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Effects of chronic 2,4,6,-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene exposure on developing bullfrog (*Rana catesbeiana*) tadpoles

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ABSTRACT

Chronic aqueous exposures were conducted using bullfrog (*Rana catesbeiana*) tadpoles (8 d old) exposed to TNT (0–4 mg/L), 2,4-DNT (0–4 mg/L), and 2,6-DNT (0–8 mg/L) for 90 d. Survival of tadpoles examined using Cox proportional hazard models was reduced at all concentrations tested. Percent of abnormal swimming and other morphological abnormalities after sublethal exposure to TNT, 2,4-DNT, and 2,6-DNT at 2 mg/L were also evaluated. The effects of TNT, 2,4-DNT, and 2,6-DNT on wet body mass, snout vent length (SVL), and developmental stage of surviving tadpoles were examined. Only 2,4-DNT did not have a significant effect on body mass or SVL, but all three compounds tested had significant effects on survival. Long-term continuous exposure to these compounds at concentrations of 0.25 mg/L could lead to significant changes in growth and survival of larval amphibians.

1. Introduction

The global use of nitroaromatic compounds has resulted in contamination of terrestrial and aquatic ecosystems and is currently a serious concern in the United States, Germany, and Canada (Pennington, 1999; Fritsche et al., 2000). The toxicity of 2,4,6-trinitrotoluene (TNT) is associated, in part, with the symmetric location of the nitro group in the aromatic ring, limiting enzymatic metabolism typically observed for aromatic compounds (Rieger et al., 1999). Because of this special structural feature, exposure to TNT often results in toxicity, mutagenicity, and carcinogenicity in humans and animals (Won et al., 1976; Whong and Edwards, 1984; Snellinx et al., 2002).

Trinitrotoluene has been detected in the vicinity of munitions facilities at concentrations ranging from 0.074 to 0.998 mg/L in lagoon water (Triegel et al., 1983) and 1–178 mg/L in waste water from load, assemble, and packing (LAP) plants (Patterson et al., 1976). Both 2,4-DNT and 2,6-DNT were found as major components in wastewaters from TNT manufacturing facilities at concentrations ranging from 0.04 to 48.6 mg/L and 0.06 to 14.9 mg/L, respectively (Spanggord and Suta, 1982).

There is substantial data describing the toxicity of TNT, 2,4-DNT, and 2,6-DNT in fish and aquatic invertebrates (Liu et al., 1984; Bailey et al., 1984; Bailey et al., 1985; Lang et al., 1997; Nipper et al., 2001; Ownby et al., 2005; Belden et al., 2005; Lotufo and Lydy, 2005; Neuwoehner et al., 2007; Sensini et al., 2008). However, studies addressing amphibian species are limited to the analysis of TNT exposure in African clawed frog (*Xenopus laevis*) embryos (Saka, 2004), tiger salamanders (*Ambystoma tigrinum*) (Johnson et al., 2000), and red-backed salamanders (*Plethodon cinereus*) (Bazar et al., 2008). Similarly, the current literature on toxicity of chemicals to bullfrog (*Rana catesbeiana*) tadpoles is limited to the effects of pesticides (Fordham, 2001; Boone and Semlitsh, 2003; Costa et al., 2008) and endocrine disruptors, such as nonylphenol and tetrachlorodibenzo dioxin (TCDD), associated with the inhibitory effect on metamorphosis and tail resorption (Beatty et al., 1976; Christensen et al., 2005). Little is known regarding the effects of chronic exposure of TNT and DNT in early developmental stages of North American species, such as the bullfrog. Thus, the purpose of the present study was to examine the effects of chronic exposure to TNT and DNT on bullfrog tadpole survival, growth, gross morphology, and incidence of abnormal swimming, morphological abnormalities.

2. Materials and methods

2.1. Test materials

2,4,6-TNT (CAS # 118-96-7), 2,4-DNT (CAS#121-14-2), and 2,6-DNT (CAS# 606-20-20) were purchased from ChemService and Alfa Aesar with 98%, 97%, and 97% purity, respectively. The exposure water (hereafter, media) consisted of carbon filtered, UV sterilized, reverse osmosis (RO), and de-ionized (DI) water

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supplemented with 0.33 mg/L Instant Ocean sea salts from Aquarium Systems (Mentor, OH, USA).

2.2. Test organisms

Adult male and female bullfrogs (*R. catesbeiana*) were purchased from Rana Ranch Bullfrog Farm (Twin Falls, ID, USA). Animals were maintained in an 888 L tank containing 118 L of media and acclimatized for one week on a 12:12 h light:dark regime at 21 ± 1 °C. Four animals were then transferred to 112 L glass tank containing 28 L of the same media and fed three live adult crickets per frog three times a week. Media changes were performed weekly. The average body weight ± standard deviation (SD) of adult female and male frogs was 370 ± 14.17 and 224 ± 7.04 g, respectively.

Naturally fertilized eggs were obtained from two pairs of adults and transferred to 112 L glass tanks containing 84 L of the media and maintained at 21 ± 1 °C on a 12:12 h light:dark regime. Each clutch was evenly distributed among replicates. Starting on post hatch day eight (Gosner stage 20), larvae were fed rabbit pellets from LabDiet (St. Louis, MO, USA) every 72 h, immediately after a 50% change in media. Lettuce was also provided as an additional source of food in order to avoid cannibalism. Animal care and maintenance followed protocol approved by the Institutional Animal Care and Use Committee of Texas Tech University (ACUC # 05049-09).

2.3. Experimental design

Larvae were maintained in a 112 L tank with 84 L of the media. On post hatch day eight, thirty tadpoles were randomly transferred to each of the 9 L tanks containing 6 L of the media. The experiment consisted of a 90 d exposure with three replicate tanks for each concentration per chemical and six replicate tanks for controls. Tanks were considered experimental units, with each experimental unit containing thirty tadpoles. All three chemicals were examined in the same experiment to minimize any temporal or clutch effects. Exposure concentrations were selected by evaluation of results from a 96 h range finding tests on bullfrog larvae for each test compound run in our laboratory. Previously determined LC_{50}'s and 95% confidence intervals were 40.34 (29.91–60.61), 40.29 (30.66–52.96), and 80.32 (79.32–92.46) mg/L for TNT, 2,4-DNT, and 2,6-DNT, respectively. Thus, the maximum dose for the chronic study was 1/10 of the calculated 96 h LC_{50} for each compound. The nominal exposure concentrations were the same for TNT and 2,4-DNT (0, 0.125, 0.25, 0.5, 1, 2, and 4 mg/L), but increased by a factor of two for 2,6-DNT (0, 0.25, 1, 2, 4, and 8 mg/L).

Tanks were placed in two living streams (Frigid Units Inc., Toledo, OH, USA) with a water depth of 12 cm. Water heaters were installed to maintain the temperature within a range of 25–29 °C. Continuous aeration was maintained throughout the study period in order to provide adequate oxygen for the tadpoles. Aeration and water bath temperatures were recorded daily. TNT has a short half life due to direct photolysis (i.e., reported half life of 14–84 h (Mabey et al., 1982)), a 50% media change was performed every 72 h. Water volume in the living streams was checked during water changes and replenished if needed. All test tanks, nets, and glassware were color coded, to properly identify the treatment tanks and avoid cross contamination of test compounds during media changes.

Developing tadpoles were monitored daily for health and condition. Animals found dead or moribund were recorded, removed from the study, and preserved in 10% buffered formalin. Gross morphological observations included dorsal flexure, curved tail, incomplete coiling, deformed head, and mild optic and abdominal edema. Abnormal swimming (defined as a tadpole swimming in a circle, upside down, or on its side) and forelimb emergence were monitored and recorded each day.

After 90 d of exposure, survivors were removed from tanks and euthanized by immersion in 3 g/L MS-222 (3-amino benzoic acid ethyl ester), rinsed in distilled water, blotted dry, and immediately weighed and measured. Effects of the three contaminants on tadpole body weight (BW) and snout-vent length (SVL) were examined for concentrations in which all three replicates had at least three surviving tadpoles after the 90 d exposure. Animals were given an identification number that included the study number, tank number, and animal number. A middle incision was made in large specimens to facilitate penetration of the fixative.

2.4. Analytical procedures

Temperature, conductivity, salinity, dissolved oxygen, and pH were recorded for each tank once a week using a YSI model 556 multiprobe water quality meter (MPS) (Yellow Springs, OH, USA). Ammonia was determined using a Hach spectrophotometer model DR 2800 (Ames, IA, USA).

Mean water temperature and conductivity in tanks were 22.2 °C (range=21.0–23.5 °C) and 0.68 mS/cm (range=0.65–0.73 mS/cm), respectively. Mean salinity and dissolved oxygen for all tanks were 0.33 mg/L (range=0.32–0.35 mg/L) and 7.5 mg/L (range=6.1–8.4 mg/L), respectively. Mean pH and ammonia for all tanks were 6.8 (range=6.5–7.1) and 0.58 mg/L (range=0.15–1.29 mg/L), respectively.

The stock solution (100 mg/L) for each chemical was prepared every 72 h. Concentrations of stock solutions were measured using high performance liquid chromatography with ultraviolet light detection (HPLC-UV). Similarly, concentrations of aliquots from tanks were monitored throughout the study before each media change. Mean ± standard error (n=45) stock concentrations for the three compounds were 84.4 ± 2.6 mg/L for TNT, 94.4 ± 2.9 mg/L for 2,6-DNT, and 92.2 ± 3.1 for 2,4-DNT.

Water samples were collected weekly (one sample per treatment) for each compound throughout the study period prior media change to verify proper toxicant concentrations in each tank. No samples were taken after all tadpoles in these tanks succumbed to treatment. Mean measured concentrations for TNT (0.12–4.1 mg/L), 2,4-DNT (0.13–3.84 mg/L), and 2,6-DNT (0.21–7.1 mg/L), as well as the corresponding nominal concentrations, are presented in Table 1. Measured concentrations were similar to the calculated nominal concentrations for two of the compounds tested ranging 72–104% for 2,4-DNT and 73–88.8% for 2,6-DNT. Measured concentrations for TNT were slightly different and varied from 34% to 102.5% of nominal concentrations; however the standard deviation was 27.5%. Therefore, nominal concentrations are reported hereafter.

2.5. Statistical analyses

The Cox proportional hazards model (Cox, 1972) was used for tadpole survival analysis for each compound over the study period using the R statistic program. One-way ANOVA with Dunnett's multiple comparison test was used to test for the effects of TNT, 2,4-DNT, and 2,6-DNT on BW and SVL of surviving tadpoles. In this analysis, individuals were treated as subsamples and averaged to derive a value for the experimental units (tanks). Thus, tank values were used for assessing treatment effects. Alpha was set at 0.05. Percent of abnormal swimming and morphological abnormalities were monitored daily throughout the study; however, due to the increase in mortality of tadpoles throughout the experiment, the data could not be analyzed with inferential statistical tests. The one-way ANOVA was performed using the NCSS statistic program (NCSS, version 2004, NCSS Statistical and Power Software Analysis).

3. Results and discussion

3.1. Survival analysis

Tadpole survival in general was low for the three compounds at the highest concentrations tested. Values provided are mean survival values. The percentages of surviving tadpoles exposed to

Table 1
Nominal and measured concentrations of TNT, 2,4-DNT, and 2,6-DNT of water samples from bullfrog (*Rana catesbeiana*) tadpoles exposure experiments.

TNT (mg/L)		2,4-DNT (mg/L)		2,6-DNT (mg/L)	
Nominal	Measured[a]	Nominal	Measured[a]	Nominal	Measured[a]
0.125	0.12 ± 0.03 (n=12)	0.125	0.13 ± 0.03 (n=12)	0.25	0.21 ± 0.01 (n=12)
0.25	0.2 ± 0.02 (n=12)	0.25	0.18 ± 0.01 (n=12)	0.5	0.38 ± 0.01 (n=12)
0.5	0.28 ± 0.06 (n=12)	0.5	0.38 ± 0.02 (n=12)	1	0.73 ± 0.04 (n=12)
1	0.34 ± 0.08 (n=12)	1	0.79 ± 0.22 (n=12)	2	1.58 ± 0.1 (n=12)
2	1.98 ± 0.05 (n=10)	2	1.83 ± 0.09 (n=10)	4	3.3 ± 0.2 (n=9)
4	4.1 ± 0.03 (n=10)	4	3.84 ± 0.1 (n=10)	8	7.1 ± 0.3 (n=6)

n: number of samples.

[a] Mean ± standard error.