Ecotoxicology and Environmental Safety 153 (2018) 45–53

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Enhancing the fathead minnow fish embryo toxicity test: Optimizing embryo production and assessing the utility of additional test endpoints

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ARTICLE INFO

Keywords:
Fish embryo toxicity test
Fathead minnow
Animal alternatives
Pericardial edema
Sublethal endpoints

ABSTRACT

The fathead minnow fish embryo toxicity (FET) test has been identified as a potential alternative to toxicity test methods that utilize older fish. However, several challenges have been identified with the fathead minnow FET test, including: 1) difficulties in obtaining appropriately-staged embryos for FET test initiation, 2) a paucity of data comparing fathead minnow FET test performance to the fathead minnow larval growth and survival (LGS) test and 3) a lack of sublethal endpoints that could be used to estimate chronic toxicity and/or predict adverse effects. These challenges were addressed through three study objectives. The first objective was to optimize embryo production by assessing the effect of breeding group composition (number of males and females) on egg production. Results showed that groups containing one male and four females produced the largest clutches, enhancing the likelihood of procuring sufficient numbers of embryos for FET test initiation. The second study objective was to compare the performance of the FET test to that of the fathead minnow LGS test using three reference toxicants. The FET and LGS tests were similar in their ability to predict the acute toxicity of sodium chloride and ethanol, but the FET test was found to be more sensitive than the LGS test for sodium dodecyl sulfate. The last objective of the study was to evaluate the utility and practicality of several sublethal metrics (*i.e.*, growth, developmental abnormalities and growth- and stress-related gene expression) as FET test endpoints. Developmental abnormalities, including pericardial edema and hatch success, were found to offer the most promise as additional FET test endpoints, given their responsiveness, potential for predicting adverse effects, ease of assessment and low cost of measurement.

1. Introduction

In response to legislative demands, including the United States (US) Frank R. Lautenberg Chemical Safety for the 21st Century Act (US Congress, 2016) and European Union (EU) Directive 2010/63/EU (EU, 2010), there has been a push to develop aquatic toxicity methods that meet the 3Rs-replacement, reduction and refinement of animal use. A zebrafish (*Danio rerio*) fish embryo toxicity (FET) test (OECD guideline 236, OECD 2013), developed as a replacement for the fish acute toxicity test using juveniles or adults (OECD guideline 203, OECD 1992), was recently validated and adopted as a standard test method by the EU (Braunbeck et al., 2015). However, national and regional species preferences are likely to hinder adoption of the zebrafish FET test in areas outside of the EU (Embry et al., 2010), including the US where the fathead minnow (*Pimephales promelas*) is often used in acute and chronic toxicity assessments (USEPA, 2002). Efforts to develop and evaluate the utility of the fathead minnow as a FET test organism are underway. Braunbeck et al. (2005) successfully transferred the zebrafish FET test procedure to the fathead minnow through slight modifications aimed at accounting for differences in temporal development (*e.g.*, use of ≤ 32-cell embryos rather than ≤ 16-cell embryos, longer test duration). More recently, Jeffries et al. (2014, 2015) compared the performance of a 120-h fathead minnow FET test to that of the 7-d fathead minnow larval growth and survival (LGS) test (USEPA, 2002), a short-term test designed to assess the toxicity of effluents. These initial comparative studies provided data suggesting that the fathead minnow FET test may be a viable replacement for the 7-d LGS test. However, a suite of potential challenges and limitations associated with the fathead minnow FET test have emerged and need to be addressed to maximize the feasibility and utility of a fathead minnow FET test.

A major challenge associated with fathead minnow FET test is the procurement of appropriately-staged embryos. The initiation of a single FET test requires a minimum of 168 embryos at the 32-cell stage or less. Though female fathead minnows are known to lay numbers of eggs well in excess of this in a single spawning event, both Jeffries et al. (2014) and Böhler (2012) found the precise time of spawning to be unpredictable making the collection of ≤ 32-cell-stage embryos an

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https://doi.org/10.1016/j.ecoenv.2018.01.042
Received 4 August 2017; Received in revised form 16 January 2018; Accepted 19 January 2018

obstacle for the initiation of FET tests using this species.

A second challenge associated with the fathead minnow FET test is an overall lack of data comparing its performance to other test types, particularly the LGS test. To date, the sensitivities of these two testing strategies have only been compared for two chemicals (3,4-dichloroaniline and ammonia) and two simulated effluents (mock wastewater treatment plant effluent and mock oily effluent) (Jeffries et al., 2014, 2015). The test types were similar in sensitivity for ammonia and mock oily effluent, but differed in their sensitivity for 3,4-dicholoraniline and mock wastewater treatment plant effluent. If a predictive relationship between the toxicity values generated by the FET and LGS tests is to be determined, a larger number of chemicals with different modes of action needs to be evaluated.

A third limitation of the fathead minnow FET test is its inability to estimate chronic toxicity and predict sublethal adverse effects. Because the FET test was designed to assess acute toxicity, the only endpoint measured is mortality, though documentation of developmental abnormalities is encouraged in OECD test guideline 236 (OECD 2013). The lack of sublethal FET test endpoints is problematic if the FET test is to be considered as a potential alternative to the LGS test, as the inclusion of growth as a LGS test endpoint allows for the estimation of chronic toxicity. The previous study by Jeffries (2015) identified growth, growth-related gene expression and pericardial edema as potential sublethal endpoints for FET test inclusion; however, the responsiveness of these metrics was only evaluated for the two simulated effluents.

The overall goal of the current study was to enhance the feasibility and utility of the fathead minnow FET test by addressing the challenges and limitations outlined above. The first objective was to identify the optimal composition of fathead minnow spawning groups for the procurement of appropriately-staged embryos for use in FET tests. This was accomplished by examining the effects of breeding group structure, specifically the number of males and females per group, on embryo production. The second objective was to compare the sensitivity of the FET and LGS tests for three reference chemicals with different modes of action in an effort to supplement the existing FET-LGS test comparison data sets. The third objective was to evaluate not only the responsiveness, but also the practicality, of several sublethal metrics as FET test endpoints. Endpoints such as growth, developmental abnormalities (*e.g.*, edema, inability to hatch, etc.), growth-related gene expression and stress-related gene expression were evaluated for their responsiveness, sensitivity and cost to measure.

2. Materials and methods

2.1. Animal husbandry

All experimental procedures involving animals were approved by Texas Christian University's (TCU's) Institutional Animal Care and Use Committee (protocol #14/05). Brood stock utilized in the breeding study, and to produce embryos and larvae for use in the FET and LGS tests, were maintained at densities ≤ 0.4 fish/L in 30 L aerated glass aquaria containing 26.0 ± 0.9 °C dechlorinated municipal water (mean ± standard deviation, pH 8.0 ± 0.5, conductivity 376.2 ± 96.1 µS/cm, alkalinity 102.7 ± 29.0 mg/L, hardness 101.0 ± 32.0 mg/L) under a photoperiod of 16:8 h light: dark. Each breeding group was provided with one spawning structure (10-cm-long pieces of 7.6-cm polyvinyl chloride schedule 40 pipe, split longitudinally) per male. Commercially available flake food (TetraMin) was provided *ad libitum* twice daily along with supplements of freshly-hatched *Artemia* nauplii. One-third water exchanges were conducted daily to maintain water quality.

2.2. Optimization of fathead minnow embryo production

To assess the effect of breeding group composition on embryo production, sexually mature adult fathead minnows (~ 6 months old) obtained from Aquatic Research Organisms (ARO, Hampton, NH) were randomly sorted into groups consisting of 1 male and 4 females (1:4), 1 male and 8 females (1:8), 2 males and 4 females (2:4) and 2 males and 8 females (2:8). Each group type was replicated four times for a total of 16 groups. Males and females were separated from each other using a plastic aquarium divider for one week, then dividers were removed and groups were allowed to spawn for three weeks. During the spawning period, eggs were collected and counted three times daily at 2, 4 and 8 h following the onset of light. Eggs collected at 2 h were staged on a Leica DMi1 inverted microscope to determine their suitability for use in FET tests (≤ 32-cell stage). No adult mortalities occurred over the course of the breeding study.

2.3. Comparison of LGS and FET test performance

To compare the test performance, LGS and FET tests were conducted using three common reference toxicants with different modes of action: sodium chloride (NaCl), sodium dodecyl sulfate (SDS) and ethanol (EtOH) (Table 1). Test organisms for the LGS and FET tests were collected from fathead minnow breeding groups, consisting of sexually mature adults (~ 6–12 months old) maintained in the TCU Aquatic Facility. Embryos and larvae utilized in tests featuring NaCl were produced from breeding groups consisting of fish supplied by ARO and from fish produced in-house, while those used in tests with SDS and EtOH were produced from groups consisting of fish supplied by Environmental Consulting and Testing (ECT, Superior, WI) and from in-house fish. The males and females comprising each individual breeding group were from different sources to prevent inbreeding. Upon collection, embryos were gently removed from spawning structures using a metal spatula. Those designated for use in FET tests were transferred directly into glass Petri dishes containing the appropriate test solutions, while those designated for use in LGS tests were transferred to 1 L beakers containing dechlorinated tap water. Beakers were maintained in a 27 °C incubator and heavily aerated to prevent fungal growth. Daily water exchanges of > 75% were conducted until hatch at ~ 4 days post fertilization (dpf).

For each test, a minimum of five toxicant concentrations were utilized (Table 1), in addition to a negative control (exposed to dilution water) and a positive control (exposed to 3,4-dichloroaniline at 1.5 and 16 mg/L in the LGS and FET tests, respectively (Jeffries et al., 2015)). Each FET test was repeated three times per chemical, whereas LGS tests were conducted four times per chemical.

2.4. General LGS procedures

Fathead minnow 7-d LGS test methods were based upon US Environmental Protection Agency (USEPA) test method 1000.0 (USEPA, 2002) and are described in Jeffries et al. (2014). Briefly, 10 eleutheroembryos (< 5 h post hatch) were transferred to 100 mm x 50 mm glass crystallizing dishes containing 250 mL of test solution.

Table 1
Concentrations of the chemical solutions used in the FET and LGS test exposures.

Chemical	Mode of action	Test concentrations
Sodium chloride (NaCl)	Osmotic	FET and LGS tests: 16, 8, 4, 2 and 1 g/L
Sodium dodecyl sulfate (SDS)	Polar narcosis	FET tests: 30, 15, 7.5, 3.8 and 1.9 mg/L LGS tests: 60, 30, 15, 7.5, 3.8 and 1.9 mg/L
Ethanol (EtOH)	Nonpolar narcosis	FET tests: 31.6, 15.8, 7.9, 3.9, 2.0, and 1.0 g/L LGS tests: 15.8, 7.9, 3.9, 2.0, and 1.0 g/L