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Physiological effects and bioconcentration of triclosan on amphibian larvae

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ABSTRACT

We examined the acute effects of triclosan (TCS) exposure, a common antimicrobial found as a contaminant in the field, on survival and physiology of amphibian larvae. LC50 values were determined after 96 h for North American larval species: *Acris crepitans blanchardii*, *Bufo woodhousii woodhousii*, *Rana sphenocephala*, and for a developmental model: *Xenopus laevis*. Amphibian larvae were most sensitive to TCS exposure during early development based upon 96-h LC50 values. Heart rates for *X. laevis* and North American larvae exposed to TCS were variable throughout development. Metabolic rates of *X. laevis* and *R. sphenocephala* larvae exposed to TCS were significantly affected in larvae exposed to [50% LC50] and [LC50]. Tissue uptake and tissue bioconcentration factor (BCF) of TCS were investigated in *X. laevis*, *B. woodhousii woodhousii*, and *R. sphenocephala*. In general, a significant increase was observed as exposure concentration increased. Tissue BCF values were dependent upon stage and species. While TCS concentrations used here are higher than environmental concentrations, exposure to TCS was dependent upon species and developmental stage, with early developmental stages being most sensitive to TCS exposure.

1. Introduction

Over the past few decades, concerns have increased over potential adverse ecological and toxicological effects from the use, production and disposal of household products (Daughton and Ternes, 1999). Little is known about the toxicological effects these contaminants have after their uses and disposal. In particular, knowledge of the environmental occurrence, transport, and fate of pharmaceuticals and personal care products (PPCPs) is just now emerging in the literature. One class of heavily used environmental contaminants is antimicrobials, including the widely used triclosan (TCS, 5-Chloro-2-(2,4-dichlorophenoxy)phenol). Ninety-five organic wastewater contaminants were surveyed in 139 streams across 30 states over the course of two years (1999–2000) and organic wastewater contaminants were found in 80% of the streams sampled (Kolpin et al., 2002). Of the 95 contaminants surveyed, TCS was among the top five most frequently detected compounds (Kolpin et al., 2002).

Triclosan is considered an important bacteriocide used in personal care and consumer products such as shampoo, soaps, deodorants, toothpaste, and plasticware. The fate and behavior of TCS in the environment is not fully understood, although photolysis has been identified as a major degradation pathway in surface waters (Lindstrom et al., 2002). Triclosan has an estimated half-life of 60 days in water (Halden and Paull, 2005) and TCS was detected in 58% of the streams investigated with a maximum concentration of 2.3 μg L^{-1} (Kolpin et al., 2002). Triclosan is a relatively stable lipophilic compound (log K_{ow} of 4.8) and is expected to bioaccumulate in aquatic organisms (Halden and Paull, 2005). Triclosan is not currently regulated by the EPA.

Environmentally safe concentrations of contaminants are typically based on levels determined from only a handful of organisms. Amphibians are not usually included in toxicity tests because chemical registration does not currently require amphibian tests (Cooney, 1995). Many amphibians breed within or around agricultural areas that are routinely exposed to pesticides and other contaminants. Some consider amphibians more sensitive to aquatic contaminants than other species because they readily absorb chemicals through both their gills and permeable skin (Boyer and Grue, 1995). The timing of contaminant exposure during development can be crucial for amphibian larvae survival (Bridges, 2000). Amphibian larvae are known to reduce their activity in the presence of low concentrations of various contaminants, including metals, polycholorinated biphenyls, ammonia, and nitrates (Bridges and Semlitsch, 2005). In contrast, recent studies suggest that amphibians may not be more sensitive than other aquatic species (Schiesari et al., 2007; Bernanke and Köhler, 2009; Kerby et al., 2010). Because of a lack of toxicity data for amphibians it is important to assess the role contaminants might play in the decline of amphibian populations.

Physiological studies should provide a better understanding as to how contaminants are toxic to aquatic organisms. Behavioral responses of amphibians to exposure to TCS have been investigated. Triclosan was found to negatively affect activity and larvae exposed to TCS had increased mass from the controls (Fraker and Smith, 2005).

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Triclosan was found to affect hind limb development in amphibian larvae by affecting thyroid hormone induced metamorphosis (Veldhoen et al., 2006). Because TCS has caused effects in amphibian larvae, it is important to further investigate TCS in order to determine an underlying mechanism for these effects. In the presence of adverse environmental conditions, aquatic organisms will try to escape or activate physiological and biochemical responses to counteract the stress. Aquatic organisms can thus display large variations in cardiac and metabolic functions in response to adverse environmental conditions.

The objectives of this study were to measure acute toxicity (96-h LC50 values) to TCS in early and late larval developmental stages of *Xenopus laevis* (African clawed frog) and to determine if sensitivity changes as the larvae mature. The larval stages of *X. laevis* were chosen as test organisms for this study because they are a widely used developmental model. Four developmental stages were investigated to determine if the toxicity of TCS on *X. laevis* was dependent upon developmental stage. *X. laevis* is often a model system for amphibian studies, but contaminant sensitivity can vary between species. Therefore, the effects of TCS on an early developmental stage of three North American amphibian species (*Acris crepitans blanchardii, Bufo woodhousii woodhousii*, and *Rana sphenocephala*) were also investigated in this study. The acute effects of TCS exposure were determined on the heart rate and metabolic rate to determine if the sensitivity differs between species when compared at the same developmental stage. Triclosan body burden assessments for all the exposed species were made on the basis of empirical bioconcentration factors (BCFs).

2. Materials and methods

2.1. Test animals and stages

X. laevis larvae were obtained from Xenopus Express (Brooksville, FL) and were staged according to Nieuwkoop and Faber (1967). Four larval stages were investigated throughout development; Nieuwkoop and Faber stages 41, 49, 54, and 66. *A. crepitans blanchardii* (Blanchard's cricket frog), *B. woodhousii woodhousii* (Woodhouse's toad) and *R. sphenocephala* (Southern leopard frog) larvae were collected locally in Denton and Tarrant Counties, Texas under Scientific Permit Number SPR-0604-389 issued by the Texas Parks and Wildlife Department. Larvae were staged according to Gosner (1960). Laboratory hatched and field hatched larvae were included in this study. Water samples were taken from field sites to determine if TCS was present. There were no detectable levels of TCS in the field. Larvae were allowed to acclimate for one week in the lab and were treated in the same fashion as those which were hatched in the lab. Triclosan exposure experiments were conducted on larvae at stage 30 of *A. crepitans blanchardii*, *B. woodhousii woodhousii*, and *R. sphenocephala*. All experiments were approved by the University of North Texas IACUC. All tadpoles were weighed on a balance (Ohaus Explorer, Model E12140) prior to use.

2.2. LC50 values

Dilutions of TCS were made using artificial pond water mixed with TCS, which was dissolved in ethanol. All studies were conducted in artificial pond water (6 mM NaCl, 0.6 mM $CaCl_2$, and 12 μM $NaHCO_3$ in distilled water). Water quality measurements were measured and monitored over the course of each experiment: pH 7.5–8.0, water hardness 100–120 mg $L^{-1}CaCO_3$, and dissolved oxygen 7–10 mg L^{-1}. Triclosan was dissolved in ethanol and then added to artificial pond water for a final concentration of 1000 μg L^{-1} TCS. The effects of ethanol as a carrier were investigated in preliminary trial runs and no significant differences in heart rate or metabolic rates were observed between the highest ethanol concentration and control. For determining LC50 values, larvae were placed into beakers containing 250 ml nominal test concentrations (ranging from 31 to 750 μg L^{-1}) of TCS for 96 h. Control beakers containing no TCS were also included in the study. The solutions remained static and unaerated for the 96-h exposure period. The beakers were placed into an environmental chamber with a 12D:12L photoperiod and maintained at a temperature of 24 ± 1 °C. There were 10 larvae per beaker, 4 replicate beakers per concentration, and one experiment was carried out for each species. Triclosan treatments varied for each species and concentrations were altered depending upon their sensitivity to TCS determined in preliminary trials. More treatments were included for *A. crepitans blanchardii* (8), *B. woodhousii woodhousii* (9), and *R. sphenocephala* (10) as no data exists on the effects of TCS in these North American species, while *X. laevis* had 5 treatments per developmental stage. There was one experiment for each species. Amphibian larval survival was observed daily and the LC50 concentration was determined using the Trimmed Spearman Karber Method, where acceptability of the test results required 90% survival of controls.

2.3. Heart rates

Those larvae surviving the 96 h were then used for heart rate measurements. Heart rates were measured by inverting the larvae in a low melting point agar (Sea Plaque agarose) containing 0.1 mg ml^{-1} MS-222. The animals were maintained at 24 °C and visualizing them with a Nikon dissecting microscope. A 30-s video clip of the beating heart ventricle was recorded with an Olympus DP70 camera using ImagePro Plus software. Heart rate videos were played back in ImagePro Plus and ventricle contraction was counted for 30 s and multiplied by two to obtain a measure of heart rate in beats per minute (bpm).

2.4. Metabolic rates

Triclosan concentrations for measuring metabolic rates were based on the calculated LC50 values. Ten to twelve larvae were placed into one of three concentrations, 0 μg L^{-1}, [50% LC50], and [LC50]. Following 96-h exposure, individual metabolic rates were measured using a closed respirometry system. Larvae were placed into 50 ml Erlenmeyer flasks containing the same experimental concentration of TCS the larvae had been exposed to over the last 96 h. The flask had two ports, one near the top and the other on the bottom. One empty 10-ml syringe was connected to the port at the top of the flask, while another 10-ml syringe containing 10 ml of water with the experimental concentration was connected at the bottom. Every 30 min, the water inside the flask was mixed by pushing 2 ml of water from the bottom syringe into the flask and removing 2 ml of water with the top syringe a total of four times. After mixing, a 2-ml water sample was removed and injected into an oxygen electrode (Microelectrodes, Inc.) that was calibrated and connected to an ADinstruments 8SP Powerlab. Water samples were taken every 30 min for 3 h. The calculated metabolic rates were averaged for each individual. Weight specific metabolic rates were determined for each larva and compared across concentrations.

2.5. TCS bioconcentration

North American amphibian larvae were investigated for TCS exposure at developmental stage 30 (according to Gosner, 1960), while *X. laevis* larvae were investigated at three developmental stages: 49, 54, and 66. Larvae were exposed to nominal TCS concentrations of 0, 31, 94, 250, and 500 μg L^{-1} for 96 h. After 96 h, the larvae were euthanized by placing them in a solution of 0.1 mg ml^{-1} MS-222, placed in foil, and stored at −20 °C until tissue analysis was performed. *B. woodhousii woodhousii* larvae at 250 and 500 μg L^{-1},