We only used 96-h LC50s to minimize the variation due to exposure duration.

2.5. Chemical and statistical analyses

Chlorothalonil exposure concentrations were quantified using a gas chromatograph equipped with an electron capture detector. Measured chlorothalonil concentrations were reported throughout with correction for extraction efficiency. Data were analyzed using JMP Statistical Analysis Software (Ver 9.0.0, Statistical Analysis System Institute, Cary, NC, USA) and results were considered significant at $\alpha = 0.05$ (see Supplemental materials for more information on chemical and statistical analyses).

3. Results and discussion

3.1. Chemical analyses

Measured water concentrations of chlorothalonil ranged from 58 to 142% of the nominal concentrations with an average of 96% (Table 1). In Experiment 3, the mean chlorothalonil concentrations were 84, 84, and 76% of the initial concentrations for 3.2, 9.0, and 15.0 μg/L (nominal) treatments, respectively, after 96 h (data provided in Supplemental materials). Water quality data were also provided in Supplemental Materials.

Table 1
Nominal and measured chlorothalonil concentrations (μg/L) at the beginning of exposures, percent mortality at each concentration, 96-h median lethal concentrations (LC50s, 95% confidence interval), and model parameters associated with LC50 estimates for *Xenopus laevis* and *Spea multiplicata*.

Species	Stage	Nominal (μg/L)	Measured (μg/L)	Mortality (%)	LC50 (95% CI)	Slope	Intercept
X. laevis[a]	Embryo	Control	0	0	42.4	0.13	−5.36
		7.8	8.8	8	(39.8–45.3)		
		13.0	12.8	3			
		21.6	19.3	6			
		36.0	36.4	16			
		60.0	60.7	99			
	Larvae	Control	0	10	8.2	0.31	−2.57
		10.2	5.9	29	(7.1–9.2)		
		12.8	8.3	52			
		16.0	14.4	82			
		20.0	17.0	98			
		25.0	23.8	100			
X. laevis[b]	Embryo	Control	0	0	22.9	0.25	−5.68
		7.8	6.1	5	(21.4–24.6)		
		13.0	9.5	2			
		21.6	18.8	23			
		36.0	26.8	75			
		60.0	60.3	100			
	Larvae	Control	0	3	14.4	0.56	−8.02
		10.2	8.6	10	(13.6–15.4)		
		12.8	11.1	0			
		16.0	13.4	27			
		20.0	16.3	86			
		25.0	22.5	100			
S. multiplicata	Larvae	Control	0	0	10.7	0.58	−6.26
		3.2	4.6	0	(9.7–12.2)		
		5.4	7.0	0			
		9.0	11.0	7			
		15.0	20.5	93			
		25.0	34.6	100			

[a] Experiment 1.
[b] Experiment 2, verification experiment.