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Effects of α-cypermethrin enantiomers on the growth, biochemical parameters and bioaccumulation in *Rana nigromaculata* tadpoles of the anuran amphibians

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ABSTRACT

Populations of many amphibian species are declining worldwide in part because of pesticide contamination. As a surface water contaminant, α-cypermethrin may have severe ecological impacts on amphibians. Here, we examined the acute toxicity of α-cypermethrin enantiomers to dark-spotted frog *Rana nigromaculata* tadpoles at 24, 48, 72 and 96 h, finding that the tadpoles were indeed sensitive to α-cypermethrin. The (S)-(1R, 3R)-enantiomer was approximately 29 times more toxic than the (R)-(1S, 3S)-enantiomer at 96 h. A significant delayed growth in *R. nigromaculata* tadpoles after exposure to 0.5 μg L^{-1} of S-(1R, 3R)-cypermethrin was observed. Additionally, increased superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and malondialdehyde (MDA) levels indicate the presence of oxidative stress in the tadpoles. Further, tadpoles exposed to sublethal concentrations of α-cypermethrin enantiomers exhibited enantioselective growth and oxidative damage. Bioaccumulation experiments showed that the tadpoles could rapidly accumulate α-cypermethrin. The (R)-(1S, 3S)-enantiomer was preferentially accumulated over the (S)-(1R, 3R)-enantiomer, and it was also eliminated more quickly, as evidenced in the subsequent depuration experiments.

1. Introduction

Amphibian populations have become a major concern because of their rapid decline and extinctions (Houlahan et al., 2000). Factors contributing to the amphibian crisis are diverse, including climatic changes, increased exposures to ultraviolet-B (UV-B) radiation, increased numbers of emerging infectious diseases, habitat losses and environmental contaminations (Blaustein et al., 2003; Carey and Bryant, 1995; Davidson et al., 2002; Houlahan et al., 2000; Kiesecker et al., 2001). Many studies have shown that chemical pesticides may have detrimental effects on the health of amphibians since these agricultural contaminations runoff into aquatic ecosystems. Amphibians have exhibited a variety of physiological, histological, and biochemical alterations, leading to morphological abnormalities, delayed development, retardation in growth and a reduction in numbers (Attademo et al., 2014; Mann et al., 2009; Yadav et al., 2013; Yin et al., 2014). Amphibians may be especially sensitive to pesticides because of their highly permeable skin, unshelled eggs, and exposure to terrestrial and aquatic environments at different life stages (Kerby et al., 2010). Therefore, amphibians have been regarded as ideal bio-indicators of aquatic and agricultural ecosystems (Feng et al., 2004). Tadpoles, which are amphibian larvae, provide key links from lower to higher trophic positions because they are omnivores and are often preyed upon by other species (Junges et al., 2012). Dark-spotted frog is a widespread and abundant species in China that lives in a variety of habitats, including agricultural sites. Dark-spotted frog larvae grow and develop at the same time as the pesticide is used in spring. Thus, damage caused by the uptake of the toxicants begins from birth. In this study, we selected the dark-spotted frog tadpole as a test animal to evaluate the toxic effects of chemicals in aquatic environments.

Cypermethrin is widely used in agricultural and non-agricultural areas because of its high efficiency in pesticide control and its low mammalian toxicity. It was often detected at levels of 0.01–9.8 μg L^{-1} in water column, but its concentration can be as high as 194 μg L^{-1} in the runoff of some farmed areas following pesticides applications (Laabs et al., 2002; Marino and Ronco, 2005; Vryzas et al., 2011; Xing et al., 2012). Since its chemical structure contains two chiral carbons in the cyclopropyl ring and one chiral position at the R-cyano carbon, cypermethrin is a combination of eight stereoisomers. α-Cypermethrin is one racemate of cypermethrin that includes two cis diastereomers, (+)-(S)-(1R, 3R)-enantiomer and (-)-(R)-(1S, 3S)-enantiomer (Fig. 1). Individual enantiomers of a chiral compound may

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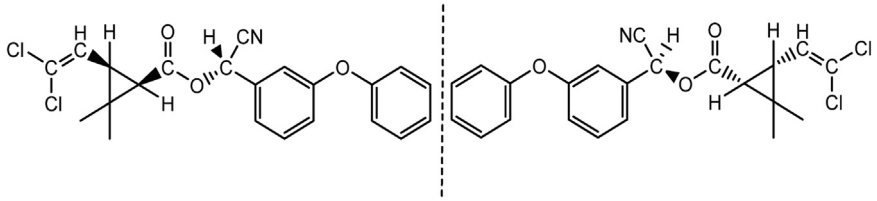

Fig. 1. Chemical structure of α-cypermethrin enantiomers.

display differences in biologically mediated environmental processes, including bioaccumulation, biodegradation, bioactivity and toxicity (Diao et al., 2011; Liu et al., 2004). However, most previous studies concerning risk and environmental fate assessments considered only cypermethrin rather than its specific forms or different stereoisomers, presumably because of the inherent difficulties in separating and identifying the various stereoisomers (Greulich and Pflugmacher, 2004). Therefore, it is crucial to assess the enantioselectivity of α-cypermethrin in an aquatic environment.

The toxicity data of cypermethrin for aquatic non-target organisms are abundant and include fishes (e.g., 96 h LC_{50} was 0.952 μg L^{-1} for Great sturgeon, 0.917 μg L^{-1} for Silver carp and 0.627 μg L^{-1} for Caspian roach) (Jahanbakhshi et al., 2012; Shaluei et al., 2012) and aquatic invertebrates (96 h EC_{50} of 0.03 μg L^{-1} for Crustaceans *Diaptomus forbesi*) (Saha and Kaviraj, 2008). Several studies have shown that cypermethrin is extremely toxic and can induce physiological alterations, delayed development, and morphological abnormalities in tadpoles (Agostini et al., 2010; David et al., 2012; Yu et al., 2013). However, little is known about the enantioselective toxic effects of α-cypermethrin in tadpoles.

In this study, a short-term exposure assessment was developed to investigate the enantioselective toxicokinetics of α-cypermethrin in dark-spotted frog tadpoles at environmentally relevant concentrations. More specifically, the objectives of this study were to (i) determine the acute toxicity of α-cypermethrin enantiomers to tadpoles, (ii) examine the differences between the enantiomers on the tadpoles' growth and the antioxidant enzyme activities of SOD, CAT, GST and MDA, and (iii) evaluate the enantioselective behaviours on the uptake and elimination of α-cypermethrin in tadpoles.

2. Materials and methods

2.1. Chemicals and reagents

The α-cypermethrin analytical standard (CAS 67375-30-8, 99.0%) was provided by the Aladdin Reagent Co. Ltd., Shanghai, China. (+)-(S)-(1R, 3R)-Cypermethrin (purity ≥ 98.0%, optical purity ≥ 98.5%) and (-)-(R)-(1S, 3S)-cypermethrin (purity ≥ 98.0%, optical purity ≥ 98.5%) were prepared by HPLC based on a cellulose tri-(3,5-dimethyphenyl-carbamate) chiral stationary phase (CDMPC-CSP) in our laboratory (Diao et al., 2011). α-Cypermethrin and its enantiomers were dissolved in dimethyl sulfoxide to produce stock solutions. Dimethyl sulfoxide and 3-aminobenzoic acid ethyl ester (MS-222) were obtained from Sigma-Aldrich (USA).

2.2. Animals

Dark-spotted frog tadpoles were obtained from the State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Beijing, China). Tadpoles were held in the glass aquaria containing 30 L of continuously aerated dechlorinated tap water (22 ± 1 °C). The dechlorinated tap water quality was measured daily, and the operating parameters used for the experiments were as follows: dissolved oxygen > 5 mg L^{-1}, pH 7.3–7.8, and water hardness ($CaCO_3$) of approximately 150 mg L^{-1}. The test animals were reared under controlled laboratory conditions of 12:12 h, light:dark cycles. Tadpoles were fed with Labdiet Frog Diet (Labdiet, America) twice daily in the bioaccumulation experiments, and tadpoles were not fed in the acute toxicity assay. Healthy tadpoles (Gonser stage 25) (Gosner, 1960) were selected randomly for the exposure experiments.

2.3. Acute toxicity assay

Tadpoles were exposed to various concentrations of rac-α-cypermethrin (i.e., 1, 2, 3, 4, 5, 6 and 7 μg L^{-1}), (+)-(S)-(1R, 3R)-enantiomer (i.e., 0.4, 0.6, 0.8, 1, 2, 4 and 8 μg L^{-1}) and (-)-(R)-(1S, 3S)-enantiomer (i.e., 30, 35, 40, 45, 50, 55 and 60 μg L^{-1}) to conduct the mortality experiments. Test solutions were prepared by diluting the stock solutions with dechlorinated tap water containing 0.01% (v/v) dimethyl sulfoxide. The control solvent group received the same amount of dimethyl sulfoxide. Ten tadpoles for each experiment were treated in a glass beaker containing 500 mL of the solution. Each concentration was tested in three parallel experiments. The medium was changed daily using freshly prepared solutions. Dead tadpoles were recorded and removed. The assays were performed for a total of 96 h.

2.4. Tadpole growth

To compare the effects of the α-cypermethrin enantiomers on the dark-spotted frog tadpoles, a short-term test was conducted with 0.5 μg L^{-1} of the α-cypermethrin enantiomers for 14 days. The tadpoles did not exhibit an acute toxic response with this concentration. Each tank was assigned 20 tadpoles randomly in 20 L of water. Sampling was conducted at 0.5, 2, 4, 8 and 14 d. At each sampling point, the tadpole was placed into the euthanasia solution (100 mg L^{-1} tricaine methanesulfonate (MS-222)). The tadpole was removed from the solution when it was unresponsive to external stimuli. The fresh body weight (mg) and snout-to-vent length (SVL, mm) were then determined using a digital electronic balance (0.1 mg precision) and digital callipers (0.01 mm precision), respectively. Before recording the body weight, excess water adhering to the tadpoles was removed with blotting paper. After determined the length and weight, the tadpoles were stored at −20 °C until the biochemical analyses were performed.

2.5. Biochemical analyses

All tadpole samples were thawed at room temperature and then homogenized on ice in 3 mL of phosphate buffer (50 mmol L^{-1} PBS, pH 7.0). The supernatant was collected after centrifugation for 10 min at 10,000 rpm at 4 °C and stored at −20 °C prior to analysis. Protein contents were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

SOD activity was assayed based on its ability to inhibit nitroblue tetrazolium reduction by O_2^- generated by the xanthine/xanthine oxidase system (Huang et al., 2006). One unit of SOD activity is defined as the amount of enzyme that elicits 50% inhibition of NBT reduction in 1 mL of reaction solution per mg of tissue protein. The specific activity is expressed in U mg^{-1} protein.

CAT activity was measured by the spectrophotometric method described by Aebi (1984). The disappearance rate of H_2O_2 was measured at 240 nm at 20 °C for 3 min. These results are expressed in U mg^{-1} protein.

The terminal product formed in the decomposition of polyunsatu-