Standard Operating Procedure (SOP)

Aim of SOP (tick box)

☐ Munition detection or identification	☒ Toxicity
☐ Sampling	☐ In situ exposure studies
☐ Chemical analysis	☐ Bioassays
☐ Bioindicators/biomarkers	

21. Zebrafish embryo acute toxicity test (ZFET)

version 1.1

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Scope

This Standard Operating Procedure describes the Zebrafish embryo acute toxicity (ZFET) test using zebrafish embryos (*Danio rerio*) as test species. This method is used to determine the acute toxicity of chemicals on embryonic stages of fish and can be applied to single substances or mixtures of chemicals[1].

The test was developed as an alternative to the acute toxicity tests with adult fish[2,3] and is used for risk assessment and described in OECD guideline No. 236[4].

Summary of the method/SOP

The ZFET is based on the chemical exposure of freshly fertilized zebrafish eggs for up to 96 hours. By monitoring both lethal and sublethal endpoints every 24 hours, a so-called median lethal concentration (LC_{50}) and median effective concentration (EC_{50}) can be calculated for the tested chemical. Four apical endpoints are considered as indicators for lethality: *1)* coagulation of fertilized eggs, *2)* lack of somite formation, *3)* lack of detachment of the tail-bud from the yolk sac, and *4)* lack of heartbeat[4].

Before starting the test, information on the substance tested, including the structure, purity, stability in water, solubility in water, K_{ow} and vapour, pressure is required. In addition, in the absence of information on expected toxicity, a preliminary test to determine the appropriate test concentrations is necessary. Finally, a suitable analytical method for the tested chemical is recommended to measure the tested nominal concentrations with respect to their actual concentrations.

For a sound statement on the embryo toxicity of the tested substance, at least 5 different concentrations are required, evenly distributed over the expected toxicity range. Three independent replicates per test concentration (using 24 eggs) are preferred.

Safety aspects

Depending on the chemicals to be tested, the corresponding risk assessments have to be considered before starting work. If further instructions are required, contact the local safety officer or the laboratory manager.

Documentation

According to OECD guideline No. 236[4] the test report should include the following information.

Test chemical:

- Information on the test chemical, including water solubility, IUPAC or CAS name, CAS number, purity and additional relevant physico-chemical properties.
- For mixtures: Chemical identity where possible, relevant physico-chemical properties of the constituents.

Test procedure:

- Information on the test conditions, including test procedure (e.g. semi-static renewal), photoperiod, test layout (e.g. number of test chambers, types of controls), water quality criteria (e.g. pH, temperature).
- Nominal concentrations and, where appropriate, results of the measured concentrations.
- Information if solvent was used.
- Indication whether controls have fulfilled the validity criteria and the hatching rate of treatments and controls.

Results:

- Detailed listing of effects and mortality in each well/embryo at each examination time, including controls.
- Cumulative mortality and effect rate for each concentration tested.
- The LC_{50}/EC_{50} values at 96 hours and optionally at 48 hours, including dose-response curve and information on which regression was applied.
- Information on incidents in the course of the test that may have influenced the results.

Methods

Equipment:

- Fish tanks of suitable capacity for brood fish (1 L water per fish).
- Glass or plastic jar with mesh (1 - 2 mm) with artificial plants for egg collection.
- Incubator (26 °C) with photoperiodical illumination. It should be possible to adjust the room in which the microscope is used to about 26 °C.
- Binocular and/or inverted microscope (40x - 80x).
- pH-meter, oxygen meter, equipment to determine hardness of water.
- Glass beakers/96-well polystyrene plates for exposure.
- Pipettes, volume adjustable including tips.
- Measuring cylinders (20 – 1000 mL).
- DURAN bottles of suitable volumes.
- Compressed air supply.
- Disposable plastic Pasteur pipettes (3 mL).
- Parafilm.

Maintenance of brood fish:

For egg production, a breeding stock of non-exposed, wild-type zebrafish with well documented fertilization rates of the eggs is used. For breeding, fish free from apparent symptoms of disease

and not older than 12 to 18 months should be used. The maintenance water should be kept between 26° - 28 C and pH 6.8 – 7.3. The photoperiod should be set to 12-16 hours daylight and a male to female ratio of 2:1 has been found to be good for breeding success[5,6]. Feeding takes place twice a day from Monday to Friday and once at the weekend. The fish are usually fed with flake food in the morning and brine shrimp *(Artemia spec.)* as living food in the afternoon.

Preparation of ISO water and test solutions:

The reconstituted water, so called ISO water, which is used for to dilute the tested chemicals, has to be prepared one day before starting the test. ISO water is prepared according to OECD guideline No. 203, Annex 2[2] and needs to be aerated till oxygen saturation overnight.

Depending on the type of chemical to be tested, the test solutions can be prepared by diluting a stock solution with aerated ISO water. In case of the use of solvents (should be avoided) the solvent concentration in the final test solution should not exceed 100 µl/L and should be the same in all test solutions. Before using the test solutions for egg exposure all solutions need to be tempered at 26° C. If the tested chemical is instable, it is recommended to renew the test solutions in the test vessels every 24 hours (semi-static test design). In this case new test solutions must be prepared daily.

Egg collection:

The evening before, the collecting trays with mesh are placed in the fish tanks with the spawning groups in order to collect the eggs for the experiment the next morning after switching on the light. Spawning takes place within 60 min after switching on the light and fish should be left undisturbed during this time. After removing the collecting trays, rinse the eggs with fresh aquarium water and place them into a glass beaker filled with tempered ISO water.

Test procedure:

The first cleavage in fertilized eggs is taking place after 30 min to 45 min and the following cleavages will form 4, 8, 16 and 32 cell blastomers[4,7]. Collected eggs should be exposed to the test solution in a petri dish as fast as possible and no later than 90 min post fertilization. In the meantime, the eggs can be separated from unfertilized, damaged or malformed eggs. During selection of the eggs, care should be taken not to damage the eggs, therefore it is recommended to draw up the eggs in the test solution with a disposable Pasteur pipette (the end cut off for a larger opening).

If possible the exposure is carried out in 96-well polystyrene plates. Eggs are placed one by one into the wells filled with 200 µL of each test solution. The plates are then sealed with Parafilm and incubated at 26° C with a 14:10 hour light-dark cycle.

The following controls are required for a single ZFET:

- Negative control/dilution water control: 24 eggs as negative control and 12 eggs as internal control on each plate using ISO water.
- Positive control: 24 eggs as positive control using 4 mg/L 3,4-dichloroaniline.
- Solvent control (in case solvent is used): 24 eggs as solvent control exposed to the same concentration of solvent used in the test solutions.

Assessment:

The test plates are assessed under a binocular or inverted microscope after 24 hours and then sealed again with Parafilm. In the case of a semi-static test design, approximately 180 µL of test solution is renewed in the well without harming the embryo.

The following validity criteria should be applied:

- Overall survival of the embryos in the negative control and when used in the solvent control should be ≥ 90% until the end of exposure.
- Overall mortality of the embryos in the positive control should be ≥ 30% at the end of exposure.
- Hatching rate of the embryos in the negative control (and solvent control) should be ≥ 80% at the end of exposure (when running a 96 h scenario).

The apical endpoints to determine lethality of the embryo are shown in Table 1. The sublethal endpoints shown in Table 2 are used to detect any other non-normal development during embryogenesis.

Table 1: Apical endpoints of acute toxicity in zebrafish embryos according to the exposure time (hours).

	24 h	48 h	72 h	96 h
Coagulation of the embryo	+	+	+	+
Lack of somite formation	+	+	+	+
No detachment of the tail	+	+	+	+
No heartbeat		+	+	+

Table 2: Sublethal endpoints used to detect non-normal development in zebrafish embryos according to the exposure time (hours)[8].

Endpoint	24 h	48 h	72 h	96 h
Altered formation of somites	+			
Eye development	+	+	+	+
Spontaneous movement	+	+	+	+
Blood circulation/heartbeat frequency		+	+	+
Lack of pigmentation		+	+	+
Edema		+	+	+
Malformations	+	+	+	+
Chorda deformation	+	+	+	+

Yolk deformation	+	+	+	+
Growth retardation	+	+	+	+

Data evaluation:

For deriving the LC_{50} and EC_{50} values, the cumulative percentages of apical and sublethal endpoints are plotted against the tested concentrations. Appropriate statistical methods such as non-linear regression including the confidence limits are used for calculating the LC_{50} and EC_{50} values, according to OECD Document 54[9].

References

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