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Short communication

Comparison of embryo toxicity using two classes of aquatic vertebrates

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ABSTRACT

Toxicity tests of musk ketone (MK) and tetrabromobisphenol-A (TBBPA) on embryos were conducted in two amphibian species, *Xenopus* (*Silurana*) *tropicalis* and the Swedish native species *Rana arvalis*. TBBPA was also tested on fish embryos of *Danio rerio*. All species were tested in similar experimental setup. Musk ketone caused decreased heart rates at concentrations from 10 and 100 μg/L in *R. arvalis* and *X. tropicalis*, respectively. TBBPA caused effects at 1000 μg/L in all three species. The responses were comparable between all three species which supports the relevance for using data from non-native species in national risk assessment.

1. Introduction

The fish embryo toxicity (FET) test (OECD, 2013) which is based on individual exposure of eggs in well-plates has been frequently used with zebrafish (*Danio rerio*) in routine effluent monitoring and in testing of single chemicals (Braunbeck et al., 2005; Carlsson and Norrgren, 2004). Studies have shown promising results with comparable data when adapting two other fish species recommended by OECD, the Japanese medaka (*Oryzias latipes*) and the fathead minnow (*Pimephales promelas*) to the same protocol (Braunbeck et al., 2005).

D. rerio as well as *Xenopus* frogs are aquatic animals from two different classes of aquatic vertebrates which allows for regular studies of embryo toxicity due to simple ways of obtaining eggs. Young stages of both species have been used widely in development and toxicity models and standardised guidelines are available (OECD, 2013; ASTM, 1999). However, performing risk assessment for regional conditions, based on the results from toxicity studies on non-native species, can be questioned.

The aim of the present study was to compare the responses and the sensitivity between embryos from two different classes of aquatic vertebrates, fish and amphibians when exposed to two environmentally relevant substances, musk ketone (MK) and tetrabromobisphenol-A (TBBPA). This was accomplished by using the same embryo toxicity method to test both *D. rerio* and west-African clawed frog (*Xenopus* (*Silurana*) *tropicalis*) in identical experimental setup. Further, to explore the relevance of the result from the two tropical species to a native Swedish species, also embryos of moor frog (*Rana arvalis*) were exposed to these chemicals. Data for MK

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on *D. rerio* were obtained from an earlier study (Carlsson and Norrgren, 2004).

2. Materials and methods

Tetrabromobisphenol-A (TBBPA) was kindly provided by Professor Åke Bergman, Stockholm University and Musk Ketone (MK) were obtained from Sigma Aldrich Sweden AB. Stock solutions were prepared by dissolving chemicals in dimethylsulfoxide (DMSO) and stored at 4 °C until mixed with exposure water to a final DMSO concentration of 0.1%. In all tests, exposure concentrations of both chemicals were 1.0, 10, 100 and 1000 μg/L including a control with 0.1% DMSO.

Fish water was prepared according to ISO (1999) and continuously aerated at a temperature of 26 °C. Reproduction of *D. rerio* was performed as previously described (Carlsson and Norrgren, 2004). Briefly, adult fish of both sexes were mixed in cone shaped reproduction funnels, provided with nets, separating adults from their eggs. Eggs at homogenous developmental stages were collected 30 min after onset of light and immediately exposed to a concentration series of TBBPA in 50 mL solution. The eggs were examined for selection of eggs that were between the 4 and 16-cell stages. Selected eggs were transferred to 96-well plates, one egg in each well, together with 250 μL solution. Each exposure group included 24 eggs. The plates were kept at a temperature of 26 °C and a photoperiod of 12:12 h for 48 h. The eggs were examined at different observation times as summarised in Table 1.

The water used for *X. tropicalis* throughout the experiment, was made according to the FETAX protocol (ASTM, 1999), diluted 1:1 with deionised water and continuously aerated at 26 °C. One female and one male adult frog were injected in the dorsal lymph-sac with 20 international units (IU) of human chorionic gonadotropin, followed by 100 IU injections 2 days later to induce spawning. After spawning, frogs were removed from the aquarium and the eggs were collected. The jelly-coat of the eggs was removed by swirling the eggs in L-cysteine solution (1.25 g L-cysteine in 50 mL frog water). Eggs were then exposed to concentration series of MK and TBBPA. The following procedure was the same as for *D. rerio* with transfer to 96-well plates. Studied endpoints at different observations times are shown in Table 1.

Newly laid *R. arvalis* eggs from 12 different clutches were collected immediately after spawning from a natural pond where no other species were observed, and stored a week in a refrigerator (+4 °C) until the onset of the experiment. Eggs were carefully separated from each other and individually placed in 50 mL beakers containing the series of TBBPA or MK. The water used for the *R. arvalis* embryos was made according to the FETAX protocol (ASTM, 1999) and continuously aerated at 17 °C. The test was initiated with 25 individuals in each concentration. Different clutches contributed to the same number of eggs in each treatment. Remaining eggs, not used in the test were left in water in the laboratory for evaluation of fertilisation rate in different clutches. The beakers were placed in a water bath with a temperature of 17 °C and the photoperiod was 12:12 h. Exposure media was renewed after 2 and 4 days of exposure and the study was terminated after 7 days when the tadpoles had reached the corresponding stage in development as in the *X. tropicalis* experiment. Endpoints and times of observations are shown in Table 1. The heart was not visible due to pigmentation so heart rate was determined by counting pulsation of the external gills after hatching at 4 days of exposure. Eggs recorded as undeveloped after one day, were excluded from the study.

Heart rates were analysed using one-way ANOVA followed by Dunnett's post hoc test, comparing exposed groups with controls. Total number of affected embryos and number of unfertilised eggs were analysed comparing each treatment with respective control using Fisher's exact-test with Bonferroni adjustment. The significance level was set at 0.95 ($p < 0.05$). The analyses were made in Minitab 16.

3. Results and discussion

Nitro musks and brominated flame retardants (BFRs) are examples of chemicals that have been detected in environmental samples in relatively high concentrations (Gatermann et al., 1999; de Wit, 2002). Musk ketone (MK) and tetrabromobisphenol-A (TBBPA), belongs to the nitro musks and BFRs, respectively. MK has previously been studied using embryos from *D. rerio* with the same method as in the present study (Carlsson and Norrgren, 2004). The two substances studied on the amphibian species, their effects, and the concentrations where these effects where observed, correlated well with the results obtained from *D. rerio* in the present study and in Carlsson and Norrgren (2004).

All three species had a lowest observed effect concentration (LOEC) of 1000 μg/L of TBBPA, where oedema, absent circulation and mortality were the main findings at the last observation times. 100% of *D. rerio* and *R. arvalis* and 85% of *X. tropicalis* were affected when exposed to 1000 μg/L of TBBPA. In *D. rerio*, an increased number of embryos (68%) exposed to 1000 μg/L TBBPA showed lack of spontaneous movement already after 24 h and a decline in heart rate (61% of the control heart rate, $p < 0.01$) after 48 h exposure. These findings were not observed in the two frog species. Concentrations of TBBPA are often below limit of detection in natural water (Kuiper et al., 2007). Thus, there seems to be low risk for an acute toxic impact caused by water exposure to TBBPA.

Both *X. tropicalis* in the present study, and *D. rerio* (Carlsson and Norrgren, 2004) exposed to MK showed oedema and absent blood circulation in the highest tested concentration, 1000 μg/L. Further, heart rates were decreased in all three species. For *X. tropicalis*, heart rate decreased at 100 μg/L and higher (Fig. 1). For *R. arvalis* exposed to MK, a clear concentration-response relationship on decline in heart rate was recorded for individuals exposed to 10 μg/L and higher (Fig. 1). MK has been detected in water up to μg/L levels but is generally in lower concentrations (European Union, 2005). The fact that *R. arvalis* show the same low LOEC as *D. rerio* (Carlsson and Norrgren, 2004) when exposed to MK, raises the concern for the effects of synthetic musk contamination previously discussed by Carlsson and Norrgren (2004).

The present study shows that *D. rerio* and *X. tropicalis* might be used in the same experimental setup. The opportunity to use aquatic vertebrate species from different classes with the same basic methodology might provide more information of