Lessons learned running a Zebrafish Extended One Generation Reproduction Test (ZEOGRT) as part of a validation exercise

Becky Cahill[1], Richard Maunder[1], Matthias Teigeler[2]

[1] Scymaris Ltd., Brixham Laboratory, Freshwater Quarry, Brixham Devon TQ5 8BA UK, United Kingdom, [2] Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Auf dem Aberg 1, 57392 Schallenberg, Germany

Introduction

An update to the Classification, Labelling and Packaging (CLP) regulation to include new hazard classes including those relating to Endocrine Disruption (ED) aligns this with the existing regulations for biocides and plant protection products whereby substances require assessment for their ED properties as part of their environmental risk assessment. The OECD provides guidance for ED testing under a conceptual framework with tests being assigned a level based on the comprehensiveness of the testing. The highest level 5 tests include multigeneration testing, such as the Medaka Extended One Generation Reproduction Test (MEOGRT; OECD 240, Ref 1). This test has been reported as difficult to perform, with tests often failing to meet validity criteria, meaning interpretation of results can be difficult. An alternative EOGRT option using zebrafish (*Danio rerio*; ZEOGRT) was proposed in 2015 and is currently at the ring test stage of validation as part of an OECD workplan. In this project a ZEOGRT study was conducted at Scymaris, Brixham Laboratory, UK as part of the validation.

Methods

- Nominal temperature of 26 ± 2°C.
- Flow-through conditions.
- Photoperiod of 16 hours light: 8 hours dark with 20-minute dawn/dusk transition.
- 25 L glass aquaria, 4 replicates per concentration.
- Metal spawning trays with a removable lattice and green glass beads were added to stimulate breeding.
- When required, 2 fry cages (glass tubing with nylon-mesh bottoms) were suspended in each replicate to house the juvenile F1 generation.
- Prochloraz selected as the test reference substance over Tamoxifen citrate as it had more reliable dosing and solubility. Prochloraz is a known ED with multiple modes of action within the EATS modalities.
- Triethylene glycol was utilised as a carrier solvent, DWC and SC employed.
- Nominal test concentrations of 3.2, 10, 32, 100 and 320 µg/L.
- Prochloraz concentrations were measured in the test vessels weekly, using an analytical method validated to SANTE/2020/12830, rev. 1 guideline (Ref 4).
- The test design, adapted from the MEOGRT procedure (Ref 1), began with a Filial 0 (F_0) parental adult group in spawning status, followed by a complete F_1 generation derived from the parental eggs and finally a F_2 generation derived from the F_1 eggs, limited to the hatching period.

Figure 1. Test set up including breeding trays.

Figure 2. Suspended fry cages and breeding trays in control aquaria.

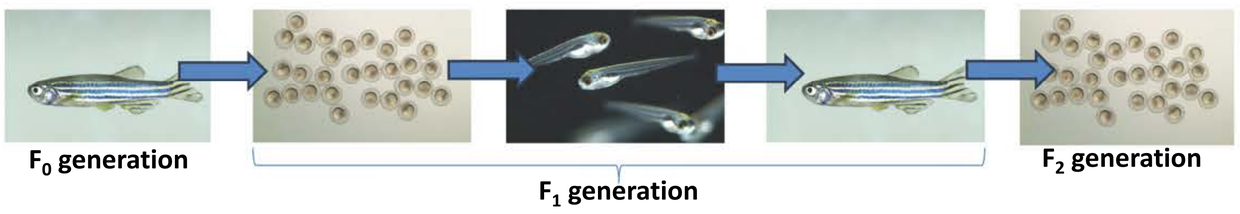

Figure 3. Flow diagram of generations exposed in test design.

F_0 generation fish were 6-month old zebrafish (*Danio rerio*), sourced from Scymaris broodstock.

Endpoints

Phase	Fish generation	Endpoints
1	F_0	Total egg number/day/female (fecundity) Fertilisation rate (fertility) Survival Total length for each sex Total weight for each sex Blood plasma VTG concentration for each sex
2	F_1	Hatching rate Post-hatch survival, up to day 21 post-fertilisation Post-hatch survival, up to day 35 post-fertilisation
3	F_1	Survival, up to day 63 post-fertilisation Total length, day 63 post-fertilisation Time to first spawning Total egg number/day/female (fecundity) Fertilisation rate (fertility) Adult survival, up to test termination Total length for each sex Wet weight for each sex Sex ratio Blood plasma VTG concentration for each sex
4	F_2	Hatching rate

Validity criteria

Criterion	Acceptable limits
Dissolved oxygen	≥60%
Water temperature	26±2°C
F_0 and F_1 control fertility	>80%
F_1 control post-hatch survival in early life stages	≥75%
F_1 control sex ratio (% males or %females)	30-70%
F_0 and F_1 control survival in juveniles and adults	>90%
F_0 and F_1 control spawning phase	'Should spawn regularly'

Results

Analytical results

As for other chronic fish studies (Refs 1-3) the consistency of the test substance analytical chemistry is a proposed validity criterion (± 20% of mean measured). In this test the arithmetic mean measured Prochloraz concentrations ranged from 103 to 112% of nominal and were consistent throughout the exposure.

F1 generation initiation

Upon exposure start the F_0 generation were successfully bred and fertility & fecundity were recorded daily for 21 days. At the end of the 21-day period eggs were collected and pooled over 2 days to avoid genetic drift and bottle-neck and used to initiate the F_1 generation.

Initially it proved challenging to hatch the eggs under flow-through conditions in the fry cages as recommended (Ref 5), with fungus infecting the eggs prior to hatch. Additional cleaning to the aquaria/breeding trays prior to egg collection improved this. To mitigate fungus transfer between eggs, it was also found to be beneficial to house the eggs individually in well plates containing test solution. Eggs were held individually until hatch, then in two crystalising dishes per replicate until day 5 post-hatch when they were transferred to the fry cages suspended in the aquaria. While under semi-static conditions test solutions were renewed daily to maintain test concentrations.

To maintain true replication throughout the test, individual replicates supplied their own eggs into the F_1 generation with no pooling across treatments. Replicate egg production was pooled over 2 days to initiate the F_1 generation, but limitations were experienced with the quality and quantity of the eggs required across all replicates in all concentrations over these 2 days. Improvements in egg quality were seen after the adult fish had a break from daily breeding and we would suggest that the eggs used to initiate the F_1 generation be collected closer to the start of the 20-day breeding period rather than the end.

Following fry mortalities post-hatch, investigations were also made into the feeding regime and a mix of a commercial liquid artemia and fry powder was found to be the most successful.

With the improvements implemented, the F_1 generation was successfully initiated (mean hatching rate of 88 and 77% and day 21 post-fertilisation fry survival rates of 79 and 75% in the DWC and SC, respectively.)

F1 breeding and evaluation

Following the F_0 generation first spawn (~day 71 post-fertilisation), fecundity & fertility were monitored daily. However, some replicates did not meet the regular spawning definition of >15 eggs and >80% fertilised for 2 consecutive days (Ref 5), which was used a starting point for the counting period, until day 96 post-fertilisation. As a result, breeding was extended to ensure reliable data was captured when the fish were regularly spawning.

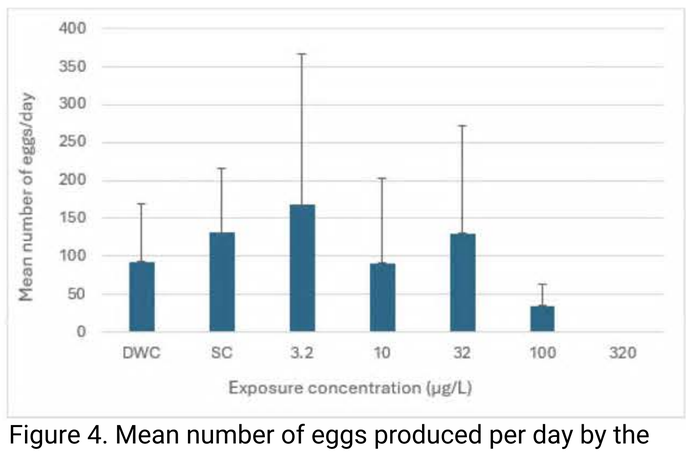

Figure 4. Mean number of eggs produced per day by the F_1 generation and standard deviation. Data not adjusted for number of females as histopathology analysis is ongoing.

Given the laboratory practicalities of monitoring eggs daily for an extended period and to ensure reliable data, we suggest the F_1 breeding phase could be postponed until regular spawning is certain, especially as egg numbers produced per day increased as the fish aged.

Conclusions

The ZEOGRT generational test design was successful in producing reliable data for a variety of endpoints, and strong ED effects were demonstrated.

The validity criteria set for the study, based on the draft standard operating procedure produced by Fraunhofer IME (Ref 5) were met, with the exception of some small deviations in the dissolved oxygen concentration and temperature, which were not considered to impact the outcome or validity of the test.

Learnings made during the test, including the initiation of the F_1 generation and evaluation of breeding have assisted in refining the test method. The study findings also support the use of the ZEOGRT test as a suitable alternative to the MEOGRT.

References (1) OECD (2015). Guidelines for the Testing of Chemicals. TG 240: Medaka Extended One Generation Reproduction Test (MEOGRT). OECD, Paris. (2) OECD (2012). Guidelines for the Testing of Chemicals. TG 229: Fish Short Term Reproduction Assay. OECD, Paris. (3) OECD (2011). Guidelines for the Testing of Chemicals. TG 234: Fish Sexual Development Test. OECD, Paris. (4) SANTE/2020/12830, rev.1 (24/02/21) - Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. (5) Teigeler M. (2018). Draft standard operating procedure. Zebrafish extended one generation reproduction test (ZEOGRT). Fraunhofer institute for Molecular Biology (IME).